

**ASPECTS OF THE ENDOCRINE CONTROL OF  
OVARIAN FUNCTION**

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## DECLARATION CONCERNING THESIS

The papers presented in this thesis were published between 1975 and 1991 and represent the outcome of my research investigations over a 16 year period. I certify that this thesis has been composed by myself. The first five papers listed in this thesis were also presented as part of my submission to the University of Edinburgh for the degree of PhD in 1977.

Of the 58 papers presented in this thesis, I am the sole author of 4, the first author of 26, the second author of 12 and the third or subsequent author of 16. I certify that I have made a substantial contribution to all of the papers presented. In those papers of which I am the sole or first author, I made the major contribution to the works described in those papers. This means that I was primarily responsible for initiating the study, had a major role in designing the experiments undertaken and performed at least some of the experimental work. While much of the routine, established laboratory procedures were often performed by technical staff, under my supervision, I was responsible for the development/establishment of any new methodologies or assays required for the study. I also undertook the data collation and analysis, wrote the paper and 'steered' it through to its final acceptance for publication. For those papers on which I was the second or subsequent co-author, my contribution to the study was less substantial than that of the first author. The closer my name appears to that of the first author, in the list of authors, the greater my contribution to the study. As a secondary author I made a substantial contribution to either the experimental design of the study and/or developed/performed a specialized technique required in the study and/or contributed substantially to the collection, analysis and/or interpretation of data.

## ABSTRACT OF THESIS

This thesis consists of papers, published by the candidate between 1975 and 1991, describing the results of investigations into the endocrine control of ovarian function. The papers are presented in chronological order in each of five sections. Each section deals with a different aspect of the research undertaken by the candidate during the sixteen year period.

Section 1 contains papers describing *in vitro* and *in vivo* studies of progesterone production by the corpus luteum, with particular regard to how prostaglandins may influence steroidogenesis. It is proposed that prostaglandin  $F_{2\alpha}$  may initiate luteolysis through a biochemical action on the luteal cell, whereby it inhibits cellular progesterone production by blocking the stimulation of adenylate cyclase by luteinizing hormone (LH). The effects on ovarian and luteal steroidogenesis of Danazol, a synthetic analogue of ethinyl testosterone having several clinical applications, was studied *in vivo* and *in vitro*. Danazol could inhibit steroidogenesis directly, and it is suggested that this action might mediate, at least in part, some of its therapeutic effects.

Section 2 is concerned with investigations of follicle development and follicle steroidogenesis. A variety of studies have been undertaken including an investigation of local effects of steroids on the ovarian response to gonadotrophins; studies of the mechanism of stimulation of steroidogenesis by LH; measurement of steroids and gonadotrophins in follicular fluids; comparisons of the ability of thecal tissue obtained from follicles of varying size and health to bind LH, and produce cyclic AMP and steroids when challenged with LH; comparisons of the steroidogenic capacity of granulosa cells obtained from follicles of varying size and health, and of their relative capacity to produce cyclic AMP when challenged with follicle stimulating hormone (FSH) or LH; studies of the influence of season on follicle characteristics.

Papers concerned with inhibin are presented in section 3. Studies are described which show that granulosa cells from large, non-atretic follicles are the major source of ovarian inhibin. Steroids and FSH are shown to be capable of regulating inhibin production by

granulosa cells. Inhibin production by male and female gonads is compared and several similarities are shown to exist. Evidence that inhibin might be physiologically important in the regulation of follicle development is provided from studies in which active immunization of ewes with an inhibin preparation increased mean ovulation rates.

The papers in section 4 describe studies to investigate the mechanism(s) responsible for the high ovulation rates observed in Booroola ewes carrying a fecundity (F) gene. The presence of the F-gene results in the development of a larger number of smaller sized preovulatory follicles, relative to ewes without the gene. Mean plasma concentrations of FSH and LH were also found to be significantly higher in F-gene bearing ewes. This was not due to genotype differences in the negative feedback effects of gonadal hormones, or the amounts of ovarian steroids secreted. The highest mean amounts of inhibin produced by granulosa cells *in vitro* was also similar for ewes with and without the F-gene.

Section 5 contains papers related to the manipulation of ovulation rate. FSH is shown to play a key role in determining ovulation rate. By raising ovarian exposure to FSH through increasing plasma concentrations of FSH, ovulation rates could be increased. Lowering plasma FSH concentrations, and thereby reducing ovarian exposure to FSH, caused anovulation. Studies of the potential usefulness of active immunization with crude follicular fluid, a rich source of inhibin, as a means of increasing the proportion of multiple ovulations in sheep indicated that it offered no advantage over the use of steroid-immunogens in this regard. Studies of several commercial gonadotrophin preparations indicated that the relative FSH and LH content of each preparation markedly influenced its superovulatory efficacy. For maximum superovulatory effectiveness, optimization of the FSH:LH ratio seems important, either too little or too much LH being deleterious to oocyte production.



**Section 1: Papers related to studies of steroid  
production by the corpus luteum**

**Paper no.:** 1

**Authors:** K.M. Henderson & K.P. McNatty

**Title:** A biochemical hypothesis to explain the mechanism of luteal regression.

**Reference:** Prostaglandins (1975) 2, 779-797

A BIOCHEMICAL HYPOTHESIS TO EXPLAIN  
THE MECHANISM OF LUTEAL REGRESSION

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ABSTRACT

It seems likely that luteal regression may involve a direct biochemical action of prostaglandin  $F_2\alpha$  ( $PGF_2\alpha$ ) on the luteal cell since there are now several reports that  $PGF_2\alpha$  can directly inhibit steroidogenesis *in vitro*. However, the mechanism of such an action of  $PGF_2\alpha$  remains obscure.

This article initially reviews the central role of adenosine 3,5-bisphosphate (c-AMP) in initiating and maintaining the structural and functional changes occurring on luteinisation. A mechanism is suggested, supported by results obtained using granulosa cells in tissue culture, in which  $PGF_2\alpha$  initiates functional luteolysis by inhibiting further synthesis of c-AMP. This mechanism is then used in conjunction with further *in vitro* observations to provide a possible explanation for the inability of  $PGF_2\alpha$  to regress newly formed corpora lutea. Finally, the possible mechanisms of structural regression are discussed.

ACKNOWLEDGEMENTS

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There is now substantial evidence to suggest that exogenously administered prostaglandin  $F_2\alpha$  ( $PGF_2\alpha$ ) acts as a luteolysin by causing the cessation of progesterone secretion and structural regression of the corpus luteum in the rat (1), hamster (2), rabbit (3), cow (4), pig (5), guinea pig (6) and in particular the ewe (7), in which species it is also the natural luteolysin secreted by the uterus (8). However, attempts to show that  $PGF_2\alpha$  has a similar luteolytic effect in primates (rhesus monkey (9), and human female (10)) have so far been unsuccessful.

In those species in which  $PGF_2\alpha$  is luteolytic, hysterectomy will prolong the life of the corpora lutea. It has been shown that  $PGF_2\alpha$  is synthesised in the uterus, enters the uterine vein, and passes from this vein directly into the ovarian artery, which is tightly adherent and coiled on it (11). Thus  $PGF_2\alpha$  can travel directly from the uterus to the ovary, where it exerts a local effect. Although women appear to have a different pattern of venous drainage (12) when compared to the sheep (13) the utero-ovarian vasculature is not entirely incompatible with a uterine luteolytic substance. Nevertheless there is convincing evidence to suggest that the luteolytic substance in women does not originate from the uterus or other mullerian duct tissue since hysterectomy or the congenital absence of uterus, Fallopian tubes and vagina does not prolong the life of the corpus luteum (14-18). Since hysterectomy also has no effect on the menstrual cycle of the rhesus monkey (19), it seems that the lytic substance in primates is not uterine in origin. Large amounts of  $PGF_2\alpha$  have been found in the ovary of the rhesus monkey (20) and in corpora lutea of women (21), therefore it is possible that  $PGF_2\alpha$  is the luteolysin in primates, but that it is synthesised locally in the ovary, rather than peripherally in the uterus, and thus it can exert a direct local effect.

The mechanisms by which  $PGF_2\alpha$  exerts its lytic action in non-primates is obscure, though several have been postulated, the most popular being those involving a vascular effect of some sort. One suggestion was that luteolysis arose from a constriction of the utero-ovarian vein which resulted in a reduced ovarian blood flow; luteolysis being a consequence of anoxia (3). However, many experiments have now shown that luteolysis does not arise as a result of reduced total ovarian blood flow, and Janson (22) has recently shown that the observed

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reduction in ovarian blood flow following  $\text{PGF}_2\alpha$  administration is probably an artefact, resulting from the concomitant fall in systemic arterial pressure. It has also been suggested that  $\text{PGF}_2\alpha$  may cause a vascular redistribution within the ovary, with selective reduction in the blood flow to the corpus luteum and a subsequent increase in blood flow to the follicles and interstitial tissue (22). Thus  $\text{PGF}_2\alpha$  would still produce luteolysis by anoxia, although simultaneously, follicular development would be enhanced by the increased blood flow.

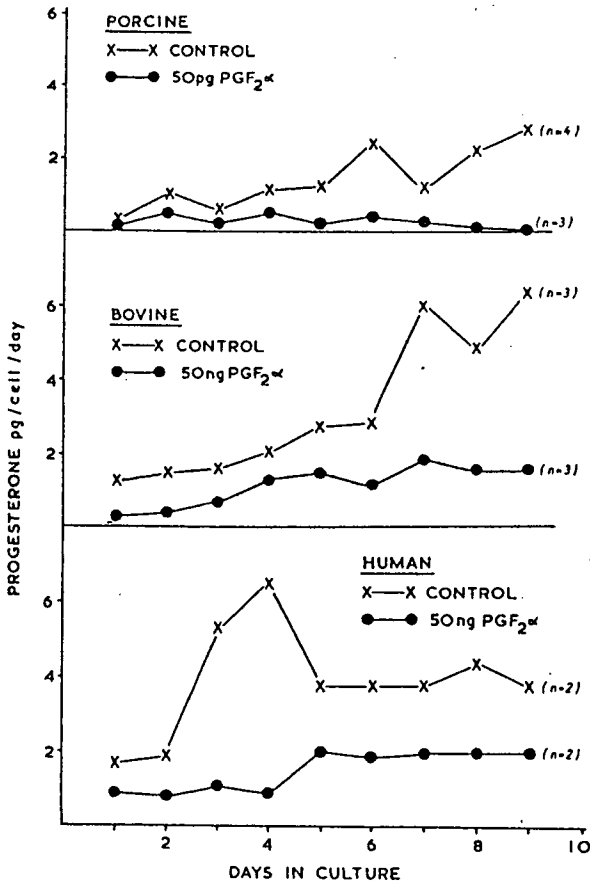
Vascular theories became particularly attractive when it was found that  $\text{PGF}_2\alpha$  actually stimulated the secretion of progesterone by luteal tissue *in vitro* (23, 24) thus seeming to rule out the possibility of a direct luteolytic action on the luteal cell. Although some kind of vascular effect, such as a vascular redistribution within the ovary seems plausible, there are now convincing reports of  $\text{PGF}_2\alpha$  inhibiting the secretion of progesterone by luteal tissue *in vitro* (25, 26). Indeed, we have found that only minimal amounts of  $\text{PGF}_2\alpha$  (50pg-50ng) are required to inhibit the secretion of progesterone by human, porcine and bovine granulosa cells grown in tissue culture, as shown in Figure 1. This strongly suggests that  $\text{PGF}_2\alpha$  is capable of exerting a direct biochemical effect on the luteal cell, thereby directly inhibiting steroidogenesis.

To understand how  $\text{PGF}_2\alpha$  may be acting at a biochemical level, it is worthwhile reviewing the mechanism of steroid production by the luteal cell. Following the pre-ovulatory surge of luteinising hormone (LH), LH enters the follicular fluid (29) and the plasma membrane LH receptors on the granulosa cells (30) will become saturated; morphological and functional luteinisation ensuing. The steroidogenic response to LH is mediated indirectly by the activation of the adenylate cyclase system (31, 32) in the cell membrane to produce the "2nd messenger", adenosine 3',5'-monophosphate (c-AMP), an activation system common to many hormones such as ACTH, glucagon, vasopressin, catecholamines and some prostaglandins (33), e.g.  $\text{PGE}_2$  (34). Although each of these hormones can activate adenylate cyclase, specificity of response depends on the existence of a specific plasma membrane receptor for each hormone, and the final effect produced by the c-AMP is dependent upon the cell type, i.e. a granulosa cell will secrete progesterone whereas lipolysis will be induced in adipose tissue. The morphological changes occurring on luteinisation are probably also mediated by c-AMP since Channing has shown that addition of c-AMP (or the dibutryl derivative) to cultures of porcine (35) and monkey (36) granulosa cells stimulates both morphological luteinisation and progesterone secretion.

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Figure 1

PGF<sub>2</sub>α induced inhibition of progesterone production by porcine, bovine and human granulosa cells *in vitro*. The PGF<sub>2</sub>α treated cultures are significantly different from the controls (P<0.01). n = the number of experiments. The technique of tissue culture was identical to that previously described (27). A minimum of 5x10<sup>4</sup> cells were cultured in 1 ml of culture medium consisting of 20% calf serum and 80% Medium 199 supplemented with HEPES buffer, 1% glutamine and antibiotics (27). The culture medium was replaced daily and stored at -20°C until assayed for progesterone by radioimmunoassay (28).



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It has been shown that c-AMP interacts with a protein receptor (37) consisting of a regulatory unit and a catalytic unit, the regulatory unit acting to suppress the protein kinase activity of the catalytic unit. Cyclic AMP in binding to the regulatory unit causes the dissociation of this receptor moiety from the catalytic unit, thereby producing an activated protein kinase. Should the stimulation of adenylate cyclase cease, the intracellular pool of c-AMP will be rapidly depleted since the conversion of c-AMP to AMP involves a large free energy change due to the highly strained and unstable ring structure of c-AMP, combined with the fact that phosphodiesterase activity was limited only by the availability of substrate. As a consequence of c-AMP depletion, protein kinase activity will diminish since the regulatory unit will recombine with the catalytic unit and so suppress its activity.

The c-AMP dependent protein kinases are non-specific phosphorylators (38). They can phosphorylate particular protein components of the nucleoprotein complex, and in so doing can conceivably produce a local disruption within the complex which allows new regions of DNA to be exposed to the transcription machinery, resulting in new m-RNA, and hence new protein (39). Cyclic-AMP dependent protein kinases can also phosphorylate many different enzymes, and in so doing, modify their activity (38). In this respect the most important enzyme in the luteal cell is cholesteryl esterase. Studies on bovine adrenal cortex show that c-AMP dependent protein kinase is capable of phosphorylating an inactive form of the cholesteryl esterase and converting it into an active form (40). From this, it is not unreasonable to assume that the luteal cell cholesteryl esterase is also capable of being phosphorylated by a c-AMP dependent protein kinase to produce an active form which will then convert the cholesterol ester, stored in lipid droplets (41), into free cholesterol which on entering the mitochondria is readily metabolised to progesterone, but, should protein kinase activity diminish due to c-AMP depletion, then a phosphatase enzyme, which has been found in all tissues containing a c-AMP protein kinase (38), will reconvert the cholesteryl esterase into its original, non-phosphorylated inactive form, and the resultant effect will be a drastic reduction in progesterone synthesis by the luteal cell. However, mammalian m-RNA is very stable (42), and the disappearance of c-AMP will not immediately affect the production of the new proteins induced

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by phosphorylation of the nucleoprotein complex. Thus, although removal of c-AMP will inhibit progesterone synthesis through inactivation of cholesteryl esterase many changes initiated during luteinisation will be unaffected following the phosphatase dephosphorylation of the nucleoprotein complex. The biochemical mechanism of luteinisation is summarised in Figure 2, where it can be seen that c-AMP can also stimulate the conversion of cholesterol to pregnenolone by inducing the synthesis of a protein with a rapid turnover rate, which is involved in the supply of cholesterol to the metabolic enzyme systems in the mitochondria (43).

How then is  $\text{PGF}_2\alpha$  most likely to interfere with progesterone synthesis by the luteal cell at the biochemical level? Behrman *et al.* (44) have suggested that  $\text{PGF}_2\alpha$  acts by inhibiting the conversion of cholesterol ester to free cholesterol. However, in view of the central role of c-AMP in stimulating steroidogenesis, it is perhaps more likely that  $\text{PGF}_2\alpha$  acts by directly inhibiting the synthesis of c-AMP, and so acts on the plasma membrane, the site of c-AMP synthesis; specific receptors for  $\text{PGF}_2\alpha$  have in fact been found in the plasma membrane of the human (45), bovine (45) and ovine (46) corpus luteum.

Studies by Rodbell *et al.* (47) on the membrane-bound adenylate cyclase system have led them to propose a three component model of the hormone-responsive membrane-bound adenylate cyclase as shown in Figure 3. On binding of a hormone to its specific regulatory unit, the intermediate coupler serves to transmit a signal, initiated by the binding event, to the catalytic site, resulting in the activation of the adenylate cyclase and an increase in intracellular levels of c-AMP.

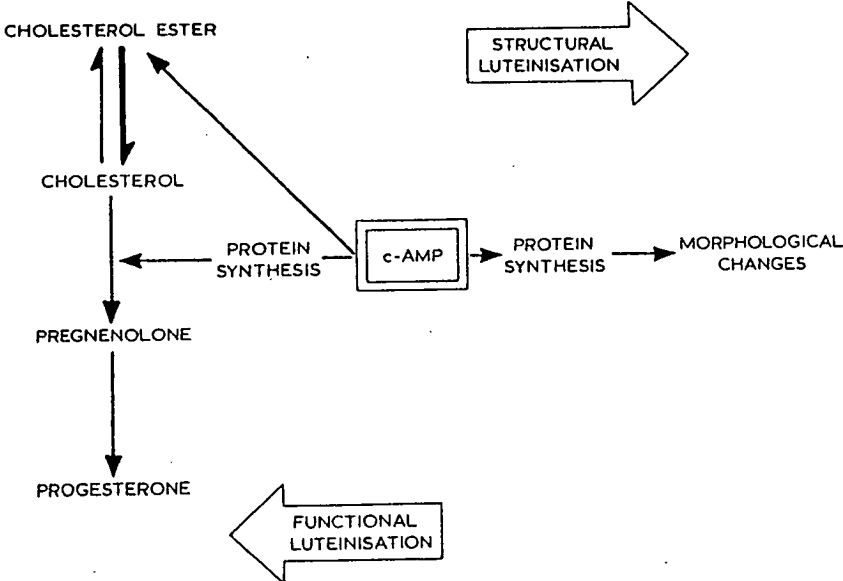
It is suggested that  $\text{PGF}_2\alpha$  acts on the coupling component, either directly or indirectly, to prevent transmission of the signal required to activate the catalytic site (see Figure 3). Consequently cellular c-AMP will rapidly disappear, resulting in a fall in progesterone secretion. This hypothesis is supported by recent studies on the pig corpus luteum, where Andersen *et al.* (48) have shown that the adenylate cyclase fails to respond to LH on Days 16 or 17 of the oestrous cycle when  $\text{PGF}_2\alpha$  secretion is probably maximal. This postulated site of action of  $\text{PGF}_2\alpha$  can be readily tested both *in vitro* and *in vivo*.



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Figure 2

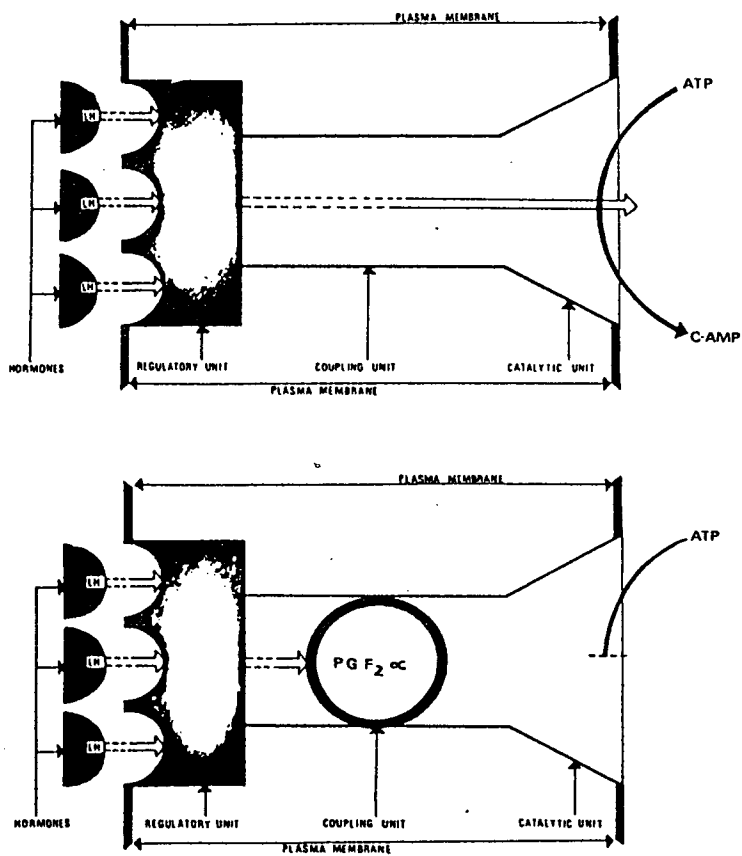
Role of c-AMP in structural and functional luteinisation.



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Figure 3

Three component model of the adenylate cyclase system showing postulated site of  $\text{PGF}_2\alpha$  action.



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By acting specifically on the LH coupling unit,  $\text{PGF}_2\alpha$  should only block the activation of adenylate cyclase by  $^2\text{LH}$ , but should not prevent its activation by any other hormone having a separate specific regulatory unit linked up to the catalytic site via its own coupling unit;  $\text{PGE}_2$  is an example of such a hormone since there is substantial evidence to show that  $\text{PGE}_2$  utilises the adenylate cyclase system (34). Thus, both in vitro and in vivo it should be possible to overcome the  $\text{PGF}_2\alpha$  inhibition of progesterone synthesis with  $\text{PGE}_2$ ,

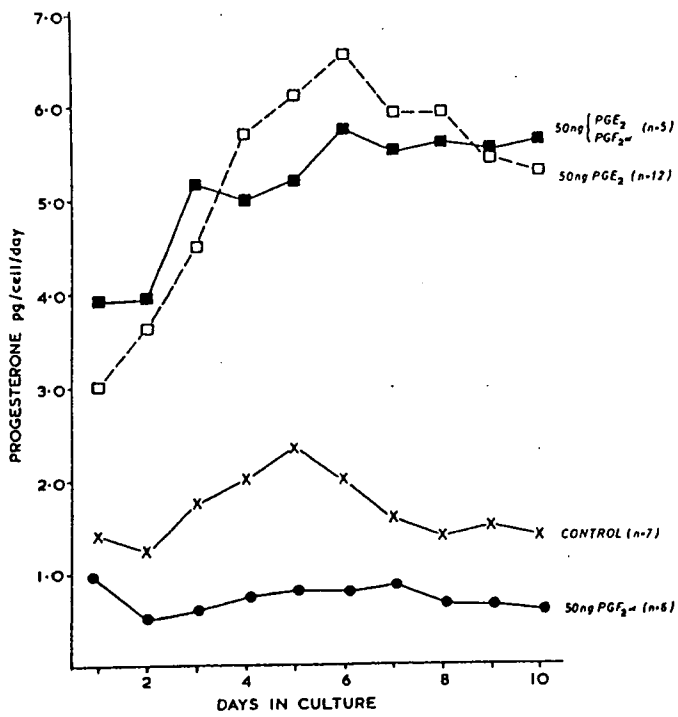
It has been previously shown that granulosa cells harvested from human Graafian follicles containing high concentrations of FSH and oestradiol but low levels of LH in follicular fluid have the biosynthetic potential to secrete maximum amounts of progesterone when cultured in vitro ( $\sim 4$  to  $7$  pg/cell/day) (27). When these cells were exposed daily to  $50$  ng of  $\text{PGE}_2$  per ml of culture medium (see details in legend to Figure 1), the maximum daily production of progesterone was achieved within 3 days (Figure 4). By contrast, the addition of  $50$  ng  $\text{PGF}_2\alpha$  per ml of culture medium markedly inhibited the daily production of progesterone (Figure 4). However the simultaneous addition of  $\text{PGF}_2\alpha$  ( $50$  ng) and  $\text{PGE}_2$  ( $50$  ng) resulted in a daily production of progesterone similar to that achieved by cells exposed to  $\text{PGE}_2$  alone. Similar results have also been obtained using intact mouse follicles maintained in organ culture (49). Thus  $\text{PGE}_2$  does appear to be capable of overcoming the inhibitory effect of  $\text{PGF}_2\alpha$  and so supports the concept that  $\text{PGF}_2\alpha$  acts specifically on LH activated cyclase.

Any mechanism of luteal regression must explain the inability of  $\text{PGF}_2\alpha$  to induce the luteolysis of the newly formed corpus luteum in many animals, cow (4), Day 4; sheep (50), Day 4; horse (51), Day 4 and in particular the pig (5) where the corpus luteum is resistant to exogenous  $\text{PGF}_2\alpha$  for the first 12 days of its life. This could be explained simply by the fact that the pre-ovulatory surge of LH saturates the regulatory units of the luteal cells and that it is this bound hormone which protects the young corpus luteum. This hypothesis is supported by recent experiments in our laboratory utilising granulosa cells in culture that had not previously been exposed to LH. If  $\text{PGF}_2\alpha$  was added from the start of the culture period, it inhibited progesterone secretion by cells subsequently exposed to LH and FSH, relative to those cells exposed to LH and FSH alone, however, a higher dose of  $\text{PGF}_2\alpha$  was required to inhibit the production of progesterone if the cells had been stimulated with LH and FSH for 6 days beforehand, as shown in Tables 1 and 2.

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Figure 4

The effect on progesterone secretion of  $\text{PGF}_2\alpha$  and  $\text{PGE}_2$  when added alone and together to human granulosa cells in culture.



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Table 1

The effect of  $\text{PGF}_2\alpha$  on the production of progesterone by cultured human<sup>2</sup>granulosa cells.

<u>Treatment</u>	<u>Progesterone (<math>\mu\text{g}</math>)</u> (mean $\pm$ s.e.m.)
a Control	18.3 $\pm$ 1.7 (n=8)
b LH + FSH	48.0 $\pm$ 1.3 (n=6)*
c LH + FSH + $\text{PGF}_2\alpha$	19.4 $\pm$ 2.1 (n=6) <sup>+</sup>

Total production of progesterone by  $1 \times 10^6$  human granulosa cells cultured in vitro for 12 days in

- a 20% calf serum + 80% medium 199 + HEPES buffer (20mm) (culture medium).
- b Culture medium + LH/FSH (36 mu./24 mu.)
- c Culture medium + LH/FSH (36 mu./24 mu. +  $\text{PGF}_2\alpha$  (50 ng/ml).

n refers to number of experiments

\* significantly different from control (P<0.01)

<sup>+</sup> not significantly different from control.

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Table 2

Effect of delaying the addition of  $\text{PGF}_2\alpha$  on the production of progesterone by cultured human granulosa cells.

<u>Treatment</u>	<u>Production of progesterone (<math>\mu\text{g}</math>)</u>	
	<u>days 1-6</u>	<u>days 7-12</u>
LH/FSH (36 mu./24 mu.) day 1-12	22.7	27.4
LH/FSH (36mu./24 mu.) days 1-12 + $\text{PGF}_2\alpha$ (50 ng/ml) from days 7-12	26.8	27.7
LH/FSH (36 mu./24 mu.) days 1-12 + $\text{PGF}_2\alpha$ (1000 ng/ml) from days 7-12)	24.1	13.7

Mean production of progesterone by  $1 \times 10^6$  human granulosa cells over two 6 day periods i.e. days 1-6 and days 7-12 respectively. Each experiment was carried out in duplicate on cells harvested from one Graafian follicle.

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It is well established that the dissociation of LH from its receptor is a slow process taking several days (52-55). Also Koch et al. (56) has shown that only a fraction of these receptors need be occupied to stimulate the adenylate cyclase system maximally. The levels of LH found in peripheral plasma during the luteal phase are very low, relative to the levels occurring at the time of the preovulatory surge, (when the granulosa cell receptors become saturated with LH) and this will encourage the dissociation of LH from its receptor. Thus throughout the luteal phase the LH receptor sites will be gradually vacated, but this will not affect the amount of progesterone being produced by the corpus luteum. However, as more and more LH dissociates from the corpus luteum, the corpus luteum would become increasingly susceptible to the luteolytic action of  $\text{PGF}_2\alpha$ .

This is quite feasible when one remembers that the plasma membrane is not a rigid, static structure, but rather a dynamic structure best described as a fluid mosaic (57), its conformation at any time being the thermodynamically most stable form. As the LH receptors on the plasma membrane fill, then it is likely that this thermodynamic stability will be upset and so consequently the membrane will undergo conformational changes in order to re-establish thermodynamic stability perhaps producing a form less congruous to the uptake of  $\text{PGF}_2\alpha$ . Similarly, as the LH receptors become vacant again throughout the luteal phase, the membrane would gradually revert to its original form, a form possibly facilitating  $\text{PGF}_2\alpha$  uptake which in turn would promote more conformational changes. This postulate that  $\text{PGF}_2\alpha$  may be inducing conformational and/or structural changes within the membrane is strengthened by the recent findings of Hichens et al. (58), where  $\text{PGF}_2\alpha$  was shown to decrease the binding capacity of luteal tissue to HCG, possibly by inducing conformational changes.

Data obtained from studies in vivo supports the concept that the onset of luteal regression is a consequence of an initial biochemical action of  $\text{PGF}_2\alpha$ . Data obtained in the ewe by Baird and Scaramuzzi (59) shows that low levels of  $\text{PGF}_2\alpha$  (<5 ng/ml) first appear in utero-ovarian venous plasma on Day 13, and progesterone levels also commence to decline from Day 13 onwards. Although these falling levels of progesterone are accompanied by some structural changes in the luteal cells (60), functional regression can be halted by hysterectomy (i.e. removal of  $\text{PGF}_2\alpha$ ) as late as Day 15 (61). Massive

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amounts of  $\text{PGF}_2\alpha$  (20 ng/ml) are then released from the uterus into the utero<sup>2</sup>ovarian vein on Days 15 and 16, and it is probably this which induces the final irreversible morphological deterioration of the corpus luteum either by directly or indirectly activating the lysosomes on Days 15 and 16 (62). Recent studies clearly show that the plasma membrane itself can influence and control lysosomal activity (63). Thus it is possible that low amounts of  $\text{PGF}_2\alpha$ , although sufficient to inhibit steroidogenesis, can only cause a partial activation of the lysosomes, but for them to be fully activated requires the plasma membrane to be completely saturated with  $\text{PGF}_2\alpha$ .

Although both functional and morphological regression could arise from a biochemical action of  $\text{PGF}_2\alpha$ , it is still possible that morphological regression may be due to a redistribution of blood flow within the ovary, since the initial structural change in the luteal cell, karyorrhexis, is indicative of anoxia damage (60). Furthermore, although  $\text{PGF}_2\alpha$  will inhibit the secretion of progesterone by luteal cells *in vitro*, there is no evidence of structural damage. However if morphological regression is mediated by a vascular effect of  $\text{PGF}_2\alpha$  then it is difficult to explain why the newly formed corpus luteum is resistant to exogenously administered  $\text{PGF}_2\alpha$  in some species.

In conclusion, it is suggested that luteal regression is initiated by a biochemical action of  $\text{PGF}_2\alpha$  on the luteal cell membrane, according to the following sequence of events.

1. Throughout the luteal period LH gradually dissociates from its specific membrane receptors on the luteal cell. This promotes a conformational change within the plasma membrane facilitating  $\text{PGF}_2\alpha$  uptake.
2. Under the influence of progesterone, increased amounts of  $\text{PGF}_2\alpha$  are secreted by the uterus or ovary.
3. This  $\text{PGF}_2\alpha$  binds to the plasma membrane of the luteal cell and either directly or indirectly prevents the LH regulatory unit of adenylate cyclase activating the catalytic unit; consequently, cellular c-AMP will diminish.
4. Cholesteryl esterase will be dephosphorylated to its inactive form and so inhibit the synthesis of progesterone.



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5. The final morphological deterioration of the corpus luteum may result from subsequent release of larger amounts of  $\text{PGF}_2\alpha$  which causes lysosomal activation through further changes in the plasma membrane. However, the possibility of morphological regression being mediated by a vascular effect of  $\text{PGF}_2\alpha$  cannot be ruled out.

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# EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ AND $E_2$ ON THE PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA CELLS IN TISSUE CULTURE

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## SUMMARY

Human granulosa cells with differing steroidogenic potentials were cultured *in vitro*. The effects of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) and  $PGE_2$  on the progesterone output and viability of these cells were investigated.

Prostaglandin  $F_{2\alpha}$  either alone or in combination with LH and FSH inhibited the production of progesterone over a wide range of concentrations (1–8000 ng/ml). However, the inhibitory effect of  $PGF_{2\alpha}$  was 200 times less effective when the cells were exposed to LH and FSH for 6 days before the addition of the prostaglandin. By contrast  $PGE_2$ , at concentrations from 1 to 500 ng/ml, markedly stimulated the production of progesterone by granulosa cells, and this was not prevented by the addition of  $PGF_{2\alpha}$ . The degree of inhibition by  $PGF_{2\alpha}$  or stimulation by  $PGE_2$  was related to the biosynthetic capacity of the cells.

These studies suggest that  $PGF_{2\alpha}$  may act directly on the adenylate cyclase system of human granulosa cells by blocking its activation by LH, and they demonstrate that functional regression of the luteal cell can be induced independently of the blood vascular system.

## INTRODUCTION

The luteolytic action of exogenous prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) has been demonstrated in the rat (Pharriss & Wyngarden, 1969), hamster (Gutknecht, Wyngarden & Pharriss, 1971), rabbit (Pharriss, 1970), cow (Rowson, Tervit & Brand, 1972), pig (C. Polge, personal communication), guinea-pig (Blatchley & Donovan, 1969) and ewe (McCracken, Glew & Scaramuzzi, 1970) – in which its physiological significance as the luteolytic hormone is well established (McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson, 1972; Scaramuzzi, Baird, Wheeler & Land, 1973; Goding, 1974). However, the mechanism(s) by which  $PGF_{2\alpha}$  induces functional and structural luteolysis is unknown; ovarian blood flow is not substantially altered during luteolysis (Baird, 1974; Bruce & Hillier, 1974; Jansen, Albrecht & Ahren, 1975), although a redistribution in capillary flow within the ovary may occur at this time (Jansen *et al.* 1975). The possibility that  $PGF_{2\alpha}$  may act directly by interfering with cholesterol ester synthetase activity (Behrman, MacDonald & Greep, 1971) or by decreasing the binding capacity of luteal tissue for human chorionic gonadotrophin (HCG) or luteinizing hormone (LH) (Hichens, Grinwich & Behrman, 1974) remains to be confirmed.

Attempts to induce luteal regression in women by short-term i.v. infusions of  $PGF_{2\alpha}$  have been unsuccessful, and there is only preliminary evidence in the monkey of a decline in

plasma levels of progesterone after similar prostaglandin treatment (Jewelewicz, Cantor, Dyrenfurth, Warren & Vande Wiele, 1972; Jones & Wentz, 1972; Lehmann, Peters, Breckwoldt & Bettendorf, 1972; LeMaire & Shapiro, 1972; Auletta, Speroff & Caldwell, 1973; Wentz & Jones, 1973). However, a specific  $\text{PGF}_{2\alpha}$  receptor was recently demonstrated in the human corpus luteum (Powell, Hammarström, Samuelsson & Sjöberg, 1974), so that the infusion experiments may have failed because rapid peripheral metabolism allowed insufficient  $\text{PGF}_{2\alpha}$  to reach the ovary (Piper, Vane & Wyllie, 1970; Granström, 1972) or because the human corpus luteum may be relatively refractory to the luteolytic effects of  $\text{PGF}_{2\alpha}$  during much of its life.

Prostaglandin  $\text{E}_2$ , in contrast to  $\text{PGF}_{2\alpha}$ , mimics the actions of trophic hormones on both primate and non-primate ovarian tissue since it stimulates the production of progesterone by the human corpus luteum (Marsh & LeMaire, 1974), bovine corpus luteum (Speroff & Ramwell, 1970), rat follicle (Lindner, Tsafiriri, Lieberman, Zor, Koch, Bauminger & Barnea, 1973), mouse follicle (Neal, Baker, McNatty & Scaramuzzi, 1975), and monkey and porcine granulosa cells (Channing & Crisp, 1972; Kolena & Channing, 1972).

Human granulosa cells cultured *in vitro* provide a convenient biochemical model system for studying some of the possible effects of prostaglandins on the human corpus luteum (McNatty & Sawers, 1975). Granulosa cells harvested from large Graafian follicles in the mid- or late follicular phase of the menstrual cycle (i.e. 'active follicles') secrete maximum amounts of progesterone within 2–6 days in culture, probably because they have been exposed to high concentrations of follicle-stimulating hormone (FSH) and oestradiol in follicular fluid (McNatty, Hunter, McNeilly & Sawers, 1975; McNatty & Sawers, 1975). By contrast cells harvested from follicles containing little or no FSH and oestradiol (i.e. 'inactive follicles') do not attain their maximum biosynthetic capacity for at least 10 days *in vitro* (McNatty & Sawers, 1975). We have therefore used human granulosa cells of differing steroidogenic potentials to investigate the possible effects of  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  on the human corpus luteum.

## MATERIALS AND METHODS

### *Patients*

Ovaries were obtained at various stages of the menstrual cycle from 12 women (aged 34–42 years) who were undergoing hysterectomy for menorrhagia due to endometriosis (2) and dysmenorrhoea or chronic pelvic pain (10). The previous menstrual cycles of these women varied in length from 20 to 29 days and five had ovulated during the cycle under study. The remaining seven women were in the follicular phase as assessed from endometrial histology and date of the last menstrual period.

### *Collection of follicular fluid and the culture of granulosa cells*

Thirty-five Graafian follicles of 4–15 mm diameter were dissected from the above ovaries. Follicular fluid was aspirated from each follicle through a 27 gauge needle into a 1 ml syringe and stored at  $-20^\circ\text{C}$  until assayed for FSH, LH and oestradiol by radioimmunoassay (McNatty *et al.* 1975). The relationship between hormone concentrations of follicular fluid and steroidogenic potential of human granulosa cells has been described by McNatty & Sawers (1975).

Granulosa cells were scraped from the collapsed follicle into Medium 199 containing Hanks' salts, HEPES buffer (20 mmol/l) with gentamicin (50  $\mu\text{g}/\text{ml}$ ), amphotericin-B (2.5  $\mu\text{g}/\text{ml}$ ) and 1% glutamine (Flow Laboratories, Irvine, Scotland).

The technique of culturing granulosa cells was identical to that previously described (McNatty & Sawers, 1975). The cells were grown for 10–12 days in 1 ml culture medium



containing 20% calf serum (v/v). The endogenous gonadotrophic activity of the culture medium was 1.7 mu. LH/ml and 1.8 mu. FSH/ml (McNatty & Sawers, 1975) (1 mu. LH  $\equiv$  11.6 ng LER 907 and 1 mu. FSH  $\equiv$  44.6 ng LER 907). The endogenous level of PGF<sub>2 $\alpha$</sub>  in the culture medium was 36 pg/ml and PGE<sub>2</sub> < 200 pg/ml (as determined by radioimmunoassay and g.l.c.-mass spectrometry). The culture medium was replaced each day and stored at -20 °C until assayed for progesterone by radioimmunoassay (Neal *et al.* 1975). Triplicate cultures for each experiment were carried out whenever possible although the low viabilities (< 10%) of some cell preparations meant that a number of studies had to be made on individual cultures. The precision attained with replicate cultures in relation to the production of progesterone for all treatments was  $7.6 \pm 0.9\%$  (S.E.M.) ( $n = 68$ ) which was similar to that reported previously (McNatty & Sawers, 1975). The number of 'live' cells at the commencement of culture together with the number remaining after 10 days of culture were determined by the technique of McNatty & Sawers (1975).

#### *Prostaglandin F<sub>2 $\alpha$</sub> and E<sub>2</sub>*

Prostaglandin F<sub>2 $\alpha$</sub>  and E<sub>2</sub> (Upjohn Company, Kalamazoo, U.S.A.) were each dissolved in 70% aqueous ethanol and stored at 4 °C in ampoules until added directly to the cell cultures in a volume of 5 or 10  $\mu$ l. A similar volume of 70% aqueous ethanol without prostaglandin was added to the control cultures.

#### *Luteinizing hormone and follicle-stimulating hormone*

The gonadotrophins which were added to the cultures were: human LH (Stockell Hartree IRC-2, 24.6.69) containing 7550 u. LH/mg and < 25 u. FSH/mg; and human FSH (MRC 73/519) containing 2200 u. FSH/mg and 8.8 u. LH/mg. These immunological potencies were assessed using the following standards: LH, MRC 68/40 assumed 77 u./ampoule; FSH, MRC 68/39, 32.8 u./ampoule (MRC National Institute for Biological Standards and Control). The concentrations of LH and FSH are expressed as mu./ml. The gonadotrophins were added in culture medium at a concentration of 300 mu. LH or FSH/ml. The medium was stored at -20 °C in ampoules and 0.1 ml samples were added to the cell cultures.

## RESULTS

### *Effect of prostaglandins on viability and mitotic activity of granulosa cells in vitro*

When cells were exposed to PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> alone or in combination there was no significant increase or decrease in cell numbers during 10 days of culture when compared with untreated control cultures with or without gonadotrophins except when PGE<sub>2</sub> was added to culture medium containing cells harvested from inactive follicles. In the latter experiments a twofold increase in cell numbers occurred ( $r = 1.91 \pm 0.22$  (S.E.M.;  $n = 4$ )) when compared with the control cultures ( $r = 1.08 \pm 0.11$ ,  $n = 4$ ) ( $r =$  no. of cells after 10 days of culture divided by the number of live cells at the start of culture).

### *Effect of differing doses of prostaglandin F<sub>2 $\alpha$</sub> on the total production of progesterone by granulosa cells in culture*

Table I shows the total production of progesterone in 10 days by granulosa cells exposed daily to PGF<sub>2 $\alpha$</sub>  in culture medium at concentrations between 1 and 8000 ng/ml medium. At all dose levels PGF<sub>2 $\alpha$</sub>  lowered the total production of progesterone by 60% or more when added to cells harvested from 'active' follicles. By contrast, a much smaller reduction in total progesterone output was observed by cells harvested from 'inactive' follicles. In both cases the higher the concentration of PGF<sub>2 $\alpha$</sub>  the greater the inhibition in progesterone output.

*Effect of prostaglandin  $F_{2\alpha}$  and gonadotrophins on the daily production of progesterone by granulosa cells*

The daily production of progesterone by granulosa cells harvested from 'active' follicles and exposed daily to  $PGF_{2\alpha}$  with or without LH and FSH was markedly inhibited (60%) compared with the control cells (Fig. 1). Although LH+FSH induced a twofold increase in progesterone secretion, the addition of 50 ng  $PGF_{2\alpha}$ /ml medium markedly inhibited this stimulatory effect. In contrast (Fig. 2) the daily production of progesterone by granulosa cells harvested from 'inactive' follicles and treated daily with  $PGF_{2\alpha}$  with or without LH and FSH was only slightly decreased (10%). Although LH+FSH induced a three- to sixfold

Table 1. Total production of progesterone by human granulosa cells after 10 days of culture in varying concentrations of prostaglandin  $F_{2\alpha}$ . (Values are means  $\pm$  S.E.M. expressed as a percentage of controls)

Source of granulosa cells	Prostaglandin $F_{2\alpha}$ (ng/ml)						
	1	10	50	100	500	1000	8000
'Inactive' follicles	93.5 (2)	88.6 (2)	85.7 $\pm$ 6.2 (4)	84.3 $\pm$ 5.7 (4)	70.0 $\pm$ 6.6 (4)	50.0 $\pm$ 4.7 (4)	45.7 (2)
'Active' follicles	39.3 (2)	33.3 $\pm$ 4.4 (4)	32.1 $\pm$ 1.1 (5)	22.0 $\pm$ 2.9 (5)	16.0 $\pm$ 3.3 (4)	—	—

'Inactive' follicles contained < 1.3 mu. FSH/ml and < 250 ng oestradiol/ml follicular fluid. 'Active' follicles contained > 1.3 mu. FSH/ml and > 250 ng oestradiol/ml. The numbers in parentheses refer to the number of experiments on individual follicles.

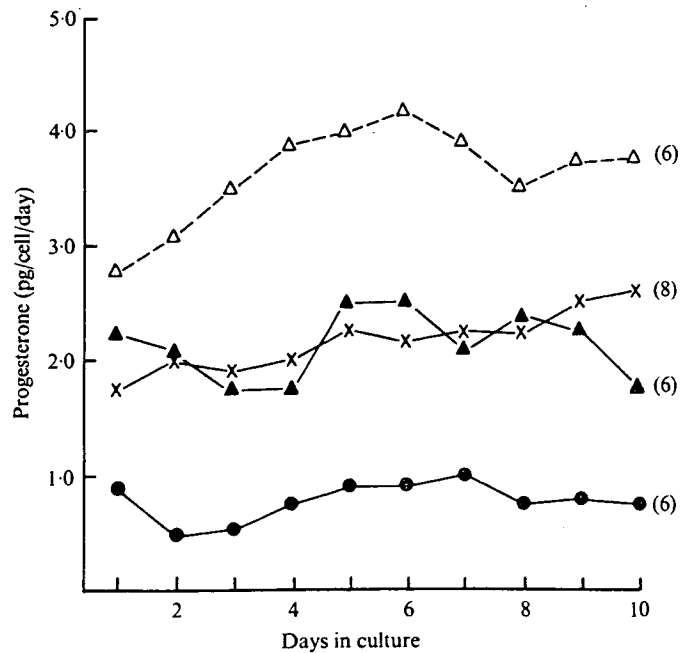


Fig. 1. Mean daily production of progesterone by human granulosa cells *in vitro* and exposed to prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). Cells were harvested from follicles containing FSH and oestradiol ( $E_2$ ): FSH, > 1.3 mu./ml;  $E_2$ , > 250 ng/ml. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH+FSH; solid circle,  $PGF_{2\alpha}$ ; solid triangle, LH+FSH+ $PGF_{2\alpha}$ .

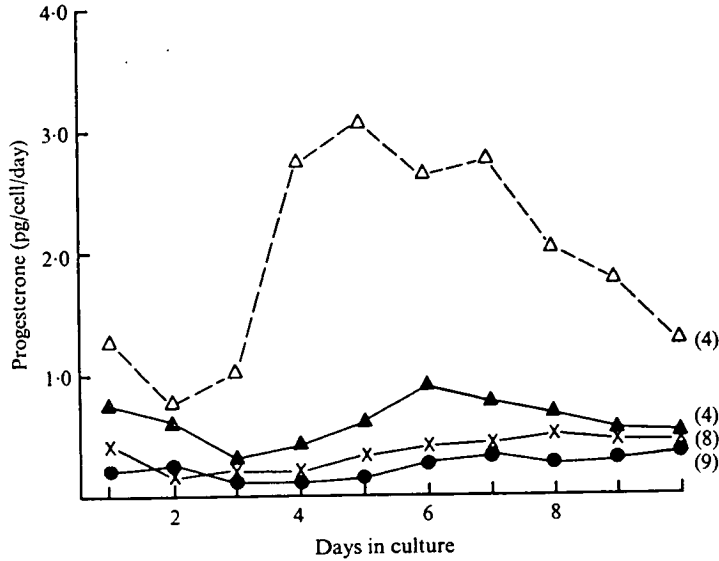


Fig. 2. Mean daily production of progesterone by human granulosa cells *in vitro* and exposed to prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). Cells were harvested from follicles containing low concentrations of FSH and oestradiol (E<sub>2</sub>): FSH, < 1.3 mu./ml; E<sub>2</sub>, < 250 ng/ml. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH+FSH; solid circle, PGF<sub>2α</sub>; solid triangle, LH+FSH+PGF<sub>2α</sub>.

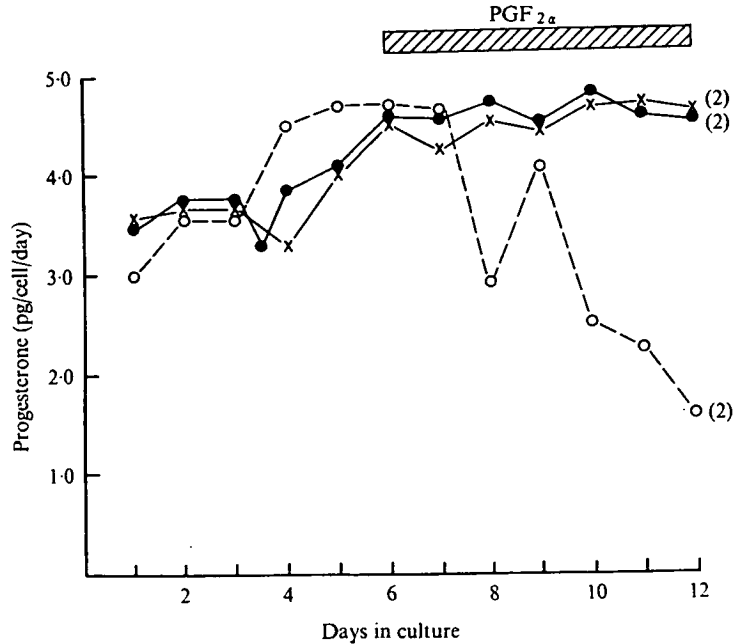


Fig. 3. Effect of delaying the addition of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) on the daily production of progesterone by human granulosa cells *in vitro*. Values are mean daily production of progesterone by cells exposed to either 50 or 1000 ng PGF<sub>2α</sub>/ml daily from day 6. Cells were harvested from a follicle containing FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; solid circle, 50 ng PGF<sub>2α</sub>/ml; open circle, 1000 ng PGF<sub>2α</sub>/ml.

increase in progesterone secretion the addition of 50 ng  $\text{PGF}_{2\alpha}$ /ml medium almost totally inhibited this stimulatory effect.

The effect of delaying the addition of  $\text{PGF}_{2\alpha}$  is shown in Fig. 3. The daily addition of 50 ng  $\text{PGF}_{2\alpha}$  from Day 6 had no effect on the production of progesterone. However, when 1000 ng  $\text{PGF}_{2\alpha}$  were added daily from Day 6 there was a progressive decrease in progesterone output, so that by Day 12 it was only one-third of that achieved by the control cells.

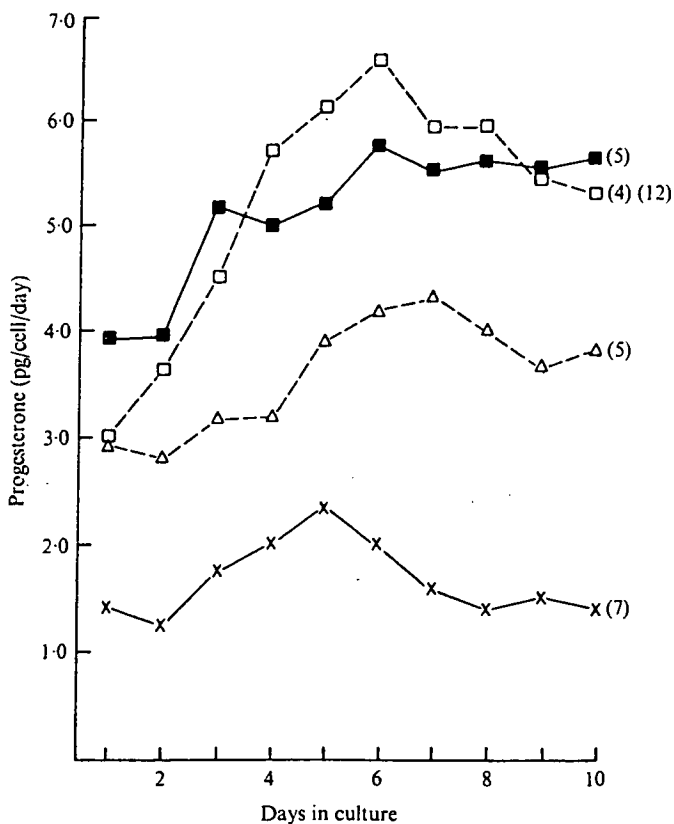


Fig. 4. Mean daily production of progesterone by human granulosa cells *in vitro* exposed to prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH; open square, LH + FSH +  $\text{PGE}_2$  (4) and  $\text{PGE}_2$  only (12); solid square,  $\text{PGE}_2$  +  $\text{PGF}_{2\alpha}$  (50 ng/ml).

*Effect of prostaglandin  $\text{E}_2$  and gonadotrophins on the daily production of progesterone by granulosa cells*

The daily production of progesterone by granulosa cells harvested from 'active' follicles and exposed daily to  $\text{PGE}_2$  with or without added LH + FSH is shown in Fig. 4. LH + FSH only stimulated a twofold increase in the production of progesterone, whilst the daily addition of 50 ng  $\text{PGE}_2$ /ml medium stimulated a three- to fourfold increase, so that the maximum secretion of about 5 pg/cell/day (McNatty & Sawers, 1975) was reached within 3-4 days in culture. The addition of LH + FSH together with 50 ng  $\text{PGE}_2$ /ml medium did not increase the daily production of progesterone above that achieved by  $\text{PGE}_2$  alone. Furthermore, there was no dose-related increase in the daily production of progesterone if the cells were exposed to  $\text{PGE}_2$  at concentrations between 1 and 500 ng/ml medium.

The daily production of progesterone by granulosa cells harvested from 'inactive' follicles and exposed to PGE<sub>2</sub> with or without LH + FSH is shown in Fig. 5. The addition of between 1 and 500 ng PGE<sub>2</sub>/ml medium to cells harvested from 'inactive' follicles failed to stimulate the production of progesterone more than LH + FSH. Furthermore, the addition of PGE<sub>2</sub> together with LH + FSH failed to produce any further stimulation.

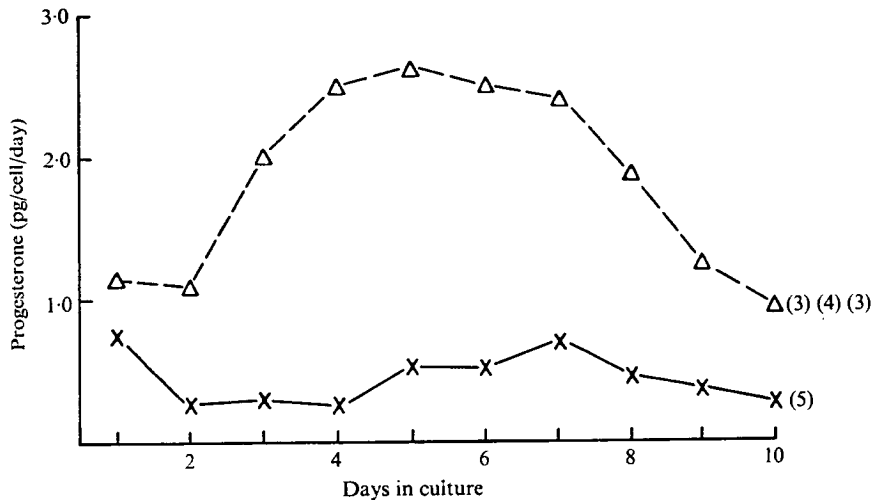


Fig. 5. Mean daily production of progesterone by human granulosa cells *in vitro* exposed to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing low concentrations of FSH and oestradiol. Numbers in parentheses refer to number of experiments. Cross, control; open triangle, LH + FSH + PGE<sub>2</sub> (3), PGE<sub>2</sub> alone (4) and LH + FSH (3).

Table 2. Effect of adding prostaglandins F<sub>2α</sub> (PGF<sub>2α</sub>) and E<sub>2</sub> (PGE<sub>2</sub>) on the production of progesterone by human granulosa cells

Prostaglandin added	Concentration (ng/ml)	Total production of progesterone during 10 days of culture (μg progesterone/10 <sup>6</sup> cells; mean ± s.e.m.)	Number of separate cultures
Control	< 0.04	51.8 ± 4.6	5
PGE <sub>2</sub>	{ 10	60.3 ± 1.9	5
	{ 500	61.9 ± 2.3	5
PGF <sub>2α</sub>	{ 10	23.2 ± 3.7	5
	{ 500	17.5 ± 2.1	5
PGE <sub>2</sub>	10	49.9 ± 4.9	3
PGF <sub>2α</sub>	10		
PGE <sub>2</sub>	50	48.8 ± 5.1	3
PGF <sub>2α</sub>	50		
PGE <sub>2</sub>	100	57.6 ± 4.3	3
PGF <sub>2α</sub>	100		
PGE <sub>2</sub>	500	57.2 ± 6.6	3
PGF <sub>2α</sub>	500		

Cells were harvested from a single 15 mm late-follicular-phase follicle with detectable levels of LH (4.8 mu./ml), FSH (3.3 mu./ml) and oestradiol (2100 ng/ml) in the follicular fluid. The concentration refers to the final concentration of prostaglandin in the medium.

*Effect of adding prostaglandins F<sub>2α</sub> and E<sub>2</sub> on the production of progesterone by granulosa cells*

The effects of adding 50 ng of each prostaglandin to granulosa cells harvested from 'active' follicles are shown in Fig. 4; the daily production of progesterone was similar to that achieved by adding PGE<sub>2</sub> alone. The total production of progesterone by granulosa cells harvested from a preovulatory follicle after 10 days of culture when exposed to varying doses of PGF<sub>2α</sub>, PGE<sub>2</sub> or PGF<sub>2α</sub> + PGE<sub>2</sub> is shown in Table 2. The addition of 10 to 500 ng PGF<sub>2α</sub> lowered the total production of progesterone by at least 60%. The addition of 10–500 ng PGE<sub>2</sub>/ml medium with PGF<sub>2α</sub> over the same dose range did not alter the total production of progesterone achieved by adding PGE<sub>2</sub> alone.

DISCUSSION

These results show clearly that PGF<sub>2α</sub> inhibits the production of progesterone by human granulosa cells *in vitro* without affecting cell viability. They also show that provided PGF<sub>2α</sub> is added at the commencement of culture it is equally effective in inhibiting the daily production of progesterone by granulosa cells from active or inactive follicles, although the former have a greater biosynthetic potential to secrete progesterone (McNatty & Sawers, 1975). Furthermore, when PGF<sub>2α</sub> was added at the commencement of culture it blocked the stimulatory effect of LH + FSH on granulosa cells at all stages of their development. By contrast, when granulosa cells were exposed to high concentrations of LH + FSH for several days before the addition of 50 ng PGF<sub>2α</sub>, the production of progesterone was not inhibited. Although a detailed dose-relationship was not established, a much higher concentration of PGF<sub>2α</sub> (1000 ng/ml medium) was required to inhibit the production of progesterone.

The addition of PGE<sub>2</sub> to human granulosa cells *in vitro* stimulated the secretion of progesterone at a greater rate than any concomitant increase in cell numbers. These experiments also showed that PGE<sub>2</sub> was far more effective than LH + FSH in stimulating progesterone secretion by cells from actively developing follicles. By contrast, the addition of PGE<sub>2</sub> to cells from 'inactive' follicles was no more effective than LH + FSH in stimulating the production of progesterone, emphasizing once more the differing biosynthetic potential of granulosa cells harvested from differing hormonal environments in follicular fluid (McNatty & Sawers, 1975). There was no evidence of any synergism between PGE<sub>2</sub> and LH + FSH.

The present studies indicate that extremely low levels (1–50 ng/ml) of both prostaglandins can cause dramatic alterations in progesterone production under *in-vitro* conditions. Similar concentrations of PGF<sub>2α</sub> are present in the ovarian artery of the sheep during luteal regression (Baird & Scaramuzzi, 1975), and preliminary studies indicate that comparable levels are also present in the human corpus luteum (I. Swanston, D. T. Baird, R. W. Kelly and K. P. McNatty, unpublished data). Prostaglandins found within the human follicle and corpus luteum could therefore have a major controlling influence on steroid secretion by these structures. Although PGF<sub>2α</sub> was extremely effective (~ 60%) in blocking the stimulatory effect of LH + FSH on progesterone secretion, it was totally ineffective in blocking the stimulatory effects of PGE<sub>2</sub>. These studies provide some clues as to the site of action of PGF<sub>2α</sub>. Both LH (Channing, 1975) and PGE<sub>2</sub> (Rao, 1973) have specific receptors on the membrane, and their steroidogenic response is mediated by activation of the adenylate cyclase system (Savard, Marsh & Rice, 1965; Dorrington & Kilpatrick, 1967; Robison, Butcher & Sutherland, 1971). This suggests that PGF<sub>2α</sub> acts directly on the adenylate cyclase system by blocking its activation by LH. Similar data have been obtained *in vitro* using porcine and bovine granulosa cells (K. M. Henderson & K. P. McNatty, unpublished) suggesting that the interaction of PGF<sub>2α</sub> with this cell type is species-independent. These studies,

however, may not be consistent with the hypothesis proposed by Kuehl (1974), since in his model  $\text{PGE}_2$  is unlikely to overcome the inhibitory effect of  $\text{PGF}_{2\alpha}$ . Such differences as exist between Kuehl's hypothesis and the present study may depend on the rates at which both  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  act on the biochemical pathway to progesterone synthesis. Since continued high levels of LH and FSH protect the granulosa cells against subsequent inhibition by  $\text{PGF}_{2\alpha}$  it seems likely that the inhibitory effect of  $\text{PGF}_{2\alpha}$  is inversely related to the amount of gonadotrophin bound to its receptor;  $\text{PGF}_{2\alpha}$  was 200 times less effective when granulosa cells had been exposed to high concentrations of LH + FSH for 6 days before its addition. These findings are in agreement with those of Hichens *et al.* (1974), who showed that  $\text{PGF}_{2\alpha}$  decreased the binding capacity of luteal tissue to HCG.

In conclusion these data suggest that functional luteal regression of the human corpus luteum could occur by a biochemical mechanism independent of the vascular system. However, the lack of cell death *in vitro* after exposure to  $\text{PGF}_{2\alpha}$  suggests that other mechanisms in addition to the inhibition of steroidogenesis may be necessary to bring about complete structural and functional regression of the gland.

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**Author:** K.M. Henderson

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INFLUENCE OF 16-ARYLOXYPROSTAGLANDINS ON THE  
PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA  
CELLS *IN VITRO*

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SUMMARY

16-Aryloxy analogues of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) are potent luteolysins in laboratory and farm animals. When their effect on progesterone production by luteinized human granulosa cells in tissue culture was investigated inhibition of both basal and gonadotrophin-stimulated progesterone production was observed, so revealing characteristics expected of potential human luteolysins. The analogues were, however, unable to inhibit progesterone production stimulated by  $PGE_2$ , suggesting that like  $PGF_{2\alpha}$  these compounds may act by specifically blocking LH-activated adenylate cyclase.

The 16-aryloxyprostaglandins similarly inhibited progesterone production by porcine granulosa cells, so that the effects observed with the 16-aryloxyprostaglandins *in vitro* may be indicative of their potential *in vivo*.

INTRODUCTION

Although prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) is luteolytic in many laboratory and domestic animals (Weeks, 1972; Inskeep, 1973), attempts to demonstrate that it is luteolytic in women have so far met with little success (Coudert, Winter & Faiman, 1974; Pharriss & Shaw, 1974). Recently, we have shown that  $PGF_{2\alpha}$  will inhibit the production of progesterone by luteinized human granulosa cells cultured *in vitro* (Henderson & McNatty, 1975; McNatty, Henderson & Sawers, 1975), and it is possible, therefore, that the relative ineffectiveness of  $PGF_{2\alpha}$  *in vivo* in women may be due to difficulty in delivering adequate amounts to the ovary via the arterial supply, since  $PGF_{2\alpha}$  is rapidly metabolized by the lungs and liver (Piper, Vane & Wyllie, 1970). However, even if  $PGF_{2\alpha}$  should ultimately prove to be luteolytic in women, its use as a contraceptive would be limited by unacceptable side-effects such as the diarrhoea, vomiting and nausea arising from its concomitant action on smooth muscle.

Some 16-aryloxy analogues of  $PGF_{2\alpha}$ , namely ICI 79,939, ICI 80,996 and ICI 81,008, have been shown to be very much more potent luteolysins than  $PGF_{2\alpha}$  without having a correspondingly increased activity on smooth muscle (Binder, Bowler, Brown, Crossley, Hutton, Senior, Slater, Wilkinson & Wright, 1974). Consequently, low doses will induce luteolysis in laboratory and domestic animals without side-effects (Dukes, Russell & Walpole, 1974). However, it is not known whether the increased potency of these compounds is due to the fact that they are metabolized more slowly than the parent substance or whether they are intrinsically more luteolytic.

The purpose of the present study was to investigate the luteolytic properties of three 16-aryloxy derivatives upon human granulosa luteal cells in tissue culture.

## MATERIALS AND METHODS

*Gonadotrophins*

Ovine luteinizing hormone (NIH-LH-S12) and ovine follicle-stimulating hormone (NIH-FSH-S4) were supplied by the National Institutes of Health, Bethesda, Maryland, U.S.A. The gonadotrophins were diluted to 500 ng/ml with culture medium, and stored in ampoules at  $-20^{\circ}\text{C}$ . Samples of each (0.1 ml) were added to the cell cultures.

*Prostaglandins*

16-Aryloxyprostaglandins, ICI 80,996, ICI 81,008 and ICI 79,939, were obtained from ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire. Each was dissolved in phosphate-buffered saline (Flow Laboratories, Irvine, Scotland) and stored at  $4^{\circ}\text{C}$  until added to the cell cultures in 0.1 ml aliquots.

Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ), obtained from the Upjohn Company, Kalamazoo, U.S.A., was dissolved in 100% ethanol and stored at  $4^{\circ}\text{C}$  until added to the cell cultures in 10  $\mu\text{l}$  aliquots. Control cultures received appropriate volumes of either phosphate-buffered saline and/or 100% ethanol.

*Culture of granulosa cells*

Porcine ovaries were obtained from pigs within 1 h of slaughter at a local abattoir. Human ovaries were obtained from patients undergoing ovariectomy for various gynaecological disorders.

The techniques for obtaining dispersed granulosa cell suspensions and the method of fissure culture of the porcine and human granulosa cells were as previously described (McNatty & Sawers, 1975). Briefly, a minimum of  $5 \times 10^4$  granulosa cells harvested from small antral follicles were cultured in 1 ml culture medium, consisting of 20% calf serum and 80% Medium 199 containing HEPES buffer (20 mmol/l) and supplemented with gentamicin (50  $\mu\text{g}/\text{ml}$ ), amphotericin-B (2.5  $\mu\text{g}/\text{ml}$ ) and 1% glutamine (Flow Laboratories, Irvine, Scotland). Culture medium was replaced daily and stored at  $-20^{\circ}\text{C}$  until assayed for progesterone by radioimmunoassay (Neal, Baker, McNatty & Scaramuzzi, 1975). At the end of the culture period the coverslips were removed, washed extensively with Medium 199, stained with haematoxylin/eosin and the cells remaining counted.

Small antral follicles were chosen since it has previously been demonstrated that granulosa cells harvested from these follicles secrete relatively low amounts of progesterone (McNatty & Sawers, 1975). However, steroidogenesis can be readily stimulated by the daily addition of gonadotrophins to the culture medium.

The endogenous level of  $\text{PGF}_{2\alpha}$  in the culture medium was 36  $\text{pg}/\text{ml}$ , and of  $\text{PGE}_2$  < 200  $\text{pg}/\text{ml}$  (as determined by radioimmunoassay and g.l.c.-mass spectrometry).

## RESULTS

Table 1 shows the effect of three 16-aryloxyprostaglandins on the total production of progesterone by human granulosa-luteal cells cultured *in vitro*. All three analogues markedly inhibited the basal secretion of progesterone. This effect would appear to be a direct biochemical inhibition of steroidogenesis since the analogues did not affect the number of cells remaining at the end of the culture period, relative to the control cultures, nor was there any observable morphological difference between the two groups on examination by light microscopy.

The limited number of human granulosa cells available at any one time makes it difficult to study the effects on steroidogenesis of differing amounts of analogue. However, large

numbers of viable porcine granulosa cells can be easily obtained. Utilizing these cells, it was found that as little as 50 pg/ml of analogue was as effective as 50 ng/ml (Table 2) a result very similar to that obtained with PGF<sub>2α</sub> (Henderson & McNatty, 1977).

Figure 1 shows the effect of ICI 80,996 on progesterone production by human granulosa cells simultaneously treated with either gonadotrophins or PGE<sub>2</sub>. Although the analogue effectively inhibited progesterone production stimulated by gonadotrophins ( $P < 0.01$ ), it had no effect on steroidogenesis stimulated by PGE<sub>2</sub>, as also observed with PGF<sub>2α</sub> (Henderson & McNatty, 1975). Similar results were obtained with the other analogues, suggesting that, like PGF<sub>2α</sub>, these compounds may act by specifically inhibiting adenylate cyclase activation by LH.

Table 1. *The effect of 16-aryloxyprostaglandins on the total production of progesterone by human granulosa cells cultured for 8 days in vitro (means ± S.E.M.)*

Treatment	Progesterone ( $\mu\text{g}/10^6$ cells)
Control	9.2 ± 2.3 (3)
ICI 79,939 (50 ng/ml)	1.6 ± 0.5 (3)
Control	3.7 ± 0.8 (4)
ICI 80,996 (50 ng/ml)	0.4 ± 0.1 (4)
Control	4.4 ± 0.1 (3)
ICI 81,008 (50 ng/ml)	1.1 ± 0.1 (4)

The numbers in parentheses refer to the number of replicate cultures. Prostaglandin-treated cultures produced significantly less progesterone than control cultures ( $P < 0.01$ ).

Table 2. *The effect of 16-aryloxyprostaglandins on the total production of progesterone by porcine granulosa cells cultured for 8 days in vitro (ng progesterone/ $10^6$  cells; means ± S.E.M.)*

Control (6)	ICI 80,996		ICI 79,939		ICI 81,008	
	50 pg/ml (6)	50 ng/ml (6)	50 pg/ml (6)	50 ng/ml (6)	50 pg/ml (6)	50 ng/ml (6)
1340 ± 280	157 ± 42	133 ± 27	120 ± 35	85 ± 12	92 ± 10	108 ± 17

The numbers in parentheses refer to the number of replicate cultures. Prostaglandin-treated cultures produced significantly less progesterone than the control cultures ( $P < 0.01$ ).

#### DISCUSSION

These results demonstrate that analogous with PGF<sub>2α</sub>, 16-aryloxyprostaglandins inhibit both basal and gonadotrophin-stimulated progesterone production by human granulosa cells in tissue culture, thus displaying in-vitro characteristics expected of potential human luteolysins.

The possible pharmacological significance of these findings is strengthened by the ability of the 16-aryloxy analogues to inhibit steroidogenesis by porcine granulosa cells; these drugs are luteolytic in the pig (Ash & Heap, 1973). Although there are differences in the control of corpus luteum function between pigs and women, it is likely that the biochemistry of the progesterone-secreting cells is similar. Therefore, the ability of 16-aryloxyprostaglandins to inhibit progesterone production by human granulosa cells *in vitro* is perhaps a good indication of their potential *in vivo*, providing that sufficient analogue can reach and interact with the receptor for PGF<sub>2α</sub> located on the corpus luteum (Powell, Hammarström, Samuelsson & Sjöberg, 1974). Since 16-aryloxyprostaglandins are very much more potent luteolysins than PGF<sub>2α</sub>, without being correspondingly more toxic, it may be possible to deliver to

the corpus luteum an amount of analogue sufficient to initiate luteolysis, but without producing the undesirable side-effects associated with infusion of  $\text{PGF}_{2\alpha}$ .

Recently, preliminary studies by Csapo & Mocsary (1976) have shown that intra-uterine administration of ICI 81,008 will effectively induce menstruation in women during early pregnancy. However, like  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , this effect depends on the vasoconstrictive properties of ICI 81,008 inducing uterine contracture, and not on its luteolytic properties. In view of our in-vitro findings, further detailed clinical studies to assess the potential of 16-aryloxyprostaglandins to act as 'menstrual inducers' by interacting directly with the corpus luteum to inhibit progesterone production seem worthwhile.

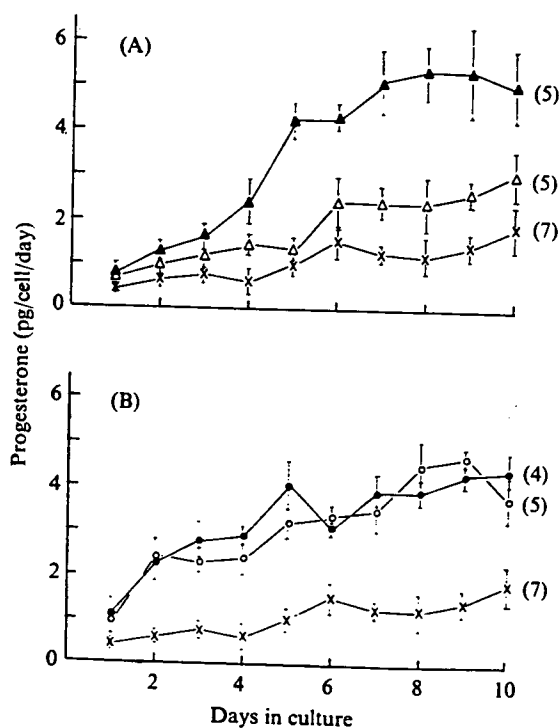


Fig. 1. The effect of ICI 80,996 (100 ng) on the production of progesterone by human granulosa cells simultaneously treated with either gonadotrophins (A) or with  $\text{PGE}_2$  (B). Numbers in parentheses refer to the number of replicate cultures. Gonadotrophin-treated cultures were exposed to 50 ng ovine LH (NIH-LH-S12) and 50 ng ovine FSH (NIH-FSH-S4). Vertical lines represent  $\pm$  S.E.M. x, control; ▲, LH+FSH; △, LH+FSH+ICI 80,996; ●,  $\text{PGE}_2$  (50 ng); ○,  $\text{PGE}_2$  (50 ng)+ICI 80,996.

Generous amounts of the 16-aryloxyprostaglandins ICI 80,996, ICI 79,939 and ICI 81,008 were kindly supplied by Dr A. L. Walpole and his colleagues of ICI Pharmaceuticals Division. K. M. Henderson is in receipt of an M.R.C. Research Studentship.

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Paper no.: 4

Authors: K.M. Henderson, R.J. Scaramuzzi & D.T. Baird

Title: Simultaneous infusion of prostaglandin E<sub>2</sub>  
antagonizes the luteolytic action of prostaglandin F<sub>2α</sub>  
*in vivo*.

Reference: Journal of Endocrinology (1977) 72, 379-383

SIMULTANEOUS INFUSION OF PROSTAGLANDIN E<sub>2</sub>  
ANTAGONIZES THE LUTEOLYTIC ACTION OF  
PROSTAGLANDIN F<sub>2α</sub> *IN VIVO*

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(Received 5 July 1976)

SUMMARY

Corpora lutea of ewes bearing ovarian autotransplants were infused for 4 h with prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) (10 μg/h), PGF<sub>2α</sub> + PGE<sub>2</sub> (10 μg/h of each), PGE<sub>2</sub> (10 μg/h) or saline on day 10 of the cycle. Ovarian venous blood obtained before, during, and up to 12 h after the infusion period, was assayed for progesterone. Prostaglandin F<sub>2α</sub> produced an immediate, rapid and sustained decline in progesterone secretion, but infusion of PGE<sub>2</sub> together with PGF<sub>2α</sub> prevented the decline until after the infusion. Progesterone secretion was unaffected by infusion of PGE<sub>2</sub> alone. Oestrous behaviour was observed in four out of seven animals infused with PGF<sub>2α</sub> but in only one out of six infused with PGF<sub>2α</sub> + PGE<sub>2</sub>. None of the animals infused with PGE<sub>2</sub> alone or saline only came into heat.

INTRODUCTION

Although prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) is luteolytic in many laboratory and domestic animals (Weeks, 1972; Inskip, 1973) its mechanism of action remains obscure. Several studies have suggested that PGF<sub>2α</sub> may initiate luteolysis through a biochemical action on the luteal cell. Prostaglandin F<sub>2α</sub> has been shown to inhibit progesterone production by ovarian tissue *in vitro* (Demers, Behrman & Greep, 1972; O'Grady, Kohorn, Glass, Caldwell, Brock & Speroff, 1972) and the onset of luteal regression is associated with biochemical changes in the luteal cell, namely a decline in adenylyl cyclase activity and loss of responsiveness to luteinizing hormone (LH) (Andersen, Schwartz & Ulberg, 1974; Hichens, Greenwich & Behrman, 1974; Greenwich, Hichens & Behrman, 1976). Previous results from our laboratory also supported this concept, and provided a clue to the possible site of action of PGF<sub>2α</sub>. Utilizing granulosa cells in tissue culture we found that although PGF<sub>2α</sub> could inhibit the stimulatory effect of LH on progesterone production, it did not inhibit the stimulatory effect of PGE<sub>2</sub> (Henderson & McNatty, 1975; McNatty, Henderson & Sawers, 1975). There is substantial evidence that both LH and PGE<sub>2</sub> interact with respective specific membrane receptors (Rao, 1973; Han, Rajaniemi, Cho, Hirshfield & Midgley, 1974) to stimulate steroidogenesis through activation of the adenylyl cyclase enzyme system (Marsh, 1970*a, b*, 1971, 1976), and so it seemed likely that PGF<sub>2α</sub> inhibits steroidogenesis by specifically inhibiting LH activation of adenylyl cyclase. More recent studies have shown directly that PGF<sub>2α</sub> can indeed inhibit LH stimulation of cyclic AMP production *in vitro* (Lahav, Freud & Lindner, 1976).

If PGF<sub>2α</sub> initiates luteolysis *in vivo* by specifically interfering with the coupling of LH and

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adenyl cyclase, the action of  $\text{PGF}_{2\alpha}$  should be overridden by simultaneous administration of  $\text{PGE}_2$ . This possibility has been tested in the present work.

## MATERIALS AND METHODS

### *Experimental animals*

Eleven ewes with the left ovary autotransplanted to a carotid-jugular skin loop as described by Goding, McCracken & Baird (1967) were used. In this preparation the corpus luteum persists due to physical separation of the ovary from the uterus, but as it was necessary to study corpora lutea of the same age, the persisting corpora lutea were caused to regress, and the ovarian cycles of the animals synchronized, by administering a single intramuscular injection of ICI 80,996 (50  $\mu\text{g}$ ), a potent analogue of  $\text{PGF}_{2\alpha}$ . This induced oestrous behaviour within 2 or 3 days; the infusion experiments being carried out 10 days after the onset of oestrus.

### *Infusion of the ovary*

The procedures associated with cannulation of the ovary, infusion through the ovarian artery, and the timed collection of ovarian venous blood samples have been described previously (McCracken, Uno, Goding, Ichikawa & Baird, 1969).

Before the infusion of prostaglandins, an infusion of 0.9% physiological saline (10 ml/h) was maintained during which time two 'control' samples of ovarian venous blood were obtained 30 and 90 min after commencement of infusion. Following this control period the ovary was infused for 4 h with  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha} + \text{PGE}_2$  or saline only; 10  $\mu\text{g}$  prostaglandin in 10 ml saline being delivered at a constant rate each hour. Timed collections of ovarian venous blood were made at hourly intervals for 6 h commencing 30 min after the start of the test period. A final sample was taken next day, approximately 18 h after the start of the infusion. At the end of the experiment the animals were run with a raddled, vasectomized ram for 4 days, and they were inspected twice daily for signs of mating (Radford, Watson & Wood, 1960).

### *Progesterone assay*

The concentration of progesterone in ovarian venous plasma was measured by radioimmunoassay (Scaramuzzi, Corker, Young & Baird, 1975). The secretion of progesterone was calculated from the plasma concentration, the haematocrit and the blood flow.

In 32 duplicate determinations performed in the same assay, the coefficient of variation, calculated by the method of Snedecor (1956), was 13%. In 15 duplicate determinations in two different assays, the coefficient of variation was 18%.

## RESULTS

### *Effect of prostaglandin infusions on progesterone secretion*

The results shown in Fig. 1 are expressed as a percentage of the mean progesterone secretion rate obtained from the two control ovarian venous blood samples. The overall rates of progesterone secretion during the control infusion period for each of the experimental groups were not significantly different from each other ( $P > 0.05$ ), the values being: saline,  $6.8 \pm 1.3$  (S.E.M.)  $\mu\text{g}/\text{min}$ ;  $\text{PGF}_{2\alpha}$ ,  $8.4 \pm 0.8$ ;  $\text{PGE}_2$ ,  $9.2 \pm 1.3$ ;  $\text{PGF}_{2\alpha} + \text{PGE}_2$ ,  $8.0 \pm 1.1$ .

Infusion of  $\text{PGF}_{2\alpha}$  alone produced an immediate, rapid and sustained fall in progesterone secretion while infusion of  $\text{PGE}_2$  alone had no significant effect. When  $\text{PGF}_{2\alpha}$  was infused together with  $\text{PGE}_2$ , the secretion of progesterone remained unaltered until the infusion period had finished but declined rapidly thereafter. By 18 h there was no significant difference in the rate of progesterone secretion resulting from either  $\text{PGF}_{2\alpha}$  or  $\text{PGF}_{2\alpha} + \text{PGE}_2$  infusion ( $P > 0.05$ ).

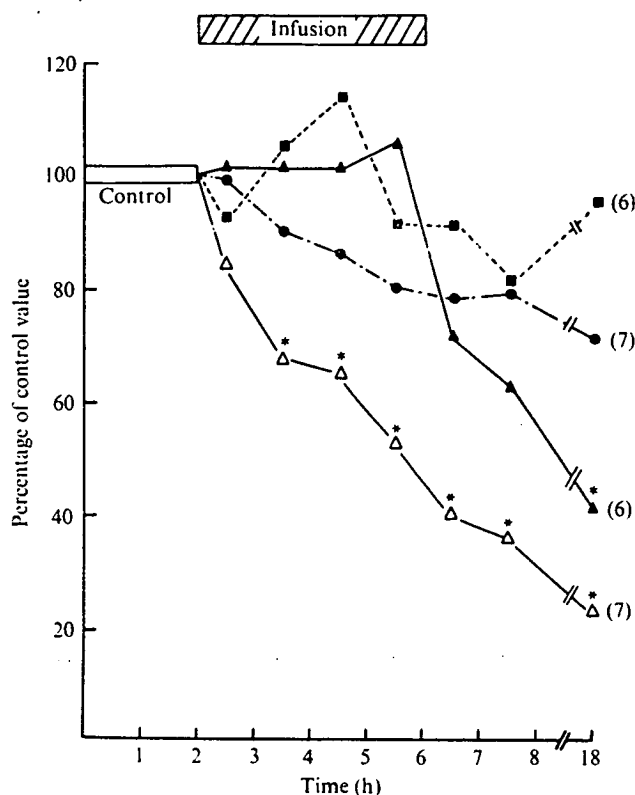


Fig. 1. Mean progesterone secretion rate during and after infusion of prostaglandins (PG) in ewes with ovarian autotransplants. The results are expressed as a percentage of the values obtained during the control period (see text). Numbers in parentheses refer to the number of animals in each group. \*  $P < 0.05$ : significantly different from the corresponding points of the control animals. ■, Saline; Δ, PGF<sub>2α</sub> (10 μg/h); ●, PGE<sub>2</sub> (10 μg/h); ▲, PGF<sub>2α</sub> + PGE<sub>2</sub> (10 μg/h of each).

Ovarian venous blood flow was significantly increased by  $13.5 \pm 1.8\%$  ( $P < 0.05$ ) during infusion of PGE<sub>2</sub> alone, but fell to control levels at the end of infusion. Infusion of PGF<sub>2α</sub> or PGF<sub>2α</sub> + PGE<sub>2</sub> did not alter blood flow ( $P > 0.05$ ).

#### Effect of prostaglandin infusions on oestrous behaviour

A higher proportion of animals showed oestrous behaviour following infusion of PGF<sub>2α</sub> alone (four out of seven) than of those animals infused with PGF<sub>2α</sub> + PGE<sub>2</sub> (one out of six). None of the animals infused with PGE<sub>2</sub> alone (7) or saline only (6) was marked by the ram.

#### DISCUSSION

These results demonstrate that PGE<sub>2</sub> when infused simultaneously with PGF<sub>2α</sub> can antagonize the luteolytic action of PGF<sub>2α</sub>, and are also consistent with the concept that PGF<sub>2α</sub> initiates luteolysis through a direct action on the luteal cell to inhibit specifically LH-activated adenylyl cyclase (Hichens *et al.* 1974; Henderson & McNatty, 1975; Greenwich *et al.* 1976; Lahav *et al.* 1976). However, in view of the observed increase in blood flow during infusion of PGE<sub>2</sub>, it could also be argued that the vasodilator properties of this prostaglandin counteract a vasoconstrictive action of PGF<sub>2α</sub> on the corpus luteum (Thorburn & Hales,

1972; Niswender, Diekmann, Nett & Akbar, 1973; Bruce & Moor, 1975). However, Bruce & Hillier (1974) have demonstrated that  $\text{PGF}_{2\alpha}$ -induced inhibition of progesterone secretion can occur without depression of corpus luteal blood flow, and there is little evidence that anoxic damage causes a reduction in progesterone secretion in the early stages of luteal regression (Umo, 1975; Gemmell, Stacy & Thorburn, 1976).

Inhibition of LH activated adenylyl cyclase by  $\text{PGF}_{2\alpha}$  infusion would cause the pool of LH-induced cyclic AMP to be depleted through the action of phosphodiesterase, so reducing steroid synthesis and depressing progesterone secretion. Infusion of  $\text{PGE}_2$  together with  $\text{PGF}_{2\alpha}$  masks this effect of  $\text{PGF}_{2\alpha}$  through the ability of  $\text{PGE}_2$  to stimulate adenylyl cyclase through separate  $\text{PGE}_2$ -sensitive receptor and coupling units, so sustaining increased levels of cyclic AMP. Consequently the rate of progesterone secretion is unaffected. On terminating the infusion, however, this pool of cyclic AMP would also be diminished by the action of phosphodiesterase, so causing a fall in the rate of progesterone secretion.

Infusion of  $\text{PGE}_2$  alone had no significant effect on the secretion of progesterone. However, it is likely that the corpus luteum on day 10 is already secreting maximally since infusion of massive amounts of LH on day 10 of the cycle fails to stimulate an increased secretion of progesterone (Land, Collett & Baird, 1974). Studies with granulosa cells in tissue culture support this view, for although both  $\text{PGE}_2$  and LH + follicle-stimulating hormone could stimulate progesterone production, cells which had been fully stimulated with gonadotrophins could not be further stimulated with  $\text{PGE}_2$  (McNatty *et al.* 1975).

In conclusion, it seems likely that  $\text{PGF}_{2\alpha}$  may initiate functional regression of the corpus luteum by specifically inhibiting LH activation of adenylyl cyclase, a process which can be overridden by the simultaneous administration of  $\text{PGE}_2$ . The ultimate morphological regression of the corpus luteum may also arise through further cellular processes being triggered by this initial action of  $\text{PGF}_{2\alpha}$ .

We are extremely grateful to Dr J. Pike, Upjohn Company, Kalamazoo, Michigan, U.S.A., for the supply of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ . ICI 80,996 was kindly supplied by Dr M. Dukes, I.C.I. Pharmaceuticals Ltd, Macclesfield, Cheshire. The skilled assistance of W. Davidson is gratefully acknowledged. K. M. Henderson is in receipt of an M.R.C. Research Studentship.

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Paper no.: 5

Authors: K.M. Henderson & K.P. McNatty

Title: A possible interrelationship between gonadotrophin stimulation and prostaglandin F<sub>2α</sub> inhibition of steroidogenesis by granulosa-luteal cells *in vitro*.

Reference: Journal of Endocrinology (1977) 73, 71-78.

A POSSIBLE INTERRELATIONSHIP BETWEEN  
GONADOTROPHIN STIMULATION AND PROSTAGLANDIN  $F_{2\alpha}$   
INHIBITION OF STEROIDOGENESIS BY GRANULOSA-  
LUTEAL CELLS *IN VITRO*

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(Received 22 May 1976)

SUMMARY

The newly formed corpus luteum of many species is refractory to the lytic action of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ). This phenomenon was studied utilizing porcine, bovine and human granulosa-luteal cells in tissue culture. The steroidogenic potential of the granulosa-luteal cells was critical in determining whether  $PGF_{2\alpha}$  could inhibit progesterone production. Since the steroidogenic potential of the granulosa-luteal cell is related to the amount of LH bound to the cell, the bound LH may protect the granulosa-luteal cells from the lytic action of  $PGF_{2\alpha}$ . Finally, a 'see-saw' type of interaction between LH and  $PGF_{2\alpha}$  is postulated to account for the resistance of the newly formed corpus luteum to  $PGF_{2\alpha}$ .

INTRODUCTION

Although prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) induces luteal regression in laboratory and domestic animals (Weeks, 1972; Inskeep, 1973), the newly formed corpus luteum of several species shows considerable resistance to  $PGF_{2\alpha}$ . In the horse (Allen & Rowson, 1973), sheep (Hearnshaw, Restall & Gleeson, 1973) and cow (Rowson, Tervit & Brand, 1972; Henricks, Long, Hill & Dickey, 1974) the corpus luteum is refractory to  $PGF_{2\alpha}$  for the first 4 days after its formation, but in the pig this refractory period is 12 to 14 days (Moeljono, Bazer & Thatcher, 1976).

Granulosa cells, harvested from the Graafian follicle, can be induced to luteinize in tissue culture and so provide a convenient model of the newly formed corpus luteum (Channing & Ledwitz-Rigby, 1974; McNatty & Sawers, 1975). Recently it has been demonstrated that  $PGF_{2\alpha}$  inhibits the production of progesterone by human granulosa-luteal cells in tissue culture (McNatty, Henderson & Sawers, 1975*b*). The purpose of this study was to utilize the tissue culture of porcine, bovine and human granulosa cells in an attempt to gain some insight into the manner by which the newly formed corpus luteum may protect itself from the luteolytic action of  $PGF_{2\alpha}$ .

MATERIALS AND METHODS

*Gonadotrophins*

Ovine luteinizing hormone (NIH-LH-S12) and ovine follicle-stimulating hormone (NIH-FSH-S4) were supplied by the National Institutes of Health, Bethesda, Maryland, U.S.A.

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Human luteinizing hormone (LH-DEAE-I) was kindly supplied by Dr A. Stockell Hartree of the Department of Biochemistry, University of Cambridge. Human follicle-stimulating hormone (FSH-73/519) was supplied by the MRC National Institute for Biological Standards and Control. The concentrations of human LH and FSH were expressed in  $\mu\text{u./ml}$  as described previously (McNatty & Sawers, 1975) (1  $\mu\text{u. LH} \equiv 11.6 \text{ ng LER907}$  and 1  $\mu\text{u. FSH} \equiv 44.6 \text{ ng LER907}$ ).

The gonadotrophins were diluted with culture medium and stored at  $-20^\circ\text{C}$  until added to the cell cultures in 0.1 ml aliquots.

#### *Prostaglandins*

Prostaglandin  $F_{2\alpha}$  was generously supplied by Dr J. Pike (Upjohn Company, Kalamazoo, Michigan, U.S.A.) and [ $9\text{-}^3\text{H}$ ]prostaglandin  $F_{2\alpha}$  (42 mCi/mg) was obtained from the Radiochemical Centre, Amersham. Each was diluted with 100% ethanol and stored at  $4^\circ\text{C}$  until added to the cell cultures in 10  $\mu\text{l}$  aliquots. Control cultures received 10  $\mu\text{l}$  100% ethanol only.

#### *Radioimmunoassay of progesterone*

The progesterone content of the culture medium was measured by radioimmunoassay using an antiserum supplied by Dr K. K. Dighe of the Department of Pharmacology, University of Edinburgh. Its specificity was similar to that reported previously (Dighe & Hunter, 1974). The assay technique was identical to that described by Neal, Baker, McNatty & Scaramuzzi (1975). In 28 duplicate determinations performed in the same assay, the coefficient of variation calculated by the method of Snedecor (1956) was 7.8%. In 20 duplicate determinations in two different assays the coefficient of variation was 12.4%.

#### *Culture of granulosa cells*

Porcine and bovine ovaries were obtained from pigs and cattle within 1 h of their slaughter at a local abattoir. Human ovaries were obtained from female patients who were undergoing ovariectomy for various gynaecological disorders.

The techniques for obtaining dispersed granulosa cell suspensions and the method of tissue culture were as previously described (McNatty & Sawers, 1975). Briefly, aliquots of granulosa cells harvested from antral follicles were cultured on 18  $\text{mm}^2$  cover-slips in 1 ml culture medium consisting of 20% calf serum and 80% Medium 199 containing HEPES buffer (20 mmol/l) and supplemented with 1% glutamine, amphotericin B (2.5  $\mu\text{g/ml}$ ) and gentamicin (50  $\mu\text{g/ml}$ ). The culture medium was replaced daily and stored at  $-20^\circ\text{C}$  until assayed for progesterone by radioimmunoassay. At the end of the culture period, the cover-slips were removed, washed extensively with Medium 199, stained with haematoxylin/eosin and the cells remaining were counted by the technique of McNatty & Sawers (1975). The endogenous level of  $\text{PGF}_{2\alpha}$  in the culture medium was 36  $\text{pg/ml}$  (as determined by radioimmunoassay).

Standardized conditions were used throughout all the culture experiments to ensure that the only differences between cultures were the cell types involved.

#### *Uptake of [ $^3\text{H}$ ]PGF $_{2\alpha}$*

Human and bovine granulosa cells were cultured in the presence of human LH and FSH (36  $\mu\text{u./ml}$  and 24  $\mu\text{u./ml}$  respectively) for 2–10 days. Thus, by varying the length of time in culture it was possible to obtain granulosa-luteal cells which were secreting varying amounts of progesterone ranging from  $< 0.5\text{--}7 \text{ pg/cell/day}$ . At the end of each culture period the cover-slips were thoroughly washed with Medium 199 and incubated for 1 h at  $37^\circ\text{C}$  in 1 ml Medium 199 containing either 5 ng [ $^3\text{H}$ ]PGF $_{2\alpha}$  ( $25 \times 10^4 \text{ c.p.m.}$ ) or 1 ng

[<sup>3</sup>H]PGF<sub>2α</sub> (5 × 10<sup>4</sup> c.p.m.). The medium was then decanted into a counting vial and the cells were washed twice with Medium 199, each washing being transferred separately into counting vials. Counting was carried out in a Packard liquid scintillation spectrometer. The cells were then re-incubated for 1 h in the same manner but with 10 μg unlabelled PGF<sub>2α</sub>. Since this procedure did not cause a significant release of radioactive PGF<sub>2α</sub> the percentage uptake of [<sup>3</sup>H]PGF<sub>2α</sub> was expressed as follows:

$$\% \text{ uptake} = \frac{A-P}{A} \times 100,$$

where *A* is the total number of counts remaining after incubating [<sup>3</sup>H]PGF<sub>2α</sub> for 1 h in the absence of granulosa cells and *P* is the total number of counts remaining after incubating [<sup>3</sup>H]PGF<sub>2α</sub> for 1 h in the presence of granulosa cells.

The cover-slips were then stained and the cells remaining counted. From a knowledge of the cell number and specific activity of the [<sup>3</sup>H]PGF<sub>2α</sub> the amount of [<sup>3</sup>H]PGF<sub>2α</sub> retained by the cells was expressed finally as ng [<sup>3</sup>H]PGF<sub>2α</sub>/10<sup>6</sup> cells.

## RESULTS

### Experiment 1

#### *Effect of PGF<sub>2α</sub> on progesterone production by porcine granulosa-luteal cells*

Porcine granulosa-luteal cells when cultured in medium containing 20% calf serum secreted approximately 0.2 pg progesterone/cell/day. Steroidogenesis could be stimulated by the daily addition of ovine LH and ovine FSH, each to a final concentration of 100 ng/ml. Progesterone production increased to a maximum of 2.5 pg/cell/day within 8 days of culture.

Table 1. *Effect of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) on the total production of progesterone by porcine granulosa-luteal cells cultured for 9 days (means ± S.E.M.)*

PGF<sub>2α</sub> was added daily from the start of the culture period. Gonadotrophin-treated cultures were exposed to 100 ng LH (NIH-LH-S12) + 100 ng FSH (NIH-FSH-S4). Numbers in parentheses represent the number of replicate cultures. Levels of significance between control and treated cultures were calculated using Student's *t*-test.

Treatment	Production of progesterone (pg/cell)
Control	1.9 ± 0.5 (4)
50 pg PGF <sub>2α</sub> <sup>1</sup>	0.7 ± 0.2 (4) <i>P</i> < 0.02
500 pg PGF <sub>2α</sub>	0.8 ± 0.1 (4) <i>P</i> < 0.02
50 ng PGF <sub>2α</sub> <sup>2</sup>	0.6 ± 0.1 (4) <i>P</i> < 0.02
500 ng PGF <sub>2α</sub>	0.9 ± 0.3 (4) <i>P</i> < 0.02
LH + FSH	10 ± 1.5 (4)
LH + FSH + 50 pg PGF <sub>2α</sub>	2.2 ± 0.9 (3) <i>P</i> < 0.01
LH + FSH + 500 pg PGF <sub>2α</sub>	3.7 ± 1.7 (3) <i>P</i> < 0.02
LH + FSH + 10 ng PGF <sub>2α</sub>	5.7 ± 2.0 (3) <i>P</i> < 0.05
LH + FSH + 100 ng PGF <sub>2α</sub>	4.0 ± 0.6 (3) <i>P</i> < 0.05

The effects of various concentrations of PGF<sub>2α</sub>, added daily from the start of the culture period, on the total production of progesterone by porcine granulosa-luteal cells cultured with or without added gonadotrophins are shown in Table 1. As little as 50 pg PGF<sub>2α</sub> significantly inhibited progesterone secretion in the presence or absence of added gonadotrophins. Furthermore, from Fig. 1, it can be seen that when the addition of PGF<sub>2α</sub> to porcine cells treated with LH and FSH was delayed until day 6, 50 pg PGF<sub>2α</sub> still inhibited steroidogenesis.



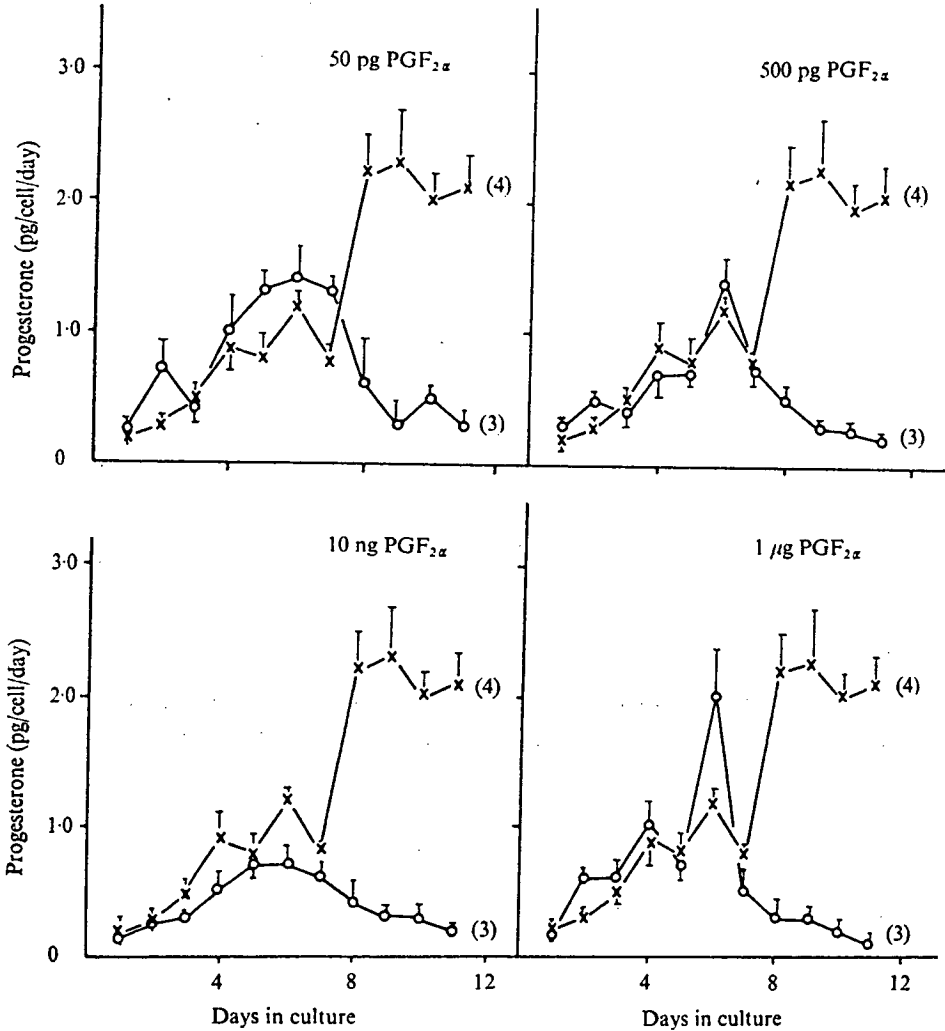


Fig. 1. Effect of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) on the daily production of progesterone by porcine granulosa-luteal cells stimulated for 6 days with ovine gonadotrophins (100 ng NIH-LH-S12 + 100 ng NIH-FSH-S4) before addition of  $PGF_{2\alpha}$ . Numbers in parentheses represent the number of replicate cultures and vertical lines represent +S.E.M. All  $PGF_{2\alpha}$ -treated cultures produced significantly less progesterone from day 8 onwards than those treated with gonadotrophins alone ( $P < 0.02$ , paired *t*-test). Crosses, LH + FSH; open circles,  $PGF_{2\alpha}$  added together with LH + FSH from day 6.

### Experiment II

#### *Effect of $PGF_{2\alpha}$ on progesterone production by bovine granulosa-luteal cells*

The daily production of progesterone by bovine granulosa-luteal cells cultured in medium containing 20% calf serum was very much greater than that of porcine cells; progesterone production gradually increased from approximately 1 pg/cell/day to a maximum of 6–7 pg/cell/day within 7 days of culture. The bovine granulosa-luteal cells were probably responding maximally to endogenous gonadotrophin in the calf serum since, unlike porcine cells, progesterone production by bovine cells could not be further increased by the daily addition of ovine LH and ovine FSH, each to a final concentration of 100 ng/ml.

Table 2 shows that the daily addition of PGF<sub>2α</sub> from the start of the culture period at concentrations of  $\geq 500$  pg/ml significantly inhibited progesterone production by bovine granulosa-luteal cells. However, although PGF<sub>2α</sub> could inhibit steroidogenesis if added from the start of the culture period, when progesterone production was low ( $< 2$  pg/cell/day), it was not possible to inhibit progesterone production with any amount of PGF<sub>2α</sub> (up to  $5 \mu\text{g/ml}$  tested) if the cells were cultured for 6–8 days, so that they were secreting maximally (6–7 pg progesterone/cell/day) before the addition of PGF<sub>2α</sub>. This result matches that previously obtained with human granulosa-luteal cells (McNatty *et al.* 1975*b*), where it was found that although PGF<sub>2α</sub> readily inhibited progesterone production by cells producing low amounts of progesterone (1–2 pg/cell/day), those cells producing larger amounts of progesterone ( $> 4$  pg/cell/day) were much less susceptible to the lytic action of PGF<sub>2α</sub>.

Table 2. Effect of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) on the total production of progesterone by bovine granulosa-luteal cells cultured for 9 days (means  $\pm$  S.E.M.)

PGF<sub>2α</sub> was added daily from the start of the culture period. Numbers in parentheses represent the number of replicate cultures. Levels of significance between the control and treated cultures were calculated using Student's *t*-test.

Treatment	Production of progesterone (pg/cell)
Control	30.2 $\pm$ 2.6 (3)
50 pg PGF <sub>2α</sub>	21.6 $\pm$ 6.8 (3) <i>P</i> $>$ 0.05
500 pg PGF <sub>2α</sub>	15.8 $\pm$ 5.0 (3) <i>P</i> $<$ 0.05
50 ng PGF <sub>2α</sub>	10.4 $\pm$ 1.3 (3) <i>P</i> $<$ 0.01
500 ng PGF <sub>2α</sub>	14.6 $\pm$ 7.1 (3) <i>P</i> $<$ 0.05

### Experiment III Uptake of [<sup>3</sup>H]PGF<sub>2α</sub>

Taking these porcine and bovine data together with our previous human data, it would seem that while those cells producing only low amounts of progesterone ( $< 2$  pg/cell/day) are readily susceptible to the lytic action of PGF<sub>2α</sub>, as the 'steroidogenic potential' of the cells increase, they become increasingly refractory to PGF<sub>2α</sub>. To determine whether the ability of granulosa-luteal cells to bind PGF<sub>2α</sub> might be inversely related to their secretory activity, uptake studies using [<sup>3</sup>H]PGF<sub>2α</sub> were carried out on bovine and human granulosa-luteal cells producing varying amounts of progesterone ( $< 0.5$ –7 pg/cell/day).

The relationship between PGF<sub>2α</sub> uptake and steroidogenesis is shown in Fig. 2. As the progesterone production increased, so the ability of the cells to retain [<sup>3</sup>H]PGF<sub>2α</sub> decreased.

### DISCUSSION

The critical factor in determining whether exogenous PGF<sub>2α</sub> induces luteolysis in the cow and pig is the age of the corpus luteum. The data in Experiments I and II utilizing porcine and bovine granulosa-luteal cells suggest that *in vitro* the most critical factor in determining the luteolytic response to PGF<sub>2α</sub> is the steroidogenic potential of the cell. Cells producing only low amounts of progesterone ( $< 2$  pg/cell/day) are readily susceptible to the lytic action of PGF<sub>2α</sub>, but as the cellular capacity to produce progesterone rises, the cell becomes increasingly refractory to PGF<sub>2α</sub>. The data previously obtained with human granulosa-luteal cells are also consistent with this concept (McNatty *et al.* 1975*b*). In Experiment III, it was found that the ability of [<sup>3</sup>H]PGF<sub>2α</sub> to bind to granulosa-luteal cells was inversely related to their steroidogenic potential. Thus as the steroidogenic potential of the cell rises, a decreased ability to bind PGF<sub>2α</sub> renders it refractory to the lytic action of PGF<sub>2α</sub>.

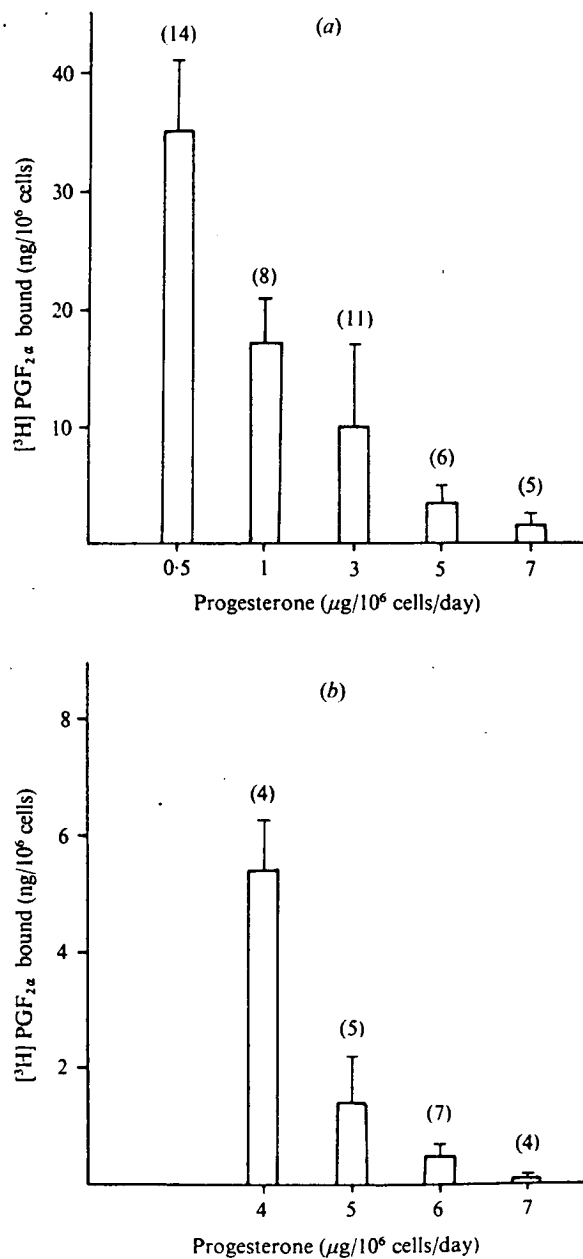


Fig. 2. Uptake of [<sup>3</sup>H]PGF<sub>2α</sub> by human (a) and bovine (b) granulosa-luteal cells of differing steroidogenic potential. The number of uptake studies is given in parentheses. The vertical lines represent +S.E.M.

The daily production of progesterone by granulosa-luteal cells *in vitro* is related to the cellular response to gonadotrophin present in the culture medium (Channing & Ledwitz-Rigby, 1974; McNatty & Sawers, 1975). In the absence of gonadotrophin, granulosa cells secrete minimal amounts of progesterone (McNatty, Bennie, Hunter & McNeilly, 1975*a*), whilst the addition of gonadotrophin stimulates production of progesterone, FSH acting to stimulate LH binding to the granulosa cells (Channing, 1975), while LH, on binding to its specific plasma membrane receptors (Han, Rajaniemi, Cho, Hirshfield & Midgley, 1974), stimulates steroidogenesis by activating adenylate cyclase (Marsh, 1970). Those granulosa-luteal cells secreting maximum amounts of progesterone will have more LH bound to the plasma membrane than those cells producing minimal amounts of progesterone. Since cells producing high levels of progesterone retain only low amounts of PGF<sub>2α</sub>, it is possible that receptor-bound LH antagonizes the uptake of PGF<sub>2α</sub>. This interpretation is consistent with the *in-vivo* results of Behrman, Yoshinaga & Greep (1971), who found that LH antagonizes the luteolytic action of PGF<sub>2α</sub> in the rat. Furthermore, the receptor for PGF<sub>2α</sub>, like the receptor for LH, is located in the plasma membrane (Powell, Hammarstrom, Samuelsson & Sjöberg, 1974; Powell, Hammarstrom & Samuelsson, 1975) so allowing for the possible existence of some interaction between the two.

The binding of human chorionic gonadotrophin to rat corpora lutea is antagonized by PGF<sub>2α</sub> (Hichens, Grinwich & Behrman, 1974). This, in conjunction with our data, suggests that a 'see-saw' type of situation could possibly exist between the receptors for LH and PGF<sub>2α</sub>. The filling of the LH receptors may prevent any subsequent interaction of PGF<sub>2α</sub> with the PGF<sub>2α</sub> receptors while, conversely, occupation of the PGF<sub>2α</sub> receptors may inhibit any subsequent interaction of LH with the LH receptors. This would offer an intrinsic mechanism to account for the resistance of the newly formed corpus luteum to the lytic action of PGF<sub>2α</sub>. Following the ovulatory 'surge' of gonadotrophins, the receptors for LH on the newly formed corpus luteum would be saturated with LH, thus causing the receptors for PGF<sub>2α</sub> to be effectively masked. Consequently, the newly formed corpus luteum would become refractory to PGF<sub>2α</sub>. The binding of LH to its receptors is extremely tight and dissociation of LH from its receptor is a slow process, in the order of several days (Channing & Kammerman, 1973; Lee & Ryan, 1973; Haour & Saxena, 1974; Hsueh, Dufau, Katz & Catt, 1976). Thus, throughout the luteal phase, there will be only a gradual dissociation of LH from its receptor sites; progesterone production by the corpus luteum would be unaffected by this dissociation since only a fraction of the LH-receptor sites need be occupied to ensure maximum stimulation of adenylate cyclase (Koch, Zor, Chobsiang, Lamprecht, Pomerantz & Lindner, 1974). However, gradual vacation of the LH receptors would also result in the gradual unmasking of the receptors for PGF<sub>2α</sub>, so causing the corpus luteum to become increasingly susceptible to the lytic action of PGF<sub>2α</sub>.

The possible mechanism of action of PGF<sub>2α</sub> on interacting with its membrane receptor has been discussed elsewhere (Henderson & McNatty, 1975).

We are indebted to Drs D. T. Baird and R. S. Sawers for their sustained efforts in providing suitable human ovarian tissue. K. M. Henderson is in receipt of an M.R.C. Research Studentship and K. P. McNatty is in receipt of a New Zealand N.R.A.C. Fellowship.

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**Paper no.:** 6

**Authors:** B.K. Tsang, K.M. Henderson & D.T. Armstrong

**Title:** Effect of Danazol on estradiol-17 $\beta$  and progesterone secretion by porcine ovarian cells *in vitro*.

**Reference:** American Journal of Obstetrics and Gynaecology (1979) 133, 256-259

# Effect of danazol on estradiol-17 $\beta$ and progesterone secretion by porcine ovarian cells in vitro

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The effect of danazol on progesterone (P<sub>4</sub>) and estradiol-17 $\beta$  (E<sub>2</sub>) secretion by cultured porcine granulosa cells was studied. Danazol markedly inhibited both basal and luteinizing hormone (LH)-stimulated P<sub>4</sub> secretion by both granulosa and luteal cells in a dose-dependent manner during 24 hour culture periods. E<sub>2</sub> secretion by granulosa cells was low (< 3 ng/mg of protein) when cultured in the absence of an exogenous aromatizable substrate but was markedly increased when testosterone (T) was added to the media. Danazol inhibited E<sub>2</sub> secretion by granulosa cells only when cultured in the presence of testosterone. E<sub>2</sub> secretion by luteal cells was low even in the presence of testosterone and was not affected by danazol. These findings support the hypothesis that danazol can directly inhibit ovarian steroidogenesis independently of its inhibitory action on gonadotropin secretion. (AM. J. OBSTET. GYNECOL. 133:256, 1979.)

DANAZOL (17 $\alpha$ -pregn-4-en-20-yn[2,3-d]isoxazol-17-ol), a synthetic analogue of ethinyl testosterone, is currently used clinically in the management of endometriosis, fibrocystic mastitis, and precocious puberty and as an antifertility agent.<sup>1-8</sup> The mechanism(s) by which danazol exerts its therapeutic effects remains unclear, though it has been suggested that they may be attributable to its inhibitory action on gonadotropin secretion.<sup>1-4, 6</sup> Recent studies, however, have suggested that danazol may also have an action on the gonads to inhibit steroidogenesis.<sup>9, 10</sup> In order to test this latter possibility, granulosa and luteal cells isolated from porcine ovaries were cultured for 24 hours in the absence or presence of danazol. The influence of danazol on the secretion of estradiol-17 $\beta$  (E<sub>2</sub>) and progesterone (P<sub>4</sub>) by these cells in vitro was assessed.

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## Material and methods

**Isolation of granulosa cells.** Ovaries were collected from individual pigs (4 to 6 months of age) within 10 to 12 minutes after they had been killed at a local slaughterhouse and were brought back to the laboratory in ice-cold Eagle's Minimum Essential Medium (MEM) containing the following: penicillin (50 U/ml), streptomycin (50  $\mu$ g/ml), fungizone (0.62  $\mu$ g/ml), and L-glutamine (2 mM) with nonessential amino acids. Medium-size follicles, with diameters of 3.5 to 5 mm, were dissected clean of connective tissue from both ovaries and cut into quarters or eights, depending on size, in a Petri dish containing Hank's balanced salt solution (BSS) (modified, Flow Laboratories) devoid of magnesium and calcium, and supplemented with HEPES buffer (20 mM). These follicular wall preparations were stirred gently over a magnetic stirrer in this medium for 5 minutes to release the granulosa cells, which were then collected with a Pasteur pipet. This stirring process was repeated and all granulosa cells were pooled and washed twice with MEM. Cells were resuspended in fresh culture media containing the appropriate hormone(s) and/or danazol. Follicles from ovaries containing corpora lutea were omitted from the present study.

**Isolation of luteal cells.** Porcine luteal cells were isolated from corpora lutea (approximately 10 mm in diameter) of both ovaries of individual pigs by the

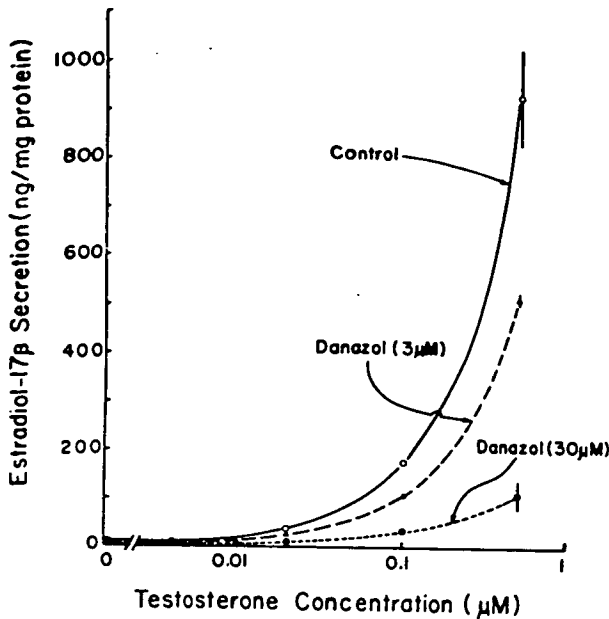


Fig. 1. Effect of danazol on estradiol-17 $\beta$  secretion by granulosa cells in the presence of exogenous testosterone in vitro. Each point represents the mean  $\pm$  SE of replicate incubations.

Table I. Effect of danazol on estradiol-17 $\beta$  secretion by granulosa cells from three individual pigs in the absence of an aromatizable substrate

Treatment	Estradiol secretion (ng/mg protein; mean $\pm$ SE)		
	Pig No. 1 (n = 2)	Pig No. 2 (n = 3)	Pig No. 3 (n = 5)
Control	2.56 $\pm$ 0.33	0.85 $\pm$ 0.05	0.70 $\pm$ 0.04
Danazol (3 $\mu$ M)	2.53 $\pm$ 0.14	1.19 $\pm$ 0.06	—
Danazol (30 $\mu$ M)	2.86 $\pm$ 0.40	—	0.57 $\pm$ 0.05

method previously described by Henderson.<sup>11</sup> Briefly, the corpora lutea were freed of adherent connective tissue and cut into small pieces, about 22 mm in diameter, with fine microdissecting scissors. The pieces were washed twice with the above-mentioned Hank's BSS and stirred over a magnetic stirrer at 37° C for 10 minutes in the same medium containing 0.2% collagenase (Type II, Sigma Chemical Co., St. Louis, Mo.). Cell dispersal was finally achieved at the end of the incubation period by repeatedly drawing any remaining tissue fragments through the tip of a 3 ml syringe. The freed luteal cells were then filtered through sterile gauze, washed three times with Hank's BSS, and finally resuspended in MEM containing the appropriate hormone(s) and/or danazol.

**Cell cultures.** All cells were cultured for 24 hours at 36° C under an atmosphere of 5% CO<sub>2</sub> in air in 1 ml of Eagle's MEM, modified as described previously.<sup>12</sup> At

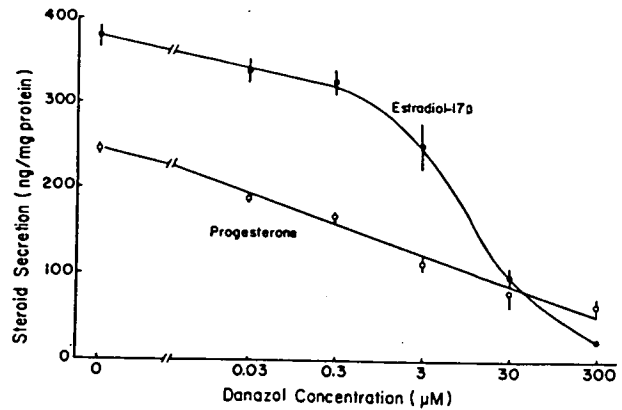


Fig. 2. Influence of danazol on progesterone and estradiol-17 $\beta$  secretion by granulosa cells cultured in the absence and presence of exogenous testosterone (5  $\mu$ M), respectively. Each point represents the mean  $\pm$  SE of triplicate incubations.

Table II. Effect of danazol on progesterone secretion by porcine granulosa cells in 24 hour culture

Treatment	Progesterone secretion (ng/mg protein; mean $\pm$ SE; n = 5)
None	28.8 $\pm$ 0.8
Danazol (30 $\mu$ M)	16.9 $\pm$ 1.8
LH (1 $\mu$ g/ml)	375 $\pm$ 71
Danazol + LH	66.7 $\pm$ 12.3

the end of the incubation period the media were collected and stored at -20° C pending P<sub>4</sub> and E<sub>2</sub> determination by radioimmunoassay. Protein content of the cells was determined by the method of Lowry and associates<sup>13</sup>.

**Radioimmunoassays of P<sub>4</sub> and E<sub>2</sub>.** E<sub>2</sub> and P<sub>4</sub> were measured in diethyl ether-extracted aliquots of culture medium by specific radioimmunoassays. E<sub>2</sub> was measured with the use of an antiserum, prepared against estradiol-17 $\beta$ -6-BSA, kindly supplied by Dr. Gordon Niswender, Colorado State University. The specificity of this antiserum and the assay technique have been described previously.<sup>14</sup> P<sub>4</sub> was assayed according to the method of Abraham and associates<sup>15</sup> with an antiserum to progesterone-11-BSA described by Orczyk and associates.<sup>16</sup> At the concentrations studied, danazol did not cross-react with the antiserum used in the P<sub>4</sub> and E<sub>2</sub> radioimmunoassays.

**Reagents.** Danazol capsules were donated by the Clinical Research Division of Sterling Drug, Ltd., Winthrop Laboratories, Aurora, Ontario. Danazol was extracted from these capsules by shaking the equivalent of 200 mg of danazol (two capsules) in 15 ml of redistilled ethanol for 30 minutes in a metabolic shaker. The ethanolic extract was filtered (Whatman No. 1 filter paper; qualitative) and the concentration of



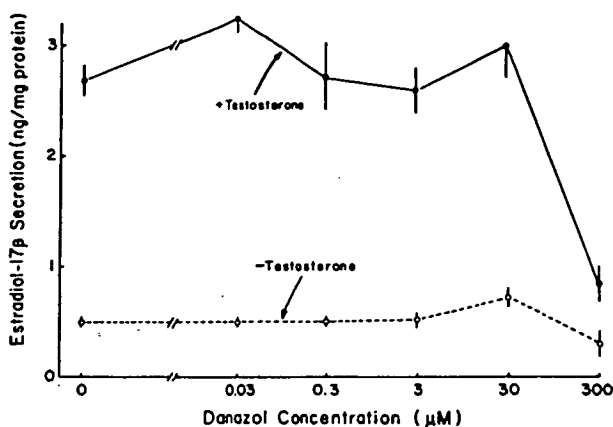


Fig. 3. Estradiol-17 $\beta$  secretion by luteal cells in the presence of various concentrations of danazol in vitro. Each point represents the mean  $\pm$  SE of four incubations.

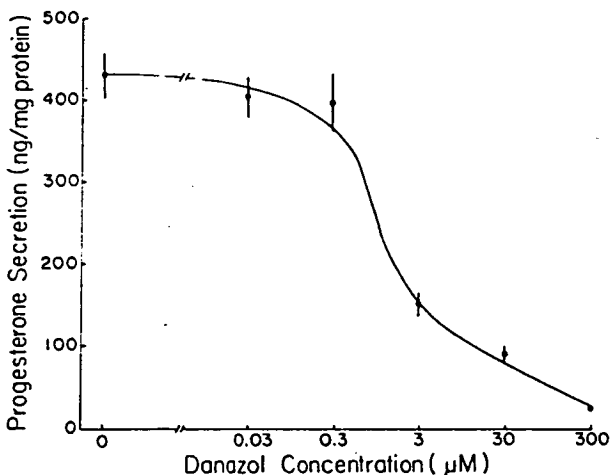


Fig. 4. Dose-response relationship between danazol concentration and inhibition of progesterone secretion by luteal cells in vitro. Each point represents the mean  $\pm$  SE of four incubations.

danazol was determined spectrophotometrically, assuming that the molar extinction coefficient for danazol at 286 nm is 11,300 (*Merck Index*). The danazol extract was diluted to the required concentrations in redistilled ethanol and added to cell cultures in a volume of 10  $\mu$ l. Control cultures received an equal volume of redistilled ethanol. Hormones added to the culture medium were testosterone (T, Sigma) and highly purified ovine LH (No. G3-222B), donated by Dr. Harold Papkoff, University of California, San Francisco.

**Statistical analysis.** Analysis of variance and Duncan's New Multiple Range Test<sup>17</sup> were used to test for statistical significance of difference due to treatment. When there was evidence of heterogeneity of variance,

statistical analysis was performed on logarithmically transformed data.

## Results

**Effects of danazol on E<sub>2</sub> and P<sub>4</sub> secretion by porcine granulosa cells.** E<sub>2</sub> secretion by porcine granulosa cells was low in the absence of exogenous aromatizable substrate, but was markedly increased when testosterone was added to the cultures (Table I, Fig. 1). Danazol, at concentrations of 3 and 30  $\mu$ M, significantly ( $P < 0.01$ ) inhibited E<sub>2</sub> secretion by granulosa cells cultured in the presence (Fig. 1) but not the absence (Table I) of T.

Table II shows the effect of danazol (30  $\mu$ M) on P<sub>4</sub> secretion by granulosa cells isolated from follicles of an individual pig. Analysis of variance of logarithmically transformed data revealed a significant ( $P < 0.001$ ) interaction between danazol and LH, brought about because danazol inhibited LH-stimulated (82%) P<sub>4</sub> secretion to a greater degree than nonstimulated secretion (42%).

The influence of various concentrations of danazol on E<sub>2</sub> (in the presence of 0.5  $\mu$ M T) and P<sub>4</sub> secretion by granulosa cells pooled from follicles of one pig is shown in Fig. 2. The antisteroidogenic effects of danazol were dose dependent and a significant ( $P < 0.05$ ) inhibition of E<sub>2</sub> secretion was detected at a concentration of 3  $\mu$ M. Inhibition of P<sub>4</sub> secretion by danazol did not deviate significantly from linearity.

**Effect of danazol on E<sub>2</sub> and P<sub>4</sub> secretion by porcine luteal cells.** E<sub>2</sub> secretion by porcine luteal cells was low in the absence of an exogenous aromatizable substrate (Fig. 3). Although addition of T (0.5  $\mu$ M) to the culture medium significantly increased this steroidogenic activity fivefold, it was comparable to that observed with granulosa cells cultured in the absence of the exogenous androgen. Danazol had no effect on E<sub>2</sub> secretion by these cells at concentrations in the range of 0.03 to 30  $\mu$ M. In contrast, these cells secreted large amounts of P<sub>4</sub> during the 24 hour culture period and this steroidogenic activity was inhibited by danazol in a dose-related manner (Fig. 4).

## Comment

Although it has been stated on numerous occasions that danazol is a selective inhibitor of gonadotropin secretion and/or release, the conclusion that the therapeutic effects of this compound may be attributed to its antigonadotropic action,<sup>1-4, 6, 18</sup> however, has not been unequivocal.<sup>5, 19, 20</sup> Recent studies by Barbieri and associates<sup>9</sup> on the effects of danazol in male rats have demonstrated that danazol, at a dose of 5 mg/kg of

body weight, significantly suppressed serum testosterone levels but concomitantly elevated serum LH. These *in vivo* findings suggest that danazol may also inhibit gonadal steroidogenesis independent of its antigonadotropic action. Results from the present studies support this possibility in that danazol inhibited P<sub>4</sub> secretion by granulosa and luteal cells *in vitro*. This inhibitory effect of danazol was demonstrated in both the presence and absence of gonadotropic stimulation. Whether danazol exerts its influence on P<sub>4</sub> production at the gonadotropin receptor level and/or at a later enzymatic step(s) in the biosynthetic pathway is not clear. However, the observation<sup>10</sup> that danazol is a potent inhibitor of microsomal 3 $\beta$ -hydroxy-steroid dehydrogenase of hamster ovary, rat testis, and adrenal *in vitro* supports the latter contention.

It has been demonstrated that premenopausal women and monkeys administered danazol have normal basal LH and FSH levels, but do not exhibit mid-cycle E<sub>2</sub> and gonadotropin surges.<sup>6, 8, 21, 22</sup> The present study has shown that danazol depresses E<sub>2</sub> secretion by porcine granulosa cells *in vitro* by inhibition of the aromatase enzyme system. Similar results have been obtained when human granulosa cells were cultured in the presence of danazol (Henderson, Tsang, and Armstrong, unpublished data). These observations, together with the finding that danazol inhibits ovarian 17 $\alpha$ -hydroxylase and 17,20-lyase *in vitro*,<sup>10</sup> further support the hypothesis that danazol may inhibit di-

rectly ovarian steroidogenesis and thus prevent the preovulatory E<sub>2</sub> surge which culminates in the midcycle gonadotropin peak.<sup>23</sup>

The mechanism(s) by which danazol inhibits the aromatase of granulosa cells is not known. However, Barbieri and associates<sup>9</sup> have suggested that danazol may act *in vitro* by competitively inhibiting the binding of substrates to the active sites of cytochrome P-450-dependent enzymes. It is possible that danazol may also exert a similar effect on the cytochrome P-450 system involved in aromatization in the ovaries.

The reason for the apparent inability of danazol to inhibit E<sub>2</sub> secretion by porcine luteal cells and by granulosa cells cultured in the absence of exogenous T is unknown. However, small changes in the secretion of E<sub>2</sub> may have been masked by the relatively large experimental variation within each treatment group, thus making it difficult to detect any inhibition by danazol.

The clinical implications of the present findings remain speculative. Since danazol is used clinically in the management of endometriosis and chronic cystic mastitis and since these disease states are supported at least in part by endogenous gonadal steroids, it is tempting to infer that danazol exerts its therapeutic effects at least partly by direct inhibition of ovarian steroidogenesis. The ability of danazol to suppress luteal function and to interfere with the maintenance of pregnancy is currently under investigation.

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**Reference:** Fertility & Sterility (1980) 33, 550-556

## DANAZOL SUPPRESSES LUTEAL FUNCTION IN VITRO AND IN VIVO

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*Danazol inhibited chorionic gonadotropin-stimulated progesterone production by pregnant rat luteal cells in vitro in a dose-dependent fashion. Spectral studies indicated that the inhibition was a consequence of danazol's interfering with the functioning of mitochondrial cytochrome P-450, an essential component of the enzyme system involved in progesterone biosynthesis. Danazol also suppressed luteal function in vivo, serum levels of progesterone being reduced by 50% to 70% when danazol (50 mg/kg) was administered thrice daily to rats from days 10 to 15 of pregnancy. Since danazol (30  $\mu$ M) also inhibited progesterone production by human luteal cells in vitro and was dominant to the luteotropic action of chorionic gonadotropin, it is suggested that danazol may have some potential as an interceptive agent in humans. Fertil Steril 33:550, 1980*

Danazol (17 $\alpha$ -pregn-4-en-20-yno(2,3-*d*)isoxazol-17-ol), a synthetic analog of ethinyltestosterone, is used in a number of clinical situations,<sup>1</sup> including the therapy of endometriosis,<sup>2</sup> cystic mastitis,<sup>3</sup> precocious puberty,<sup>4</sup> and menorrhagia,<sup>5</sup> and has been proposed as a male<sup>6</sup> and female<sup>7</sup> contraceptive. Its mode of action is generally attributed to a suppressive effect on pituitary gonadotropin release.<sup>8</sup> Recent studies, however, have indicated that danazol can also inhibit the activity of steroidogenic enzymes in the ovary, testis, and adrenal.<sup>9, 10</sup> Of particular interest in this respect is the observation that danazol could inhibit progesterone production by ovarian cells in vitro.<sup>11</sup> This finding raises the possibility that danazol may have some potential as an interceptive agent, i.e., that it may interrupt pregnancy after implantation if it can inhibit ovarian progesterone production when pregnancy is sup-

ported by progesterone secreted by the corpus luteum. This possibility was examined in vitro using human and pregnant rat luteal cells in culture and in vivo using pregnant rats.

### MATERIALS AND METHODS

**Reagents.** Danazol capsules (Danatrol) were donated by the Department of Clinical Research, Winthrop Laboratories, Aurora, Ont., Canada. In addition to danazol, these capsules contain starch, lactose, talc, and magnesium stearate. For the in vivo studies the encapsulated product was administered by subcutaneous (sc) injection as a suspension in sesame oil. For the in vitro studies, danazol was extracted from the encapsulated product by shaking the equivalent of 200 mg of danazol (two capsules) in 15 ml of redistilled ethanol for 30 minutes in a metabolic shaker. The ethanolic extract was filtered and the concentration of danazol was determined spectrophotometrically, assuming that the molar extinction coefficient for danazol in ethanol at 286 nm was 11,300 (*Merck Index*).

**Animals.** Adult female Sprague-Dawley rats weighing 250 to 300 gm were purchased from Bio-Breeding Laboratories, Ottawa, Ont. Pairs of fe-

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males were caged with adult male rats, the animals being housed under constant temperature and lighting conditions and fed pellet food and water ad libitum. Vaginal smears were taken each morning. Day 0 of pregnancy was designated as the day spermatozoa were found in the smear. At day 10 of pregnancy a blood sample (~1 ml) was obtained from each animal, under light ether anesthesia, from a tail vein and the animal was given its first sc injection of either danazol in 0.2 ml of sesame oil or 0.2 ml of oil alone. The injections were repeated at 8- or 24-hour intervals through to day 15 of pregnancy. Additional blood samples were obtained at 48-hour intervals through to day 18 of pregnancy. The blood samples were stored overnight at 4° C and the serum was collected and stored at -20° C until assayed for progesterone by radioimmunoassay (RIA). Serum was similarly obtained from adult ovariectomized female rats undergoing a similar regimen of injections and blood samplings. These provided a "control" group to ensure that the levels of progesterone measured in the serum of the pregnant rats by RIA could not be accounted for by cross-reactivity of danazol present in the serum with the progesterone antiserum.

Superovulation was induced in immature female rats obtained from the Small Animal Breeding Station, University of Edinburgh, by sc injection of 50 IU of pregnant mare serum gonadotropin (PMS) (Gestyl; Organon, Morden, Surrey, United Kingdom) followed 3 days later by an sc injection of 25 IU of human chorionic gonadotropin (hCG) (Pregnyl; Organon, Morden). The gonadotropins were administered in 0.2 ml of phosphate-buffered saline. The animals were used 7 to 8 days after the second injection; they were housed under constant temperature and lighting conditions and fed pellet food and water ad libitum.

**Luteal Cell Cultures.** Human corpora lutea (CL) were obtained from the ovaries of patients undergoing ovariectomy for various gynecologic disorders. Rat CL were dissected out of the ovaries of rats killed on day 10 of pregnancy. The technique for obtaining dispersed luteal cells was the same for both species. The CL were freed of any adherent connective tissue and chopped into pieces approximately 2 mm in diameter in Hanks' balanced salt solution devoid of magnesium and calcium and supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 mM), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 µg/ml), and amphotericin B

(0.625 µg/ml). All reagents were obtained from Flow Laboratories, Mississauga, Canada. The chopped tissue was washed twice with this medium (HBS-HGA) and incubated for 20 minutes at 37° C with stirring in HBS-HGA containing 0.2% collagenase (type II, Sigma Chemical Co., St. Louis, Mo.). The medium was decanted and the released cells were collected by low-speed centrifugation and stored at 4° C in HBS-HGA. The remaining fragile tissue fragments were reincubated in 0.2% collagenase in HBS-HGA at 37° C, and final dispersal of cells was achieved by drawing the suspension through a 2-ml syringe tip and a series of needles (18- to 22-gauge), the cells being collected by centrifugation. All of the released cells were pooled together, filtered through two layers of sterile gauze, and washed four times to remove any traces of collagenase. An aliquot was taken to determine the total cell number, using a hemocytometer, and cell viability was determined using nigrosin dye. Replicates of  $\sim 5 \times 10^5$  "live" cells were cultured at 36° C in a humidified incubator on 15-mm diameter round plastic cover slips (Thermanox: Lux Scientific Corporation, Newbury Park, Calif.) in 1 ml of culture medium consisting of 10% (v/v) calf serum and 90% Minimum Essential Medium (modified) with Earle's salts and supplemented with glutamine and antibiotics, as above, and non-essential amino acids. The gas phase was 5% CO<sub>2</sub>-95% O<sub>2</sub>. Exogenous danazol was added daily throughout the culture period as 0.5 µM to 150 µM in 10 µl of ethanol; control cultures received 10 µl of ethanol only. The culture medium was replaced daily and stored at -20° C until RIA of steroids. At the end of the culture period the cells were washed and stained with hematoxylin and eosin. The number of cells remaining was estimated by counting the number of cells within a 0.3-sq mm area of 12 sampling points on the cover slip, the total number of cells being estimated by extrapolation. Control cultures containing added amounts of danazol, but no cells, allowed corrections to be made for any cross-reaction of the danazol with the steroid antisera.

**Radioimmunoassays.** Progesterone was measured in petroleum ether-extracted aliquots of serum and culture medium, and 17β-estradiol was measured in diethyl ether-extracted aliquots of culture medium using specific radioimmunoassays described and validated previously.<sup>12, 13</sup> The progesterone antiserum was prepared against 11α-hydroxyprogesterone hemisuccinate conjugated to bovine serum albumin (BSA). The 17β-

TABLE 1. Effect of Danazol on hCG (1 IU/ml)-Stimulated Progesterone Production by Rat Luteal Cells Cultured for 24 Hours

Treatment	Progesterone <sup>a</sup>		% Inhibition
	ng/10 <sup>5</sup> cells		
Untreated	25 ± 2		
hCG alone <sup>b</sup>	122 ± 5		
hCG + 0.5 μM danazol	119 ± 3		None
hCG + 5 μM danazol	117 ± 6		None
hCG + 50 μM danazol	99 ± 4 <sup>c</sup>		19
hCG + 150 μM danazol <sup>b</sup>	83 ± 7 <sup>d</sup>		32

<sup>a</sup>Each value is the mean ± standard error of the mean of four replicate cultures.

<sup>b</sup>Cellular levels of progesterone in cultures treated with hCG alone and hCG + 150 μM danazol were <1 ng/10<sup>5</sup> cells.

<sup>c</sup>Significantly different from hCG-treated cultures ( $P < 0.05$ ).

<sup>d</sup>Significantly different from hCG-treated cultures ( $P < 0.01$ ).

estradiol antiserum was generated against 17β-estradiol-6-carboxymethyloxime-BSA. Danazol, at the concentrations studied, showed no cross-reactivity with either antiserum. The sensitivities of the progesterone and 17β-estradiol assays were 25 pg and 5 pg/tube, respectively. Recovery of steroids from serum and culture medium was monitored by the addition of trace amounts of tritiated steroid (~1500 cpm) to the samples prior to their extraction. The intra- and interassay variations of the assays were <10% and <17%, respectively.

**Preparation of Luteal Mitochondria.** Superovulated immature female rats were killed by cervical dislocation. The ovaries were removed, trimmed free of fat, and homogenized at 4° C in 10% (w/v) 0.25 M sucrose (pH 7.0) using a Teflon-glass homogenizer. After sedimentation of nuclei and cell debris at 650  $g_{av}$  for 10 minutes, the mitochondrial fraction was pelleted at 8500  $g_{av}$  for 15 minutes. The mitochondrial fraction was washed by resuspension in one-half the original volume of 0.25 M sucrose and resedimented as before. The final pellet was taken up in 10 mM potassium phosphate buffer (pH 7.4) containing 5 mM MgCl<sub>2</sub> and 100 mM sucrose.

**Spectral Studies.** Spectral measurements were made using a Unicam SP800 ultraviolet-visible wavelength spectrophotometer. The mitochondrial suspension was divided between sample and reference cuvettes, and a baseline of equal light absorbance was obtained. Danazol difference spectra were obtained by adding varying amounts of danazol in 5 μl of ethanol to the sample cuvette and equal amounts of ethanol to the reference cuvette and scanning the spectrum from 510 to 370 nm. The ethanol concentration in the cuvettes never exceeded 1.4% (v/v). Mitochondrial protein concentrations in these studies were approximately 1 mg/ml as determined by the method of Lowry et al.<sup>14</sup>

**Statistical Analysis.** Analysis of variance and Duncan's New Multiple Range test<sup>15</sup> were used to test for statistical significance of differences due to treatment. The data were transformed to logarithms to eliminate heterogeneity of variance when necessary, as determined by Bartlett's test.<sup>16</sup>

## RESULTS

**Effect of Danazol on Rat Luteal Cells.** Table 1 shows the effect of various concentrations of danazol on human chorionic gonadotropin (hCG; A.P.L., Ayerst Laboratories, New York, N. Y.)-stimulated progesterone production by rat luteal cells obtained at day 10 of pregnancy and cultured for 24 hours. While failing to abolish completely the stimulatory effect of hCG, danazol did significantly reduce progesterone production when added to the cultures at concentrations of 50 and 150 μM ( $P < 0.05$  and  $0.01$ , respectively). Lower concentrations of danazol (0.5 and 5 μM) had no significant effect ( $P > 0.05$ ). No detectable amounts of 17β-estradiol were produced by any of the cultures. At concentrations of 0.5 to 50 μM, danazol had no effect on either cell numbers or cellular morphology, as observable by light microscopy, and so the inhibition observed with 50 μM danazol was most likely due to a direct

TABLE 2. Effect of Danazol (30 μM) on Steroid Production by Human Luteal Cells in Vitro

Days in culture	Progesterone <sup>a</sup>			17β-Estradiol <sup>a</sup>		
	Control	Danazol	% Inhibition	Control	Danazol	% Inhibition
	ng/10 <sup>5</sup> cells			pg/10 <sup>5</sup> cells		
1	81 ± 6	58 ± 3 <sup>b</sup>	28	1419 ± 125	564 ± 47 <sup>c</sup>	60
2	63 ± 4	35 ± 2 <sup>c</sup>	44	290 ± 8	78 ± 8 <sup>c</sup>	73

<sup>a</sup>Each value is the mean ± standard error of the mean of four replicate cultures.

<sup>b</sup>Significantly different from corresponding control values ( $P < 0.05$ ).

<sup>c</sup>Significantly different from corresponding control values ( $P < 0.01$ ).

TABLE 3. Effect of Danazol (30  $\mu$ M) on Steroid Production by Human Luteal Cells Cultured in the Presence of hCG (1 IU/ml)

Days in culture	Progesterone <sup>a</sup>			17 $\beta$ -Estradiol <sup>a</sup>		
	Untreated	hCG <sup>b</sup>	hCG + danazol	Untreated	hCG <sup>c</sup>	hCG + danazol
1	53 $\pm$ 8	93 $\pm$ 3	58 $\pm$ 4 <sup>d</sup>	776 $\pm$ 98	679 $\pm$ 45	268 $\pm$ 67 <sup>e</sup>
2	41 $\pm$ 10	73 $\pm$ 7	27 $\pm$ 2 <sup>d</sup>	539 $\pm$ 82	530 $\pm$ 44	218 $\pm$ 54 <sup>f</sup>

<sup>a</sup>Each value is the mean  $\pm$  standard error of the mean of four replicate cultures.

<sup>b</sup>hCG stimulation of progesterone production was significant at  $P < 0.05$ .

<sup>c</sup>hCG did not stimulate 17 $\beta$ -estradiol production ( $P > 0.05$ ).

<sup>d</sup>Significantly different from corresponding hCG-treated culture ( $P < 0.01$ ).

<sup>e</sup>Significantly different from corresponding untreated and hCG-treated cultures ( $P < 0.01$ ).

<sup>f</sup>Significantly different from corresponding untreated ( $P < 0.05$ ) and hCG-treated ( $P < 0.01$ ) cultures.

biochemical interaction with the cellular systems responsible for progesterone biosynthesis. In those cultures receiving danazol at a concentration of 150  $\mu$ M, there were 25% fewer cells remaining after 24 hours of culture as compared with the cultures receiving hCG alone. Thus, at this high concentration, danazol was beginning to have a deleterious effect on cell viability in addition to its direct inhibition of cellular progesterone production.

**Effect of Danazol on Human Luteal Cells.** Danazol at a concentration of 30  $\mu$ M inhibited basal progesterone production and abolished hCG-stimulated progesterone production by human luteal cells (Tables 2 and 3). 17 $\beta$ -Estradiol production was also inhibited by danazol, with the inhibition being greater than that of progesterone production (Tables 2 and 3). In addition, the inhibitory effect of danazol—on progesterone production in particular—was more pronounced during the 2nd day of culture than during the 1st day of culture. Danazol at this concentration (30  $\mu$ M) did not affect the number of cells remaining at the end of the culture period, relative to the control cultures, nor were there any morphologic differences between the control and treated groups on examination by light microscopy. Thus, the inhibition of progesterone and estradiol production was most likely the result of a direct biochemical action of danazol on the luteal cells.

**Mitochondrial Spectral Changes Induced by Danazol.** Spectral studies by Barbieri et al.<sup>10</sup> indicate that danazol inhibits testicular steroid production by interfering with the functioning of microsomal cytochrome P-450, an essential component of the microsomal enzyme system involved in androgen synthesis. Cytochrome P-450 is also an essential component of the mitochondrial enzyme system involved in cholesterol side-chain

cleavage, the rate-limiting step in progesterone production. Spectral studies were therefore performed to determine whether danazol could interact with mitochondrial cytochrome P-450 and thereby provide a possible biochemical mechanism by which danazol inhibited luteal progesterone production. In view of the large numbers of animals that would have been required to prepare adequate quantities of mitochondria from luteal tissue of pregnant rats for spectral studies, it was decided to use mitochondria prepared from the ovaries of immature rats superovulated with a

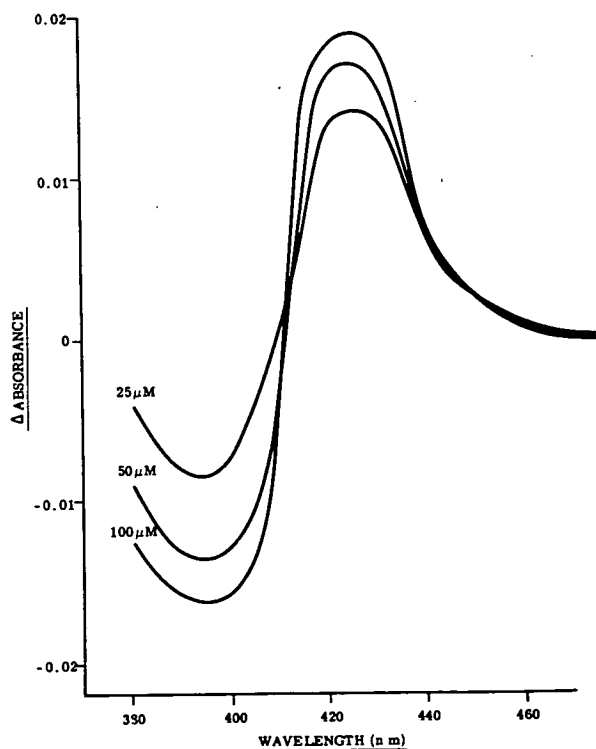


FIG. 1. Spectral changes produced by the addition of danazol (25 to 100  $\mu$ M) to rat luteal mitochondria.

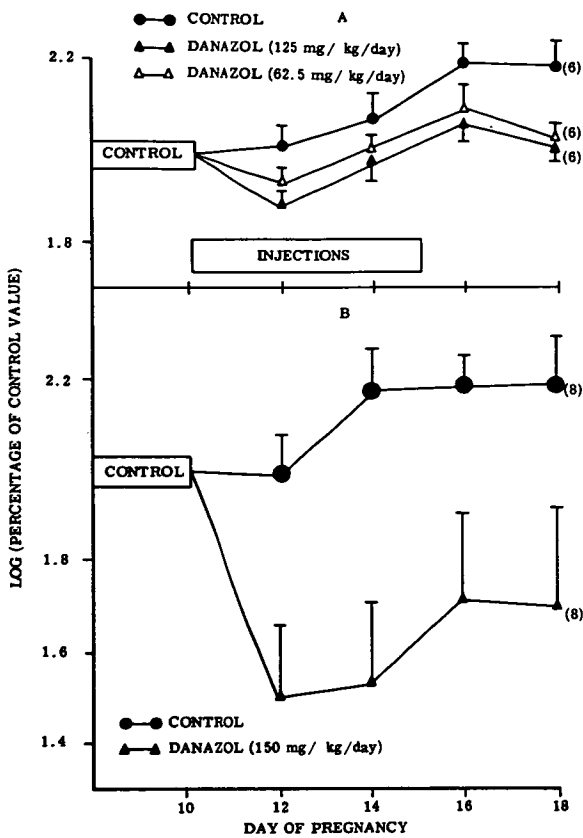


FIG. 2. Effect of danazol on serum progesterone levels in pregnant rats. A, Danazol administered once daily; B, danazol administered thrice daily (50 mg/kg/injection). Results are means  $\pm$  standard error of the mean. Numbers in parentheses refer to the number of animals in each group.

regimen of PMS and hCG. The addition of danazol to these mitochondrial preparations produced type II difference spectra characterized by a trough between 390 and 400 nm and a peak at  $\sim$ 425 nm (Fig. 1). These spectra are characteristically produced by substances which interact with the substrate-binding sites on cytochrome P-450 and thereby inhibit the binding of the natural substrate,<sup>17</sup> in this instance cholesterol. A Hanes plot<sup>18</sup> of the changes in absorbance produced by the different concentrations of danazol gave an apparent dissociation constant for the danazol binding of 25  $\mu$ M.

**Effect of Danazol Administration on Serum Progesterone Levels in Pregnant Rats.** To test the possibility that danazol might suppress luteal progesterone production in vivo, serum progesterone levels were monitored in pregnant rats receiving danazol in sesame oil or oil alone on a daily or thrice-daily basis from days 10 to 15 of pregnancy. The results, shown in Figure 2, are

expressed as a percentage of the serum progesterone concentrations found in the blood sample taken on day 10 of pregnancy immediately prior to the first injections of danazol or vehicle. These serum progesterone concentrations were not significantly different between each of the experimental groups ( $P > 0.05$ ), the values (mean  $\pm$  standard error of the mean) being as follows: control,  $61 \pm 7$  ng/ml; 62.5 mg of danazol/kg/day,  $52 \pm 4$  ng/ml; 125 mg of danazol/kg/day,  $59 \pm 4$  ng/ml for data in Figure 2A, and control,  $52 \pm 7$  ng/ml; 150 mg of danazol/kg/day,  $43 \pm 5$  ng/ml for data in Figure 2B. The data were transformed to logarithms to eliminate heterogeneity of variance.

Once-daily injections of danazol at 62.5 or 125 mg/kg of body weight produced a slight, but non-significant ( $P > 0.05$ ,  $3 \times 4$  mixed model analysis of variance), reduction in serum progesterone concentrations (Fig. 2A). Litter sizes of the control and treated groups were not significantly different from each other ( $P > 0.05$ ), their sizes (mean  $\pm$  standard error of the mean) being as follows: control,  $11 \pm 1$  pups; 62.5 mg/kg/day,  $10 \pm 1$  pups; 125 mg/kg/day,  $10 \pm 1$  pups. Similar studies with up to 500 mg/kg of danazol injected once daily also produced no significant effect on either serum progesterone levels or litter size.<sup>19</sup> When danazol was administered every 8 hours on a thrice-daily basis at a dosage of 150 mg/kg/day from days 10 to 15 of pregnancy, there was a pronounced, significant reduction in serum progesterone levels ( $P < 0.01$ ,  $2 \times 4$  mixed model analysis of variance), as is shown in Figure 2B. There was also a slight, although nonsignificant ( $P > 0.05$ ), reduction in the mean litter size of the danazol-treated animals ( $8 \pm 2$  pups) as compared with the control animals ( $11 \pm 1$  pups).

#### DISCUSSION

The results of the present study demonstrate that danazol effectively suppresses luteal function both in vitro and in vivo. The in vitro studies with human and pregnant rat luteal cells indicate that danazol can inhibit both basal and chorionic gonadotropin-stimulated progesterone production. In addition, danazol inhibited  $17\beta$ -estradiol production by human luteal cells, and it was found that the inhibition of both progesterone and  $17\beta$ -estradiol production by human luteal cells was more pronounced during the 2nd day of culture than during the 1st day of culture, indicating a cumulative effect. These inhibitory effects of



danazol on steroidogenesis *in vitro* are most likely the results of a direct biochemical interaction with the cellular systems responsible for steroid production since, in the studies with both human and rat luteal cells, danazol at concentrations of 30  $\mu\text{M}$  and 50  $\mu\text{M}$ , respectively, inhibited steroid production without affecting either cell numbers or cellular morphology. Only at the highest concentration tested (150  $\mu\text{M}$ ), in the studies with rat luteal cells, did danazol show an additional deleterious effect on cell viability as indicated by a reduction in the number of cells remaining at the end of the culture period. An insight into the possible biochemical mechanisms by which danazol inhibited luteal steroidogenesis is provided by the appearance of type II difference spectra on incubation of danazol with luteal mitochondria. This indicates that danazol can interact with the substrate-binding sites of cytochrome P-450, an essential component of the mitochondrial enzyme system involved in cholesterol side-chain cleavage, the rate-limiting step in progesterone biosynthesis. Thus danazol may inhibit luteal progesterone production through interfering with the binding of cholesterol to cytochrome P-450. Such an action would also account for the inhibition of  $17\beta$ -estradiol production by human luteal cells. Interestingly, the calculated apparent dissociation constant for danazol binding of 25  $\mu\text{M}$  is comparable to the concentration of danazol required to inhibit effectively steroid production by the luteal cell cultures.

The finding that danazol could inhibit chorionic gonadotropin-stimulated progesterone production by luteal cells *in vitro* raised the possibility that danazol might similarly interfere with luteal function *in vivo* if administered to rats during the second half of pregnancy, when luteal function is maintained by a luteinizing hormone-like chorionic gonadotropin produced by the placenta.<sup>20</sup> Moreover, the ability to over-ride the luteotropic action of chorionic gonadotropin would be an essential requirement of any potential interceptive agent designed to terminate early pregnancy in humans when it is supported only by progesterone secreted by the corpus luteum under the influence of hCG. Thrice-daily administration of danazol from days 10 to 15 of pregnancy at a dose of 50 mg/kg reduced serum progesterone levels by 50% to 70% from days 12 to 18 of pregnancy. Although one cannot state unequivocally that this arose solely from a direct inhibition of luteal function, it is perhaps the most likely explanation, being consistent with the *in vitro* findings.

Moreover, since litter sizes were not significantly reduced, it is unlikely that a toxic effect of danazol on the developing pups affected luteal function. In addition, although pituitary gonadotropin production is inhibited by danazol<sup>8</sup> this would not affect luteal function during the second half of pregnancy, pituitary gonadotropins being required only during the first half of pregnancy in the rat.

In contrast to the effect of thrice-daily administration of danazol (50 mg/kg), once-daily administration at doses of up to 500 mg/kg from days 10 to 15 of pregnancy had no significant effect on either serum progesterone or litter size. Studies in man, however, have indicated that danazol is rapidly degraded *in vivo* and that by 8 hours after administration it can no longer be detected in plasma.<sup>21</sup> The failure of the once-daily injections of danazol to reduce serum progesterone levels significantly may be due to the fact that, although high doses were administered, rapid metabolism of danazol results in circulatory levels too low to bring about a sustained inhibition of luteal progesterone production. More frequent injections of a lower dose of danazol (50 mg/kg every 8 hours) probably allows maintenance of a circulatory level of danazol sufficiently elevated to bring about the observed sustained inhibition of luteal steroidogenesis.

Although danazol administered thrice daily at a dose of 50 mg/kg reduced serum progesterone levels by 50% to 70%, pregnancy was not terminated. However, the normal circulating serum progesterone levels of pregnant rats exceeds by a wide margin the minimal requirements for maintenance of gestation, serum progesterone levels as low as 20% of initial values being compatible with undisturbed gestation.<sup>22</sup> Whether danazol could interfere with human luteal function to reduce serum progesterone levels sufficiently to terminate pregnancy in its early stages, when it is maintained by progesterone secreted by the corpus luteum alone, is speculative. However, the results of the present *in vitro* studies with human luteal cells are encouraging in this respect, since danazol is capable of effectively inhibiting both basal and hCG-stimulated steroidogenesis; further clinical studies may therefore be worthwhile. In a recent study in nonpregnant women, danazol administration failed to shorten the luteal phase, although there was some reduction in plasma progesterone levels.<sup>23</sup> The failure to reduce plasma progesterone levels sufficiently to induce premature menstruation may have been due to dana-

zol's being administered only on a once-daily basis. The results of the present study indicate that for maximal effectiveness danazol should be administered more frequently.

It could be argued that the dose of danazol required to reduce serum progesterone levels in pregnant rats (150 mg/kg/day) would be excessive if applied directly to humans. Studies with other steroids, however, indicate that dosages expressed on a milligram per kilogram basis in the rat are good predictors of the total clinical dose that would be required in humans.<sup>24</sup> By this reckoning, a dosage of 150 mg/day would be considerably less than the dosages presently used clinically (400 to 800 mg/day) to treat endometriosis.<sup>25</sup>

In conclusion, the present findings demonstrate that danazol can inhibit luteal function *in vitro* and *in vivo* and can suppress the luteotropic action of chorionic gonadotropins. Thus, danazol may have some potential as an interceptive agent by virtue of these luteolytic characteristics. Clinical studies to assess this potential seem worthwhile.

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## THE MECHANISM OF LUTEAL REGRESSION

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### INTRODUCTION

Following ovulation, the collapsed follicle differentiates into the corpus luteum (CL), the function of which is to provide the progesterone essential for the maintenance of pregnancy should the ovum be successfully fertilized. In the event that the ovum is not fertilized, there must be a return to estrous so that a fresh attempt to achieve pregnancy can be made. This requires the regression of the CL since its presence prevents any return to estrous. In many species including the sheep, cow, pig and horse, the natural luteolysin is uterine in origin while in others such as the dog, cat, monkey and perhaps most notably the human, the uterus plays no role (1). Most research has been concerned with understanding luteal regression in those species in which the uterus participates, and so will be the subject of this paper. It should, however, be borne in mind that our understanding of the mechanism of luteal regression in those species in which the uterus does not participate is still largely obscure.

### Prostaglandin $F_{2\alpha}$ - the uterine luteolysin

There is now a substantial body of evidence to indicate that prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) is the natural uterine luteolysin in several species including the ewe, cow, pig, horse, guinea-pig, rabbit and rat (see ref. 2 for review). Studies in the ewe (3) indicate that  $PGF_{2\alpha}$  reaches the CL from the uterus by a so-called "counter current" mechanism of transfer whereby on being released into the uterine vein  $PGF_{2\alpha}$  passes through it into the ovarian artery which is tightly adherent to the uterine vein and coiled on it. The anatomical structure of the utero-ovarian vasculature is such that similar transfer mechanisms may also exist in the cow, sow, rat, hamster and possibly the guinea-pig (4).

$PGF_{2\alpha}$  is synthesized and released from the uterus in a pulsatile fashion, as indicated by studies in the ewe (5). While both progesterone and estradiol are almost certainly involved in regulating this (6), the pulsatile nature of the release makes it difficult to explain by steroid action alone. Recent evidence

suggests that oxytocin released by the posterior pituitary may also be involved. The luteolytic action of oxytocin has long been known (7) and recent studies indicate that oxytocin can stimulate the release of  $\text{PGF}_{2\alpha}$  from the uterus, a process which is enhanced by estradiol, both in vivo (8) and in vitro (9). Moreover, towards the end of the luteal phase there is an increase in uterine oxytocin receptors, the formation of which is most likely regulated by estradiol (10). Since appreciable amounts of both estradiol and oxytocin are present in blood throughout the luteal phase, it has been suggested that estradiol, through interaction with its uterine receptors, induces the formation of uterine oxytocin receptors thereby sensitizing the uterus to synthesize  $\text{PGF}_{2\alpha}$  in response to the endogenous levels of oxytocin (11). Although progesterone priming of the uterus is required for maximum production of  $\text{PGF}_{2\alpha}$ , continued high levels reduce the amount of  $\text{PGF}_{2\alpha}$  released through inhibiting the formation of uterine estradiol receptors (12), inhibiting oxytocin release from the pituitary (13) and stimulating prostaglandin dehydrogenase activity (14). These actions of progesterone may explain why maximum amounts of  $\text{PGF}_{2\alpha}$  are not released from the uterus until the very late luteal phase when luteal regression has already commenced and serum progesterone levels have declined substantially.

#### Mechanism of action of $\text{PGF}_{2\alpha}$

$\text{PGF}_{2\alpha}$  is a potent vasoconstrictor and it was originally proposed that  $\text{PGF}_{2\alpha}$  might cause luteal regression through reducing blood flow to the ovary (15); luteolysis resulting from anoxia. However, there is little evidence for anoxic damage during the early stages of luteal regression (16) and studies in the rabbit (17), ewe (18) and rat (19) indicate that during  $\text{PGF}_{2\alpha}$  induced luteolysis serum levels of progesterone start to decline prior to any reductions in blood flow to the ovary or CL. Luteolysis may arise from a biochemical action of  $\text{PGF}_{2\alpha}$  on the CL since  $\text{PGF}_{2\alpha}$  can inhibit progesterone production by luteal tissue in vitro (20-23). This is illustrated in Figure 1 where it can be seen that  $\text{PGF}_{2\alpha}$  abolishes the stimulatory action of human chorionic gonadotrophin (hCG) on progesterone production by rat luteal cells during 4 hour incubation periods in vitro. A clue to the site of action of  $\text{PGF}_{2\alpha}$  is provided by the finding that  $\text{PGF}_{2\alpha}$  fails to inhibit the stimulatory action of dibutyryl cyclic AMP (DBC) on progesterone production. This suggests that  $\text{PGF}_{2\alpha}$  may be acting by inhibiting the activation of adenylate cyclase by hCG, and recent studies have demonstrated directly that  $\text{PGF}_{2\alpha}$  does indeed inhibit luteal adenylate cyclase activity, thereby indirectly suppressing progesterone synthesis (21,24). Interestingly,  $\text{PGF}_{2\alpha}$  also fails to inhibit  $\text{PGE}_2$  stimulated progesterone production (Fig. 1) which like that of hCG is mediated through cAMP. Thus  $\text{PGF}_{2\alpha}$  may act to inhibit specifically hCG and LH (luteinizing hormone) activated adenylate cyclase. While inhibition of adenylate cyclase activity is thought to be the initial event in  $\text{PGF}_{2\alpha}$  inhibition of luteal

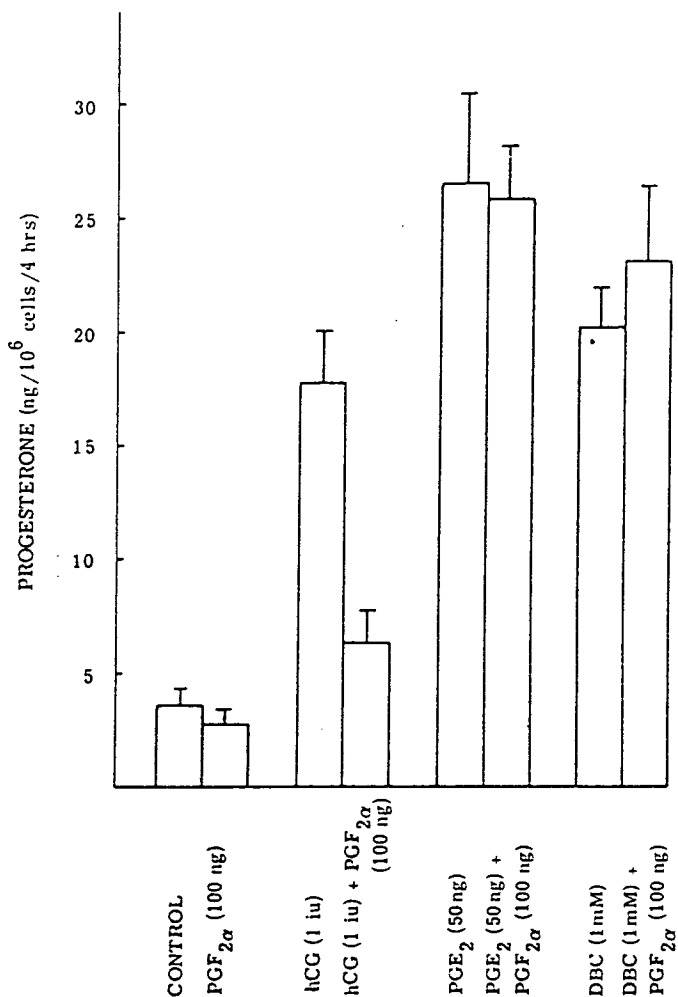


Fig. 1. Effect of hCG, PGF<sub>2α</sub>, PGE<sub>2</sub> and DBC on progesterone production by rat luteal cells in vitro. Each point is the mean + S.D. of 5 replicates.

progesterone production (21), PGF<sub>2α</sub> also has a secondary later effect of causing a loss of receptors for LH in the luteal cell (25,26). Together, these two actions of PGF<sub>2α</sub> can bring about a rapid and sustained inhibition of luteal progesterone production. The possibility that PGF<sub>2α</sub> initiates luteal regression in vivo by similar actions is strengthened by the fact that during natural and PGF<sub>2α</sub> induced luteolysis in the sow there is a reduction in luteal adenylate cyclase activity (27) and cAMP content (28), while in the ewe there is a marked reduction in luteal LH receptors (29,30). In addition, PGE<sub>2</sub> can antagonize the luteolytic action of PGF<sub>2α</sub> in vivo (31) which is also consistent with the in vitro findings.

Morphological deterioration of the corpus luteum in response to PGF<sub>2α</sub> only becomes apparent several hours after serum progesterone levels have started to

decline (16). While  $\text{PGF}_{2\alpha}$  may initiate luteal regression, as indicated by a decline in progesterone secretion, through its biochemical actions as discussed above, whether these actions also trigger morphological deterioration is uncertain. Labilization of lysosomal membranes with the consequent release of stored hydrolases is associated with morphological regression of the CL (32,33), and indeed  $\text{PGF}_{2\alpha}$  has been reported to induce some release of lysosomal hydrolase from luteal tissue in vitro (34). Morphological regression however is also associated with a reduction in blood flow to the CL (35) and while this may be a consequence of morphological regression rather than its cause, the possibility that a venoconstrictor action of  $\text{PGF}_{2\alpha}$  may be involved in causing the final irreversible morphological deterioration of the CL cannot be excluded. Interestingly, this final morphological deterioration of the CL in the ewe is coincident with the release of large amounts of  $\text{PGF}_{2\alpha}$  from the uterus, compared to the levels of  $\text{PGF}_{2\alpha}$  associated with the initiation of luteolysis (36,37). Thus, while the release of relatively small amounts of  $\text{PGF}_{2\alpha}$  from the uterus on days 12-14 of the cycle may be sufficient to initiate functional regression of the CL of the ewe i.e. to trigger the decline in progesterone production, they may be inadequate to cause complete morphological regression. The final irreversible structural demise of the CL may require the much larger amounts of  $\text{PGF}_{2\alpha}$  released from the uterus in the very late luteal phase on days 15 and 16.

#### The refractory CL

While the lytic action of  $\text{PGF}_{2\alpha}$  is well established, the newly formed CL shows considerable resistance to  $\text{PGF}_{2\alpha}$ . In the horse (38), sheep (39) and cow (40) the CL is refractory to  $\text{PGF}_{2\alpha}$  for the first 4 days after its formation, and in the pig this refractory period is about 12 days (41). In studies in which follicular granulosa cells (the precursor cells of the CL) were induced to luteinize in tissue culture, and so provide an in vitro model of the newly formed CL, it was found that while  $\text{PGF}_{2\alpha}$  could inhibit progesterone production prior to cellular luteinization when progesterone production was low,  $\text{PGF}_{2\alpha}$  had little effect on progesterone production when the cells were fully luteinized and producing maximum amounts of progesterone (42,43). This loss of response to  $\text{PGF}_{2\alpha}$  was accompanied by a decrease in the capacity of the luteinized cells to bind  $\text{PGF}_{2\alpha}$  (43). Since progesterone production by granulosa-luteal cells in vitro may be related to the cellular response to LH, it was suggested (42,43) that increasing amounts of LH interacted with the cells during luteinization and in so doing 'masked' plasma membrane receptors for  $\text{PGF}_{2\alpha}$  (44). A similar situation may exist in vivo in that following the ovulatory LH 'surge' saturation of luteal plasma membrane LH receptors may mask those for  $\text{PGF}_{2\alpha}$ , thereby rendering the CL refractory to  $\text{PGF}_{2\alpha}$ . This view is consistent with in vivo findings that LH or hCG antagonizes the luteolytic action of  $\text{PGF}_{2\alpha}$  in the rat (45,46) and guinea-pig (47) while in the sheep (48) and cow (49), both species in which  $\text{PGF}_{2\alpha}$  is thought to be the natural luteolysin, exogenous LH or hCG prolongs the life-span

of the CL. The variation in refractory periods between different species may be due to differences in the rate of unmasking of receptors for  $\text{PGF}_{2\alpha}$  as luteal bound LH is gradually depleted through either internalization and degradation by lysosomes or by gradual dissociation from its receptor sites.

#### The CL of pregnancy

Progesterone secreted by the CL is essential for the maintenance of early pregnancy, thus during a fertile estrous cycle the CL must be maintained. Studies in the guinea-pig (50) and pig (51) indicate that the levels of  $\text{PGF}_{2\alpha}$  found in uterine venous blood during a fertile cycle at the time luteolysis would normally have occurred are very much lower than the levels occurring during a non-fertile cycle. In the guinea-pig this is likely due to an inhibition of uterine prostaglandin synthesis (52), but in the pig uterine prostaglandin synthesis may not be inhibited but rather the  $\text{PGF}_{2\alpha}$  is released into the uterine lumen instead of the uterine vein (53). While uterine venous blood levels of  $\text{PGF}_{2\alpha}$  in the pregnant ewe have also been reported to be reduced compared to the non-pregnant state (5) this has been disputed (54). However, uterine venous (55) and ovarian arterial (56) blood on the side of a gravid uterus in the ewe have luteotrophic properties indicating that the CL may be maintained by an anti-luteolytic action at the ovary. Interestingly, it has been suggested that these anti-luteolytic factors may include  $\text{PGE}_2$  (57, 58) which as discussed above has the capacity to override the luteolytic action of  $\text{PGF}_{2\alpha}$ .

#### Concluding remarks

While most research, as reviewed above, has focussed attention on the role of  $\text{PGF}_{2\alpha}$  in regulating luteal regression, recent findings raise the possibility that  $\text{PGF}_{2\alpha}$  may not, after all, be the actual natural luteolysin, but a side-product, with some luteolytic activity, of the principle luteolysin. Studies in the rat (59) and sheep (60) indicate that the major PG produced by the uterus is not  $\text{PGF}_{2\alpha}$  but 6-keto- $\text{PGF}_{1\alpha}$  the stable metabolite of  $\text{PGI}_2$  (prostacyclin); both these PG's like  $\text{PGF}_{2\alpha}$  being breakdown products of the prostaglandin endoperoxides. While  $\text{PGI}_2$  is thought to play a key role in haemostasis (61) its luteolytic activity has not yet been determined. However, its metabolite 6-keto- $\text{PGF}_{1\alpha}$  has been found to inhibit luteal progesterone production in pregnant hamsters through interacting with what was thought to be the luteal receptor for  $\text{PGF}_{2\alpha}$  (44).

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## REGULATION OF CORPUS LUTEUM STEROIDOGENESIS

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Following ovulation, the collapsed follicle differentiates into the corpus luteum (CL), the function of which is to provide the progesterone essential for the maintenance of pregnancy should the ovum be successfully fertilised. In some species, such as the rat, rabbit, sheep, cow, sow and goat the progesterone secreted by the CL is necessary for the maintenance of pregnancy for the greater part, if not all, of gestation (1). In other species, perhaps most notably the human, the CL can be dispensed with early in pregnancy, the placenta then being capable of providing sufficient progesterone to sustain the pregnancy (2). Whether or not the CL can perform its function satisfactorily and secrete amounts of progesterone adequate to maintain pregnancy depends, to a large extent, on events occurring prior to ovulation, during the period of follicular growth. Histological studies indicate that it is the follicular granulosa cells which, in response to the ovulatory LH surge, differentiate into the principle progesterone secreting cells of the CL (3). It is vital, therefore, that the preovulatory follicle contain an adequate number of granulosa cells and that they be capable of differentiating into functional progesterone secreting luteal cells in response to the ovulatory LH surge.

### "Preovulatory Development" of the Corpus Luteum

The proliferation of granulosa cells during follicular development is regulated by oestradiol ( $E_2$ ) and follicle stimulating hormone (FSH), both of which are mitogenic, though FSH less so than  $E_2$  (4). Studies with hypophysectomized immature rats indicate that FSH alone cannot stimulate normal granulosa cell proliferation and follicular growth. Treatment with  $E_2$  alone, while stimulating granulosa cell proliferation, produces follicles with no antrum. Both FSH and  $E_2$  are required to produce the granulosa cell proliferation and antrum formation associated with normal follicular development (4). The ability of FSH to stimulate antrum formation is important for the proper development of the avascular granulosa cells, as the formation of follicular fluid provides a medium within the follicle where those gonadotrophins and steroids, such as FSH and  $E_2$ , essential to the proper development of the granulosa cells, can accumulate.

In order that the granulosa cells can respond to the ovulatory LH surge, it is necessary that they have functional LH receptors. These receptors are formed during follicle growth. Studies in the pig, for example, indicate that there is a 35-fold increase in the number of LH

receptor sites per granulosa cell as the follicle enlarges (5). The increase in LH receptor numbers is also accompanied by an increased capacity for the granulosa cells to produce cyclic AMP in response to LH, indicating that functional receptors are being formed (6). While LH receptor numbers increase during follicular growth, there is no change in their affinity for LH (5,6). Just as the granulosa cell proliferation and antrum formation associated with normal follicular development requires exposure to both FSH and E<sub>2</sub>, so too the increase in granulosa cell LH receptors is regulated through the combined actions of E<sub>2</sub> and FSH. Studies in the hypophysectomized immature rat, again, indicate that, while E<sub>2</sub> itself has no stimulatory effect on the formation of granulosa cell LH receptors, it markedly enhances the capacity of FSH to stimulate their formation (7). The induction of granulosa cell LH receptors by FSH has recently been demonstrated in *in vitro* studies with granulosa cells obtained from E<sub>2</sub>-primed immature hypophysectomized rats (8). Moreover, these *in vitro* studies demonstrate that this action of FSH is due to a direct effect on the granulosa cells and does not require the participation of other ovarian cell types as has previously been suggested (9). Failure to develop LH receptors during follicular growth will result in the granulosa cells being unable to differentiate into functional luteal cells in response to the ovulatory LH surge.

In addition to their proliferation and development of adequate numbers of functional LH receptors, it is also important that the granulosa cells develop the enzyme systems necessary for progesterone biosynthesis. Recent studies indicate that cholesterol derived from plasma lipoproteins is the principle source of cholesterol utilized to sustain luteal steroidogenesis (10). The metabolism of cholesterol to progesterone requires the participation of the so called "cholesterol side-chain cleavage" enzyme system, a mitochondrial mixed function oxidase which converts cholesterol to pregnenolone, and also 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase (3 $\beta$ -HSD) which metabolises the pregnenolone through to progesterone (11). Studies with isolated bovine granulosa cell mitochondria indicate that there is a 3-fold increase in the capacity of the mitochondria to metabolise both endogenous and exogenous cholesterol to pregnenolone as the follicle enlarges (see Table 1). Interestingly, there is little change in the 3 $\beta$ -HSD activity of the granulosa cell mitochondria during follicular growth. There is only a slight, but significant, increase in progesterone production by granulosa cell mitochondria obtained from large follicles in

**Table 1** Pregnenolone ( $\Delta^5$ -P) and Progesterone (P) Production by Bovine Granulosa Cell Mitochondria

	steroid production (ng/mg protein)			
	- cholesterol		+ cholesterol (100 $\mu$ M)	
	$\Delta^5$ -P	P	$\Delta^5$ -P	P
small follicles ( $<0.5$ ml)	54 $\pm$ 9 <sup>a</sup>	14 $\pm$ 3 <sup>c</sup>	513 $\pm$ 56 <sup>d</sup>	32 $\pm$ 3 <sup>f</sup>
large follicles ( $>1.0$ ml)	183 $\pm$ 24 <sup>b</sup>	22 $\pm$ 2 <sup>c</sup>	1687 $\pm$ 287 <sup>e</sup>	53 $\pm$ 6 <sup>g</sup>

(PTO for table legend).

Values are means  $\pm$  SD. Mean values with different superscripts in same column are significantly different ( $P < 0.05$ ; Wilcoxon rank sum test). Follicle sizes were classified on basis of their follicular fluid content as indicated by figures in ( ).

response to exogenous cholesterol, compared to mitochondria obtained from small follicles. Increased mitochondrial 3 $\beta$ -HSD activity occurs chiefly in response to LH during corpus luteum formation (12), as indicated in Table 2. The factors responsible for bringing about this development of the steroidogenic potential of the granulosa cell, as demonstrated by the increase in cholesterol side-chain cleavage activity, during follicular growth, are not yet fully understood. Both FSH and E<sub>2</sub> are likely involved since *in vitro* studies indicate that each can stimulate progesterone production by granulosa cells (13,14). The stimulatory effect of FSH is thought to be mediated through cyclic AMP (15). Interestingly, recent studies indicate that E<sub>2</sub> can essentially amplify FSH activation of adenylate cyclase (16), suggesting that the two hormones may act synergistically, as is the situation in their regulation of granulosa cell proliferation and LH receptor formation. FSH may also regulate intracellular progesterone formation at biochemical steps distal to the site of cyclic AMP production (13). Granulosa cell progesterone production can also be stimulated by androgens (testosterone, androstenedione, dihydrotestosterone), and these compounds can also synergise with the stimulatory effect of FSH on progesterone production (17). Moreover, *in vivo* studies in the pig indicate that intraovarian implants of anti-androgen reduce the subsequent capacity of granulosa cells to secrete progesterone, thereby further implying a role for androgens in the development of the steroidogenic potential of granulosa cells (18). It should be remembered, however, that androgens can also promote follicular atresia (19). The factors determining whether androgens promote follicular development, through their effect on granulosa cell steroidogenesis, or promote follicular atresia are not yet fully understood though FSH, through its ability to induce androgen aromatization (20) may help protect the follicle from the atretic influence of androgens.

Prolactin displays both a stimulatory and inhibitory effect on granulosa cell progesterone production (21). Recent studies in the pig suggest that the degree of differentiation of the granulosa cells and the extent of their previous exposure to E<sub>2</sub> may influence whether prolactin stimulates or inhibits granulosa cell progesterone production (21). Prolactin suppresses steroid production by cultured granulosa cells isolated from small follicles, but stimulates progesterone production by granulosa cells isolated from larger more mature follicles. Oestradiol protects granulosa cells isolated from small follicles from the inhibitory effect of prolactin on progesterone production. Studies in the rat indicate that prolactin is also involved in the maintenance of luteal LH receptors (22). Interestingly, bromocriptine treatment, both of women with normal menstrual cycles and of hyperprolactinemic amenorrhoea patients, in whom prolactin levels were suppressed below normal, was associated with CL insufficiency (23,24). This is thus consistent with prolactin being required for the proper maturation of the granulosa cells prior to ovulation, and/or for the maintenance of luteal steroidogenesis. Conversely, however, examination of follicles obtained from women with hyperprolactinemia indicate that these follicles are severely deficient in their complement of granulosa cells (25). Thus, while prolactin may be necessary for proper maturation of the granulosa cells, elevated levels are associated with abnormal follicular development.

#### Initiation of Luteal Steroidogenesis

The ovulatory LH surge terminates the phase of granulosa cell proliferation and maturation and initiates their morphological and functional

transformation into progesterone secreting luteal cells. Morphological luteinisation is associated with the granulosa cells undergoing extensive hypertrophy, with the acquisition of lipid droplets, agranular endoplasmic reticulum and mitochondria with a complex system of predominantly tubular internal cristae (26). The granulosa cells thereby assume the morphological characteristics typical of steroid secreting cells. Functional luteinisation is associated with a 'switch' in the steroid output of the granulosa cells. During follicular maturation the granulosa cells develop their potential to synthesize progesterone but do not actually secrete progesterone during this period. They do, however, make a substantial contribution to follicular E<sub>2</sub> production through their capacity to aromatize androgens, these actions stimulated by FSH (20,27,28). It has been suggested that the androgens originate predominantly from outside the granulosa cells, most likely through diffusion from thecal tissue (29) though some androgens may also be synthesized *de novo* by the granulosa cells themselves (30). During luteinisation of the granulosa cells their capacity to aromatize androgens is lost (31) and this, together with the concomitant inhibition of follicular androgen formation (32), is likely responsible for the reduction in follicular oestradiol production which occurs at this time (33). While LH inhibits aromatase activity it stimulates progesterone production by the granulosa cells. Aromatisation is localised within the microsomal fraction of the cell, while luteal progesterone biosynthesis is located predominantly in the mitochondrial fraction of the cell. Thus, luteinisation of the granulosa cells is associated with a switch in the subcellular organelles utilised for steroidogenesis, with mitochondrial steroidogenic enzymes being activated while microsomal enzymes are suppressed.

While stimulation of luteal steroidogenesis by LH is understood to be mediated through cyclic AMP (34), the intracellular mechanism(s) by which cyclic AMP actually brings about and maintains increased progesterone production is poorly understood. By analogy with the stimulation of adrenal steroidogenesis by ACTH, it has been suggested that increased progesterone production occurs in response to increased cholesterol, derived from the hydrolysis of cholesterol esters (CE), being made available for steroidogenesis (35). While such a mechanism may account for the acute stimulation of luteal progesterone by LH, it is unlikely that such a mechanism can account for the more chronic stimulation of progesterone production associated with normal luteal function. Studies in the rat indicate that cholesterol ester hydrolase (CEH) activity is not increased during CL development (36) and indeed recent studies indicate that LH treatment actually suppresses luteal CEH activity (37), which is consistent with the finding that CL development is associated with an accumulation, rather than a depletion, of CE (38). Moreover, recent studies indicate that cholesterol, derived from plasma lipoproteins and not from CE, is the principle source of cholesterol utilised to support luteal progesterone production (10). Studies with isolated ovarian mitochondria do, however, point towards the availability of cholesterol being the major limitation to steroidogenesis. Mitochondria from the ovaries of immature female rats treated with pregnant mares' serum gonadotrophin (PMSG) to induce follicular maturation do have the capacity to synthesize substantial amounts of pregnenolone ( $\Delta^5$ -P) but not progesterone (P), in response to exogenous cholesterol without prior exposure to LH (Table 2). There is only a further 3-fold increase in pregnenolone production when ovarian mitochondria from rats treated with PMSG and LH are used. Interestingly, only the mitochondria prepared from the ovaries of rats receiving LH have the capacity to synthesize progesterone, which indicates that one of the actions of LH is to induce/activate mitochondrial 3 $\beta$ -HSD. Although

increasing the mitochondrial cholesterol environment increases steroid formation (Table 2) this is not the mechanism by which LH acts *in vivo*, since increased steroidogenesis in response to LH is not associated with an increase in mitochondrial cholesterol levels (37,39).

**Table 2 Steroid Production by Rat Ovarian Mitochondria**

Additions	-LH		+LH	
	$\Delta^5$ -P	P	$\Delta^5$ -P	P
none	0.39 ± 0.06	n.d.	0.69 ± 0.11	0.34 ± 0.04
cholesterol (100 $\mu$ M)	5.9 ± 0.7	n.d.	15.8 ± 1.8	0.31 ± 0.04
24-hydroxy cholesterol (100 $\mu$ M)	11.9 ± 1.7	n.d.	10.9 ± 1.3	0.31 ± 0.03

Values are means (ng/ $\mu$ g protein/10 min) ± SD. Values have been corrected for the endogenous steroids present in the mitochondria prior to the start of a 10 min incubation period. The ovarian mitochondria were prepared 6 h after administration of saline (-LH) or LH (10 $\mu$ g, iv) to immature rats injected 48h previously with pregnant mares' serum gonadotrophin (4 iu, sc) to induce follicular development. (n.d. = non detectable)

Rather, LH may act through facilitating the intramitochondrial movement of cholesterol to the sites of steroidogenesis, which are located in the inner mitochondrial membrane, and this may be the rate limiting step in steroid production (39). Evidence for this is also shown in Table 2 where it can be seen that while LH stimulates pregnenolone formation in response to exogenous cholesterol, it does not stimulate pregnenolone formation in response to exogenous 24-hydroxy cholesterol. The hydroxy cholesterol, being more polar, is likely to traverse mitochondrial membranes with greater ease than cholesterol (39), and so its movement to the mitochondrial sites of steroidogenesis is likely not rate-limiting, and so not stimutable by LH. Thus, the principle mechanisms by which LH stimulates luteal progesterone are likely through activating mitochondrial 3 $\beta$ -HSD activity, and by promoting the utilisation of plasma cholesterol for steroidogenesis through facilitating its movement through the mitochondria to the steroidogenic enzymes.

Luteal Regression

In the event that the ovum released at ovulation is not successfully fertilized, there must be a return to oestrus so that a fresh attempt to achieve pregnancy can be made. This requires the regression of the CL, since its presence prevents any return to oestrus. In many non-primate species the natural luteolysin is uterine origin, and extensive studies, particularly in the sheep and guinea-pig, indicate it to be prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ) (see ref. no. 40 for review). The release of PGF<sub>2</sub> $\alpha$  from the uterus to terminate a non-fertile cycle is thought to be under the regulation of progesterone, oestradiol and oxytocin (41-43). PGF<sub>2</sub> $\alpha$  is a powerful vasoconstrictor and it was originally proposed that PGF<sub>2</sub> $\alpha$  may cause luteal regression through reducing blood flow to the ovary; luteolysis resulting from anoxia (44). However, there is little evidence for anoxic damage during the early stages of luteal regression (45), and



studies in the rabbit (46), ewe (47), rat (48) and guinea pig (49,50) indicate that PGF<sub>2α</sub> initiates a decline in luteal progesterone production prior to any observable reduction in blood flow to the ovary or CL. Luteolysis may be initiated through a biochemical action of PGF<sub>2α</sub> since luteal progesterone production can be inhibited by PGF<sub>2α</sub> *in vitro* (51-53). Studies on the mechanism of this biochemical action of PGF<sub>2α</sub> suggest a two-fold effect. Firstly, PGF<sub>2α</sub> causes a rapid (minutes) specific inhibition of LH activated adenylate cyclase (51), and secondly, it has a later (hours) effect of reducing the number of luteal cell receptors for LH (54). Together these two actions of PGF<sub>2α</sub> can bring about a rapid and sustained inhibition of luteal progesterone production through essentially depriving the CL of the luteotrophic support of LH (55). As discussed above, LH initiates and sustains luteal progesterone production through: (a) facilitating the movement of cholesterol to the steroidogenic enzymes in the mitochondria; and (b) activating mitochondrial 3β-HSD activity. Inhibition of LH action by PGF<sub>2α</sub> should therefore affect both these processes, and recent findings are compatible with this. Studies in the pig indicate that PGF<sub>2α</sub> inhibition of luteal progesterone production is associated with a decrease in the amount of cholesterol bound to cytochrome P450, the cholesterol binding component of the mitochondrial enzymes involved in the conversion of cholesterol to pregnenolone (56). Studies in the sheep (57) and guinea pig (58) indicate that PGF<sub>2α</sub> induced luteolysis is also associated with the suppression of 3β-HSD activity. Interestingly, these effects of PGF<sub>2α</sub> seem to be specific for LH activated steroidogenesis. Steroidogenesis maintained by prostaglandin E<sub>2</sub> which, like that of LH, is thought to be mediated through cyclic AMP, is not affected by PGF<sub>2α</sub> either *in vivo* (59) or *in vitro* (60). The cellular receptor for PGF<sub>2α</sub> and LH are both present in the plasma membrane of the luteal cell and the specificity of PGF<sub>2α</sub> towards LH activated steroidogenesis suggests that there may be close association between the two receptors, so allowing interaction to take place (60).

While PGF<sub>2α</sub> may initiate luteolysis through a biochemical interaction with the luteal cell, whether this can also bring about the final irreversible morphological deterioration of the CL is uncertain. Phase changes in the phospholipid bilayer of cellular membranes (61) and labilisation of lysosomal membranes with the consequent release of stored hydrolases (62) is associated with morphological regression of the CL. Although PGF<sub>2α</sub> has been reported to induce some release of lysosomal hydrolase from luteal tissue *in vitro* (63), which is consistent with biochemical actions of PGF<sub>2α</sub> being responsible for morphological regression also, such changes *in vivo* could arise as a consequence of anoxia. A reduction in blood flow to the CL is associated with the morphological deterioration of the CL (50, 64) and while this may merely be a consequence of the regression rather than its cause, the possibility that a vasoconstrictor action of PGF<sub>2α</sub> may also be involved in bringing about the final irreversible deterioration of the CL cannot be excluded.

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**Paper no.:** 10

**Authors:** K.M. Henderson, D.L. Willcox & N.W. Bruce

**Title:** Effect of infusion of PGI<sub>2</sub>, 6-keto-PGF<sub>1α</sub> and PGF<sub>2α</sub> on luteal function in the pregnant rat.

**Reference:** Journal of Reproduction and Fertility (1983) 69, 11-16

## Effect of infusion of PGI-2, 6-keto-PGF-1 $\alpha$ and PGF-2 $\alpha$ on luteal function in the pregnant rat

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**Summary.** Prostacyclin (PGI-2), 6-keto-PGF-1 $\alpha$  and PGF-2 $\alpha$  were infused continuously for 6 h into the dorsal aorta of rats 8 days pregnant. PGF-2 $\alpha$  (10  $\mu$ g/h) significantly reduced plasma progesterone concentrations by 66% and luteal tissue concentrations of pregnenolone and progesterone by 78% and 95% respectively. Plasma concentrations of 20 $\alpha$ -dihydroprogesterone remained unchanged whilst luteal tissue concentrations rose 2-fold. Plasma progesterone concentrations were significantly reduced to 50% by PGI-2 (10  $\mu$ g/h) but were unaffected by 6-keto-PGF-1 $\alpha$  (10 or 100  $\mu$ g/h). Neither PGI-2 (10  $\mu$ g/h) nor 6-keto-PGF-1 $\alpha$  (10 or 100  $\mu$ g/h) had any significant effect on plasma concentrations of 20 $\alpha$ -dihydroprogesterone or on luteal tissue concentrations of pregnenolone, progesterone or 20 $\alpha$ -dihydroprogesterone. Arterial blood pressure was unaffected by PGF-2 $\alpha$  and 6-keto-PGF-1 $\alpha$ , but was significantly reduced by PGI-2 at infusion rates  $\geq$  60  $\mu$ g/h.

### Introduction

Several studies indicate that prostacyclin (PGI-2) may be involved in the regulation of reproductive processes in the female. It is the prostaglandin present in greatest concentration in the rat ovary and uterus, and the major prostaglandin synthesized by homogenates of these tissues *in vitro* (Poyser & Scott, 1980). It is the major product of arachidonic acid metabolism by homogenates of sheep uteri (Jones, Poyser & Wilson, 1977) and by combined incubations of human endometrium and myometrium (Abel & Kelly, 1979). PGI-2 has been implicated in the initiation of blastocyst implantation (Kennedy & Zamecnik, 1978), the regulation of uterine blood flow and menstrual bleeding (Kelly, 1981; Smith, Abel, Kelly & Baird, 1981), and the regulation of follicular blood flow (Veldhuis, Klase & Demers, 1982). PGI-2 involvement in the regulation of corpus luteum function is possible since it is the principal product of prostaglandin endoperoxide metabolism in luteal tissue of the cow and mare (Sun, Chapman & McGuire, 1977) and it stimulates adenylate cyclase activity directly in the rabbit corpus luteum (Abramowitz & Birnbaumer, 1979).

In this study the effect of PGI-2 on luteal function in 8-day-pregnant rats was investigated because at this time the corpora lutea are particularly responsive to prostaglandins (Fuchs, Mok & Sundaram, 1974).

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### Materials and Methods

**Prostaglandins.** Prostaglandin F-2 $\alpha$  tromethamine (PGF-2 $\alpha$ ), 6-keto-prostaglandin F-1 $\alpha$  and prostacyclin sodium salt (PGI-2) were obtained from the Upjohn Company, Kalamazoo, Michigan, U.S.A. Stock solutions (2 or 5 mg/ml) of PGF-2 $\alpha$  and 6-keto-PGF-1 $\alpha$  were prepared in ethanol and acetone respectively and stored at 4°C. Test solutions were prepared on the days of infusion by diluting aliquants of the stock solutions with saline (9 g NaCl/l) or for 6-keto-PGF-1 $\alpha$  with saline containing sodium carbonate sufficient to neutralize the prostaglandin. Control rats were infused with saline containing an equivalent amount of organic solvent which never exceeded 1%. Solutions of PGI-2 were prepared immediately before the start of the infusions in 0.05 M-Tris-HCl buffer (pH 9.4) and kept on ice throughout the infusion period. Control rats in this group were infused with Tris-HCl buffer alone.

**Animals and infusion technique.** Nulliparous albino Wistar rats, 3–5 months old and weighing  $219 \pm 3$  (s.e.m.) g at mating were used. The 55 rats were kept in an environmentally controlled building (17–23°C, relative humidity 50–70%, lights from 07:00 to 21:00 h). Food and water were freely available. The rats were mated during darkness and the morning on which spermatozoa were found in a vaginal smear was called Day 1 of gestation.

On the morning of Day 8, each rat was anaesthetized with an intraperitoneal injection of pentobarbitone sodium (40 mg/kg) supplemented by subcutaneous injections (10 mg/kg) every hour or as needed to maintain the rat in a light surgical plane of anaesthesia. The trachea was cannulated to assist breathing and a cannula was inserted into the dorsal aorta through the left common carotid artery. The arterial cannula was connected to a T-piece so that prostaglandin solutions could be infused continuously, except for brief periods of about 30 sec when the flow was interrupted to withdraw blood samples through the arterial cannula. Changes in arterial pressure were monitored by connecting a pressure transducer to the side arm of the T-piece.

When prepared, the rat was placed on an electric heating pad adjusted to maintain rectal temperature at  $\sim 35^\circ\text{C}$ . A prostaglandin solution was then infused into the arterial cannula at a rate of 2 ml/h for a period of 6 h. Immediately before (0 h) and at 0.5, 1, 2, 3, 4, 5 and 6 h after the start of infusion, blood samples (0.3 ml, except for the final samples when about 1 ml was withdrawn) were taken up into heparinized microhaematocrit tubes, centrifuged, the haematocrit recorded and the plasma removed. Donor blood (1 ml) from rats in dioestrus was given after the 2 h sampling to compensate for that taken. At the end of the infusion period, each rat was killed with an overdose of pentobarbitone sodium. Both ovaries were removed and the corpora lutea were counted, dissected out, pooled, weighed and homogenized in 5 ml chloroform:methanol (2:1 v/v). A single homogenization was found to be sufficient to extract > 99% of the steroids from the luteal tissue, as assessed by the additional amount of steroid extracted following a 2nd homogenization in a further 5 ml extractant. The samples of plasma and tissue homogenate were stored at  $-16^\circ\text{C}$  until analysis.

**Radioimmunoassays.** Progesterone, 20 $\alpha$ -dihydroprogesterone and pregnenolone were measured by radioimmunoassay procedures similar to that described by Thorneycroft & Stone (1972). Progesterone and 20 $\alpha$ -dihydroprogesterone were extracted from plasma before assay with petroleum ether and diethyl ether respectively. The extraction efficiency was monitored by the addition of tritiated steroid (2000 c.p.m.) and the mean recoveries were 79% and 78% for progesterone and 20 $\alpha$ -dihydroprogesterone respectively. Preliminary studies indicated that further purification of the organic extracts of plasma and luteal tissue before assay was unnecessary. The coefficient of variation between the values for samples assayed with and without separation of steroids by LH-20 column chromatography as described by Carr, Mikhail & Flickinger (1971) ranged from 14 to 19% for all of the assays ( $n = 10$  in each instance). Samples were therefore routinely assayed without utilizing a chromatographic step.

Antisera to progesterone (Wallaceville-26) and pregnenolone (Wallaceville-18) were raised in ovariectomized ewes against progesterone-11-hemisuccinate and pregnenolone-3-hemisuccinate conjugated to bovine serum albumin. The 20 $\alpha$ -dihydroprogesterone antiserum (P20-3) was raised

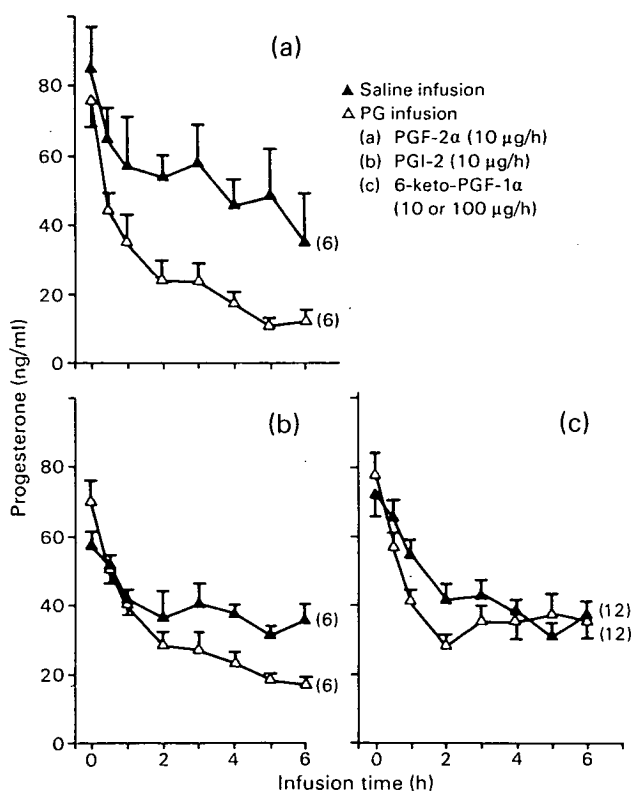
in rabbits against 20 $\alpha$ -dihydroprogesterone-3-oxime-bovine serum albumin conjugate (Endocrine Sciences, Tarzana, California, U.S.A.). Steroids showing > 1% cross-reactivity were: 11 $\alpha$ -hydroxyprogesterone (120%), 11 $\beta$ -hydroxyprogesterone (25%) and 20 $\alpha$ -dihydroprogesterone (3.5%) for the progesterone antiserum; progesterone (9%) and 5 $\alpha$ -pregnanedione (12%) for the pregnenolone antiserum; and 20 $\beta$ -dihydroprogesterone (10%) for the 20 $\alpha$ -dihydroprogesterone antiserum. The limits of sensitivity of the assays (per tube) were 25 pg for progesterone and 20 $\alpha$ -dihydroprogesterone and 50 pg for pregnenolone. The intra- and inter-assay coefficients of variation of all the steroid assays were each < 10% and < 16% respectively.

*Statistics.* Sample data derived from populations with a normal distribution, as assessed by the test of Shapiro & Wilk (1965), were subjected to parametric statistical analysis. Unless indicated otherwise, significant differences between different treatment groups were determined by analysis of variance in conjunction with Fisher's test of least significant difference. Data showing evidence of deviation from normality were analysed non-parametrically using the Kruskal-Wallis and Friedman tests in conjunction with a multiple range test based on differences between rank sums (Zar, 1974). The level of significance was set at  $P < 0.05$ .

## Results

### *Effect of 6 h infusion of prostaglandins on plasma progesterone concentrations*

Plasma concentrations of progesterone in the control groups fell substantially during the first 1–2 h of infusion (Text-fig. 1) and this decline was significant in the control groups of the 6-keto-PGF-



**Text-fig. 1.** Plasma concentrations of progesterone (mean  $\pm$  s.e.m.) for rats (no. in parentheses) during prostaglandin infusions.

1 $\alpha$  and PGI-2 studies ( $P < 0.05$ ). The trauma of surgery causes elevation of plasma progesterone due to stress-induced secretion of the hormone by the adrenal, and it requires 1–2 h for plasma concentrations to return to pre-surgical values (Meyer, Bruce & Willcox, 1982). For the final 4 h of infusion, plasma progesterone concentrations remained relatively constant in each of the control groups (Text-fig. 1). Infusion of PGF-2 $\alpha$  and PGI-2 significantly reduced plasma progesterone concentrations overall, relative to their respective matched control groups ( $P < 0.001$ ) although PGF-2 $\alpha$  was more potent than PGI-2 (Text-figs 1a & b). Plasma progesterone concentrations were reduced significantly to 70% of control values ( $P < 0.05$ ) within 30 min of starting infusion of PGF-2 $\alpha$ , but it required 4 h of infusion of PGI-2 to reduce plasma progesterone concentrations significantly ( $P < 0.05$ ), relative to its controls. In 2 rats infused with PGI-2 at 100  $\mu\text{g}/\text{h}$  there was a similar decline in plasma progesterone concentrations but these results were not analysed statistically. Only 2 rats were studied because, at this dosage, PGI-2 had a dramatic effect on arterial blood pressure, the levels falling from 144 to 43 mmHg within 5 min of starting the infusion and remaining at this level throughout the 6-h infusion period. Infusion of 6-keto-PGF-1 $\alpha$  at 10 or 100  $\mu\text{g}/\text{h}$  had no significant effect on plasma progesterone concentrations ( $P > 0.05$ ) relative to the matched controls (Text-fig. 1c). The data for these 2 treatment groups and their respective controls were each pooled because their effects on plasma progesterone concentrations were not significantly different.

No significant differences were observed between each treatment group in mean  $\pm$  s.e.m. body weight of the rats ( $217 \pm 3$  g,  $n = 50$ ), the number of corpora lutea per rat ( $13.0 \pm 0.3$ ,  $n = 50$ ) or the haematocrit based on the average of all samples taken from each rat ( $41.0 \pm 0.4\%$ ,  $n = 400$ ).

#### *Steroid concentrations in luteal tissue and plasma after prostaglandin infusion*

The effects of the prostaglandins on luteal steroidogenesis were assessed in terms of the tissue concentrations of pregnenolone, progesterone and 20 $\alpha$ -dihydroprogesterone after 6 h of infusion, and related to the circulating concentrations of progesterone and 20 $\alpha$ -dihydroprogesterone at this time (Table 1). Sufficient blood to determine the plasma concentrations of both progesterone and 20 $\alpha$ -dihydroprogesterone was obtained only at the 6 h sampling immediately before the cessation of the infusion. The control data obtained from the saline-infused rats for each prostaglandin have been combined because there were no significant differences between these groups. PGF-2 $\alpha$  significantly reduced luteal tissue concentrations of progesterone and pregnenolone by 95% ( $P < 0.005$ ) and 78% ( $P < 0.025$ ) respectively and doubled 20 $\alpha$ -dihydroprogesterone concentrations,

**Table 1.** Concentrations of steroids in luteal tissue and peripheral plasma after infusion of prostaglandins for 6 h

Treatment ( $\mu\text{g}/\text{h}$ )	Luteal tissue (ng/mg)			Peripheral plasma (ng/ml)	
	Progesterone	Pregnenolone	20 $\alpha$ -Dihydro- progesterone	Progesterone	20 $\alpha$ -Dihydro- progesterone
Saline (23 or *24)	$31 \pm 4^a$	$4.5 \pm 0.5^a$	$6.8 \pm 1.1^a$	$35 \pm 4^{*a}$	$30 \pm 4^{*a}$
PGF-2 $\alpha$ 10 (6)	$1.5 \pm 0.5^b$	$1.0 \pm 0.3^b$	$14 \pm 4^a$	$12 \pm 3^b$	$29 \pm 8^a$
6-keto-PGF-1 $\alpha$ 10 (6)	$18 \pm 5^a$	$2.1 \pm 0.3^a$	$3.0 \pm 1.1^a$	$36 \pm 7^a$	$22 \pm 4^a$
100 (6)	$16 \pm 6^a$	$2.3 \pm 0.8^a$	$7.3 \pm 2.9^a$	$33 \pm 7^a$	$30 \pm 5^a$
PGI-2 10 (6)	$15 \pm 3^a$	$3.3 \pm 0.8^a$	$4.7 \pm 2.1^a$	$17 \pm 2^b$	$22 \pm 4^a$
100 (2)	2, 18	0.6, 5.8	5.4, 12.0	17, 23	16, 24

The number of determinations ( $n$ ) is indicated in parentheses. Values are mean  $\pm$  s.e.m. except when  $n = 2$  for which individual values are given. Values with different superscripts in the same column are significantly different ( $P < 0.05$ ; non-parametric statistics). Groups with  $n = 2$  were excluded from the statistical analysis.



although this rise was not significant ( $P > 0.05$ ), Concomitantly, plasma progesterone concentrations were significantly reduced by 66% ( $P < 0.001$ ) while  $20\alpha$ -dihydroprogesterone concentrations were unaltered. PGI-2 and 6-keto-PGF- $1\alpha$  had no significant effect ( $P > 0.05$ ) on luteal tissue steroid concentrations, although mean values were generally lower than in the control groups. Plasma progesterone concentrations were significantly reduced by 50% by PGI-2 ( $P < 0.001$ ) but not by 6-keto-PGF- $1\alpha$ . Neither prostaglandin had a significant effect on plasma  $20\alpha$ -dihydroprogesterone concentrations ( $P > 0.05$ ).

#### *Effect of PGI-2 on arterial blood pressure*

To evaluate the effect of PGI-2 on arterial blood pressure, 5 rats were cannulated as described above. PGI-2 was infused 2 to 3 times per dose per rat at rates of 2, 10, 20 and 60  $\mu\text{g/h}$  in 5 min pulses interspersed by 5 min pulses of buffer lacking PGI-2 (control infusate). The changes in blood pressure associated with PGI-2 infusion were assessed by comparing them to the mean pressure during the immediately preceding control period. Average changes were determined for each rat and significance tested by Student's paired  $t$  test. Control blood pressures remained constant throughout the course of the experiment and ranged from  $155 \pm 7$  mmHg (mean  $\pm$  s.e.m.) to  $161 \pm 4$  mmHg. PGI-2 infusion at rates of 2, 10, 20 and 60  $\mu\text{g/h}$  resulted in a progressive fall in arterial blood pressure of 4, 6, 15 and 42%, respectively, although only the 42% fall was statistically significant ( $P < 0.05$ ). Maximum reduction in blood pressure occurred within the 5 min infusion period. Neither PGF- $2\alpha$  nor 6-keto-PGF- $1\alpha$  significantly affected blood pressure at infusion rates of up to 100  $\mu\text{g/h}$ .

### Discussion

The results of previous studies of the effects of PGI-2 and its metabolite 6-keto-PGF- $1\alpha$  on luteal function have been conflicting. A single subcutaneous injection of PGI-2 (0.1 mg) or 6-keto-PGF- $1\alpha$  (1 mg) into the pregnant hamster reduced serum concentrations of progesterone by 40 and 88%, respectively, after 6 h (Kimball & Porteus, 1978). In contrast, plasma concentrations of progesterone in cows were elevated for 14 h following the injection of PGI-2 (1 mg) directly into the corpus luteum while 6-keto-PGF- $1\alpha$  was without effect (Milvae & Hansel, 1980). The results of both studies, however, may represent pharmacological rather than physiological actions of the prostaglandins because of the high dosages used. The present finding that infusion of small amounts of PGI-2 (5  $\mu\text{g}$  in 5 min) provoked an immediate and substantial reduction in blood pressure is pertinent: the amounts of PGI-2 (0.1–1.0 mg) administered as a single injection in the above previous studies may have provoked circulatory changes which contributed to the differences in the results obtained.

The basis of the present study was to compare the effects on luteal function of PGF- $2\alpha$ , PGI-2 and 6-keto-PGF- $1\alpha$  under conditions as close to physiological as practicable. Therefore, prostaglandins were administered by continuous infusion rather than by bolus injection to avoid very high peak concentrations and to allow for their rapid metabolism (Piper, Vane & Wyllie, 1970; Dusting, Moncada & Vane, 1978). Moreover, infusion was into the dorsal aorta, rather than into the venous system to ensure that the prostaglandins underwent minimal metabolism before reaching the ovary. Under these conditions, PGF- $2\alpha$  had a potent luteolytic effect since its infusion caused a rapid and sustained fall in plasma concentrations of progesterone and in luteal tissue concentrations of progesterone and pregnenolone (Text-fig. 1; Table 1). Although plasma concentrations of  $20\alpha$ -dihydroprogesterone remained unchanged, luteal tissue concentrations doubled (Table 1), suggesting the start of increased  $20\alpha$ -hydroxysteroid dehydrogenase activity which is also characteristic of luteolysis in the rat (Strauss & Stambough, 1974).

In contrast to PGF- $2\alpha$ , there was no evidence that 6-keto-PGF- $1\alpha$  had any luteolytic effect, even when it was infused at a 10-fold higher rate. Plasma progesterone concentrations were not

affected and luteal tissue concentrations of progesterone and pregnenolone were not significantly altered (Text-fig. 1; Table 1). Results from the PGI-2 infusions were intermediate between those of the other two prostaglandins in that plasma progesterone concentrations fell significantly (Text-fig. 1), albeit to a lesser extent than with PGF-2 $\alpha$  infusion, while luteal tissue steroid concentrations were not significantly affected (Table 1). The effect on plasma progesterone concentrations was not related to any hypotensive action and it was not due to metabolism of PGI-2 to 6-keto-PGF-1 $\alpha$  since the latter had no apparent action.

In conclusion, the present results show that in the pregnant rat PGI-2 has only a minor suppressive effect on luteal function compared to the potent luteolytic action of PGF-2 $\alpha$ , while 6-keto-PGF-1 $\alpha$ , the immediate metabolite of PGI-2, is without apparent effect.

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**Section 2: Papers related to studies of follicle  
development and steroidogenesis**

**Paper no.:** 11

**Authors:** A.K. Goff & K.M. Henderson

**Title:** Changes in follicular fluid and serum concentrations of steroids in PMS-treated immature rats following LH administration.

**Reference:** *Biology of Reproduction* (1979) 20, 1153-1157

## Changes in Follicular Fluid and Serum Concentrations of Steroids in PMS Treated Immature Rats following LH Administration

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### ABSTRACT

The effect of an ovulatory dose (10 µg/rat) of luteinizing hormone (NIH-LH-B8) on follicular fluid (FF) and peripheral serum concentrations of steroids in pregnant mare's serum (PMS) treated rats (4 IU/rat) was studied over a 10 h period. Within 1 h of LH administration, FF and serum concentrations of progesterone (P) had risen 7- and 10-fold, respectively, the concentrations remaining elevated for the remainder of the experiment. In contrast, the FF and serum concentrations of androgen (A) and estradiol-17β (E) after rising only slightly over the first 1-2 h after LH administration, steadily declined thereafter to reach extremely low levels by 10 h. These findings not only show the range in concentrations of rat FF steroids but also demonstrate that approaching ovulation in the rat is associated with a change in follicular steroidogenesis with P production being stimulated while A and E production is suppressed. In addition, these findings indicate that the measurement of FF steroids is a useful means of assessing the steroidogenic activity of the follicle.

### INTRODUCTION

Studies in several species including the human (McNatty et al., 1975), rabbit (Younglai, 1972; Patwardham and Lanthier, 1976; Bahr, 1978), pig (Chang et al., 1976) and mare (Younglai, 1971) indicate that marked changes occur in the concentrations of steroids present in follicular fluid (FF) during follicular maturation. No information, however, is available regarding either the concentrations of steroids present in rat FF or the changes in these steroids that may occur during follicular maturation, even though the rat is widely used for research studies in reproductive biology. Both the oocyte and follicular granulosa cells are bathed in FF *in vivo* and it would therefore be of interest to know the ranges in steroid concentrations to which they are exposed because this may regulate their behavior. Moreover, knowledge of the ranges in FF steroid concentrations would be particularly

useful in assessing the possible physiological significance of *in vitro* studies examining the effect(s) of steroids on oocyte and granulosa cell function in the rat. The purpose of this study was, therefore, to determine the changes in the concentrations of P, A and E in rat FF and peripheral serum as ovulation approached. To facilitate the study, we used immature rats in which follicular development had been induced by PMS gonadotropin (4 IU/rat, s.c.). Follicular development initiated in this way mimics that of the mature animal in that following an ovulatory stimulus (in this study an ovulatory dose of luteinizing hormone) the induced ovulation closely resembles a spontaneous ovulation (Guillet and Rennels, 1964).

### MATERIALS AND METHODS

#### Animals

Immature Sprague-Dawley female rats were purchased from Bio-Breeding Laboratories, Ottawa, Ontario. The animals were housed under constant temperature and lighting conditions and were fed pellet food and water *ad libitum*. At 27 days of age the rats were injected s.c. with 4 IU PMS (Ayerst) in 0.2 ml saline to induce follicular development. Two days later, ovulation was induced by an i.v. injection through a tail vein of 10 µg LH (NIH-LH-B8) in 0.2 ml saline. The animals were sacrificed by decapitation 0, 1, 2, 4, 6, 8 or 10 h after the injection of LH (n =

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4/group). Ovulation in this model occurs ~12 h after administration of LH. Trunk blood was collected, stored overnight at 4°C and the serum collected and stored at -20°C until assayed for steroids by radioimmunoassay (RIA).

#### Collection of Follicular Fluid

Immediately after decapitation the ovaries were removed from the animals, shelled from their bursae and dissected free of oviducts and adhering fat. Under a dissecting microscope FF was aspirated and pooled from all visible antral follicles on each pair of ovaries with a lambda ( $\mu$ l) calibrated microcapillary tube attached by a 20 gauge needle and polyethylene tube to a 1 ml syringe containing 0.4 ml saline. The aspirated FF was flushed out of the microcapillary tube and connecting tubing with the 0.4 ml saline into test tubes and immediately stored at -20°C until assayed for steroids by RIA. The average amount of FF obtained from each pair of ovaries was  $4.73 \pm 0.18$  (SEM)  $\mu$ l.

#### Radioimmunoassays

Progesterone was measured in petroleum ether extracted aliquots of peripheral serum and FF using the antibody and assay technique validated and described previously by Orczyk et al. (1974). The antiserum was prepared against 11 $\alpha$ -hydroxy progesterone hemisuccinate conjugated to bovine serum albumin (BSA). The antiserum showed negligible cross reactivity (<0.1%) with androgens and estrogens. The sensitivity of the assay was 25 pg/tube. Estradiol-17 $\beta$  and A were measured in diethyl-ether extracted aliquots of serum and FF using the antisera and assay techniques validated and described previously by Dorrington and Armstrong (1975) and Auletta et al. (1974), respectively. The E antiserum was generated against estradiol-17 $\beta$ -6-carboxymethylxime-BSA. This antiserum showed low cross reactivity (<10%) with other phenolic steroids and negligible cross reactivity (<0.1%) with neutral steroids. The A antiserum was generated against testosterone-3-carboxymethylxime-BSA. This antiserum cross reacted with 5 $\alpha$ -reduced androstanes as follows, testosterone being taken as 100%: 17 $\beta$ -hydroxy-5 $\alpha$ -androstane-3-one (DHT, 53%); 5 $\alpha$ -androstane-3 $\alpha$ -17 $\beta$ -diol (30%); and 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (13%). The antiserum showed negligible cross reactivity (<0.1%) with estrogens and P. The sensitivities of the E and A assays were 5 and 12 pg/tube, respectively. Recovery of steroids from FF and serum samples was monitored by the addition of trace amounts of tritiated steroid (~1500 cpm) to the sample prior to their extraction. All 3 steroids were extracted from FF with recoveries >90%; the mean procedural recoveries from serum were P = 78%, E = 82%, A = >90%. The intra- and interassay variations of all 3 assays were <12% each.

#### Statistical Analysis

Statistical analysis of the data was performed using analysis of variance. The data were transformed to logarithms to eliminate heterogeneity of variance where necessary. Significance of differences between individual treatment groups was determined using Duncan's New Multiple Range test (Duncan, 1955).

## RESULTS

Table 1 shows P, A and E concentrations in serum and FF before and at various times after LH administration. Within 1 h, P levels in serum rose 7-fold from  $3.1 \pm 0.2$  (SEM) ng/ml to  $20.8 \pm 2.1$  ng/ml. A maximum concentration of  $49.6 \pm 6.5$  ng/ml was reached by 2 h after which the levels declined to  $26.1 \pm 3.0$  ng/ml by 6 h and then remained at this level. The FF concentrations of P increased 10-fold from  $130 \pm 14$  ng/ml to  $1256 \pm 214$  ng/ml in the first h after LH administration and remained at this level for the remainder of the time period. Although the changes in the P concentrations of FF and serum followed similar profiles, there was no significant correlation between them. Regression analysis gives the equation  $y = 22x + 551$ , the coefficient of linear correlation ( $r$ ) being 0.55 with  $P > 0.05$ .

Serum A levels rose 5-fold from  $0.95 \pm 0.5$  ng/ml to  $5.1 \pm 0.4$  ng/ml in the first h but then steadily declined to  $0.50 \pm 0.04$  ng/ml by 10 h. The FF concentrations of A followed a similar pattern in that the levels increased slightly (though nonsignificantly,  $P > 0.05$ ) from  $253 \pm 86$  ng/ml to  $582 \pm 85$  ng/ml over the first h and then steadily declined, the concentration at 10 h being only  $9 \pm 1.5$  ng/ml. There was a significant correlation between A concentrations in serum and FF ( $r = 0.91$ ;  $P < 0.01$ ;  $y = 110x - 28$ ).

Serum E concentrations changed little over the time course of the experiment. There was, however, a slight though nonsignificant ( $P > 0.05$ ) rise over the first 2 h from  $0.07 \pm 0.03$  ng/ml to  $0.11 \pm 0.04$  ng/ml, after which the levels declined significantly ( $P < 0.01$ ) to  $0.03 \pm 0.003$  ng/ml by 6 h and remained at this level. A similar slight but nonsignificant ( $P > 0.05$ ) rise in the E concentration of FF occurred over the first 2 h following LH administration, the concentration rising from  $38.7 \pm 12.8$  ng/ml to  $46.8 \pm 19.1$  ng/ml. Between 2-4 h, however, there was a pronounced decline from  $46.8 \pm 19.1$  ng/ml to  $10.5 \pm 3.2$  ng/ml and by 6 h the concentrations had fallen below the sensitivity of the assay (<5 ng/ml) where they remained for the rest of the experiment. There was a significant correlation between E concentrations in serum and FF ( $r = 0.88$ ;  $P < 0.01$ ;  $y = 491x - 11$ ).

## DISCUSSION

These findings show for the first time the ranges in concentration of steroids present in

TABLE 1. Rat serum and follicular fluid steroids before and after i.v. injection of LH (10 µg/rat; NIH-LH-B8).

Time after LH (h)	Progesterone (ng/ml)		Androgen (ng/ml)		Estradiol-17β (ng/ml)	
	Serum	Follicular fluid	Serum	Follicular fluid	Serum	Follicular fluid
0	3.1 ± 0.2 <sup>a</sup>	130 ± 1.4 <sup>a</sup>	0.95 ± 0.5 <sup>b</sup>	253 ± 86 <sup>a</sup>	0.07 ± 0.03 <sup>ab</sup>	38.7 ± 12.8 <sup>bc</sup>
1	20.8 ± 2.1 <sup>b</sup>	1256 ± 214 <sup>bc</sup>	5.1 ± 0.4 <sup>a</sup>	582 ± 85 <sup>a</sup>	0.12 ± 0.01 <sup>a</sup>	48.3 ± 7.6 <sup>b</sup>
2	49.6 ± 6.5 <sup>d</sup>	855 ± 184 <sup>b</sup>	3.4 ± 0.7 <sup>a</sup>	260 ± 80 <sup>a</sup>	0.11 ± 0.04 <sup>a</sup>	46.8 ± 19.1 <sup>b</sup>
4	40.6 ± 5.1 <sup>d</sup>	2066 ± 897 <sup>c</sup>	1.1 ± 0.1 <sup>b</sup>	31 ± 12 <sup>b</sup>	0.08 ± 0.01 <sup>ab</sup>	10.5 ± 3.2 <sup>c</sup>
6	26.1 ± 3.0 <sup>b</sup>	1196 ± 259 <sup>bc</sup>	0.58 ± 0.03 <sup>b</sup>	9 ± 2 <sup>b</sup>	0.03 ± 0.003 <sup>b</sup>	<5
8	24.2 ± 4.6 <sup>b</sup>	1424 ± 359 <sup>bc</sup>	0.53 ± 0.06 <sup>c</sup>	2 ± 0.4 <sup>c</sup>	0.04 ± 0.02 <sup>b</sup>	<5
10	24.3 ± 1.9 <sup>b</sup>	1064 ± 152 <sup>bc</sup>	0.50 ± 0.04 <sup>c</sup>	9 ± 1.5 <sup>b</sup>	0.03 ± 0.004 <sup>b</sup>	<5

Values are means ± SEM; n = 4 rats/group. Differences between means with the same superscript in the same column are nonsignificant (P > 0.05). Differences between means with different superscripts in the same column are significant at the following levels; abp < 0.01; bcp < 0.05; bdp < 0.01.

rat FF and also demonstrate that following an ovulatory dose of LH there is a marked change in follicular steroidogenesis with P production being stimulated while production of A and E is suppressed. These changes are reflected in the steroidal content of FF as well as in serum. In addition, the similar profiles and positive correlations between the concentrations of steroids in FF and serum indicate that the measurement of FF steroids is a useful means of assessing the steroidogenic activity of the follicle. These *in vivo* findings therefore confirm and extend the previous *in vitro* observations of Hillensjo et al. (1976) who demonstrated that LH stimulated P but inhibited A and E secretion by preovulatory follicles taken from PMS-primed (8 IU, s.c.) immature rats. Although the present study was carried out using PMS treated (4 IU, s.c.) immature rats, the serum concentrations of E prior to LH administration and the serum concentration of P following LH administration were similar to the corresponding levels found in the mature cycling rat immediately before and after ovulation (Schwartz, 1974; Meijs-Roelofs et al., 1975). Our study therefore supports the observation of Guillet and Rennels (1964) that follicular development induced in the immature rat with 4 IU PMS is similar to normal follicular development in the mature rat. Consequently, it appears valid to conclude that the changes in the relative concentrations of FF steroids observed in this study also occur following the ovulatory surge of LH in the normal cycling rat.

While LH administration produced a sustained increase in serum and FF concentrations of P, there were only transient increases in serum and FF concentrations of A and E after which concentrations declined, the levels in FF particularly falling to extremely low values. The physiological significance of this changing steroidal environment within the follicle in response to LH, as reflected by the rising P but falling A and E levels in FF, is uncertain. It is possible, however, that it may influence the appearance of proteolytic enzymes involved in follicular rupture (Rondell, 1974; Beers et al., 1975) and/or influence the resumption of meiosis by the oocyte (Zuckerman and Baker, 1977) both of which occur following the LH surge. It is interesting to note that the concentration of A in FF prior to LH administration (~250 ng/ml) is similar to the concentration required to stimulate P production by granulosa cells *in vitro* (Hillier et al., 1977). This, therefore, lends support to the suggestion

that intrafollicular androgens may serve to promote the development of the functional capacity of granulosa cells to secrete P (Hillier et al., 1977).

Although the cellular source of the P produced in response to LH cannot be stated conclusively, it most likely originates from the granulosa cells undergoing luteinization. This effect of LH resulting in increased P production is well established and is understood to be mediated through cyclic AMP (Channing and Tsafiriri, 1977). The inhibitory action of LH on A and E production is, however, less well understood. It has been suggested that LH may stimulate production of a protein which inhibits the enzymes involved in the cleavage of the 17-sidechain of P (17 $\alpha$ -hydroxylase and/or 17:20 lyase) thereby resulting in a simultaneous inhibition of both A and E production (Lieberman et al., 1975). In addition, Katz and Armstrong (1976) have suggested that LH may inhibit E production by reducing ovarian A aromatase activity and Henderson and Moon (1979) have recently demonstrated that luteinization of granulosa cells is accompanied by a loss in their aromatase activity. In the present study, from 0–2 h following LH administration, the A concentration in FF greatly exceeded that of E (5.5- to 12-fold) suggesting that aromatase activity rather than the availability of A precursor was rate limiting in the production of E. The dramatic fall in A commencing 4 h after LH administration could in itself account for the simultaneous fall in E, although the possibility of a concomitant reduction in ovarian aromatase activity cannot be excluded. Studies with rat thecal and granulosa cells in tissue culture indicate that thecal tissue is the principle source of follicular A while granulosa cells are the principle source of follicular E, the granulosa cells acting to aromatize the A produced by thecal tissue (Fortune and Armstrong, 1977, 1978). Thus, although LH presumably exerts an inhibitory effect on thecal cells to suppress A production, LH may have a dual effect on granulosa cells acting to stimulate their production of P while inhibiting their capacity to aromatize A.

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## GONADOTROPHIC REGULATION OF OVARIAN ACTIVITY

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- 1 Follicular growth
  - 2 Granulosa-cell maturation
  - 3 Ovulation
  - 4 Corpus luteum function
  - 5 Prolactin
- References

While changes in blood concentrations of pituitary gonadotrophins throughout the menstrual cycle have been well documented (see Coutts, 1976), the mechanisms by which gonadotrophins act at the cellular level to regulate ovarian activity in women remain unclear. This is not only because of the complexity of the problem, but also because, for ethical reasons, the human female is a rather poor experimental subject for studies at the cellular level. As a consequence, much of our knowledge concerning the mechanism of action of gonadotrophins is based on studies using experimental animals. While any extrapolation of these findings to humans must be viewed with extreme caution, much of the information originating from studies on animal reproduction has proved to be highly relevant to the human female. Thus, in discussing the present understanding of gonadotrophic regulation of human ovarian activity at a cellular level, reference will be made, where pertinent, to information obtained using other species.

### 1 Follicular Growth

One of the most basic events within the ovary is follicular growth which, in the human, is a continuous process beginning before birth and proceeding throughout childhood and adulthood, uninterrupted by pregnancy or other periods of non-ovulation (Peters *et al.* 1975). Follicular growth is an irreversible process which, once initiated, must continue until the follicle either ovulates or undergoes atresia. At birth the human ovary contains approximately 2 million primordial follicles, but by the menopause few can be found (Baker, 1971). During the reproductive life of a woman, only about 400 of these 2 million primordial follicles undergo ovulation; this indicates that the vast majority (over 99.9%) are destined to become atretic. Those few follicles that do attain ovulatory maturity do so only because they are protected from atresia by exposure to the correct gonadotrophic environment.

The initiation of primordial follicle growth and the formation of a small growing primary follicle (defined as a follicle containing a fully grown oocyte, two or more layers of granulosa cells and a basement lamina) occurs independently of pituitary gonadotrophins and is subject only to intra-ovarian controls (Peters *et al.* 1975). At about the time the growing primary follicle acquires theca interna cells, further growth and

maturation of the follicle become completely dependent on the pituitary gonadotrophins—luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and, to a lesser extent, prolactin. In common with other protein hormones LH and FSH interact with ovarian cells through specific plasma membrane receptors. Autoradiographic studies with rat ovarian tissue indicate that LH binds to thecal tissue and to receptors that develop on granulosa cells during the final stages of follicular maturation (Richards & Midgley, 1976). The binding capacity of thecal tissue for LH is related to the stage of follicular development, studies in animal species indicating that thecal cells from pre-ovulatory follicles bind considerably more LH than do thecal cells from small developing follicles (Channing & Kammerman, 1974; Richards, 1979). Interaction of LH with thecal receptors stimulates steroidogenesis. Studies with isolated thecal tissue preparations from several species (including the human) indicate that the predominant steroids secreted in response to LH stimulation are testosterone and androstenedione, thecal cells having only a relatively limited capacity to aromatize these androgens to oestradiol-17 $\beta$  (Makris & Ryan, 1975; Erickson & Ryan, 1976; Fortune & Armstrong, 1978; Tsang *et al.* 1979). In contrast to LH, FSH binds exclusively to granulosa cells (Richards & Midgley, 1976). Studies with rat granulosa cells indicate that FSH receptors are present by the time the primary follicle has developed and, although the number of granulosa cells in the follicle increases during subsequent follicular maturation, the number of FSH receptors per cell and their binding affinity do not change (Nimrod *et al.* 1976).

Interaction of FSH with granulosa-cell receptors stimulates the induction of aromatizing enzymes which convert androgens to oestradiol-17 $\beta$  (Armstrong & Papkoff, 1976; Erickson & Hsueh, 1978; Moon *et al.* 1978). Human granulosa cells, in common with those of other species, have only a limited capacity for *de novo* oestradiol-17 $\beta$  synthesis, relative to their aromatizing potential under FSH stimulation, because of their limited capacity to synthesize androgens (Channing, 1969; Fowler *et al.* 1978; Moon *et al.* 1978; Tsang *et al.* 1979). Since LH stimulates androstenedione and testosterone synthesis by thecal cells it has been proposed that, as well as being secreted into the ovarian vein, thecal androgen may also diffuse through the basement membrane into granulosa cells, thereby providing additional substrate for the FSH-induced aromatizing enzymes (see review by Armstrong & Dorrington, 1977). According to this so-called "two-cell concept" oestradiol-17 $\beta$  synthesis, which is the hallmark of the developing follicle, results from a co-operative interaction between the cellular actions of LH and FSH; LH stimulates predominantly androgen synthesis by thecal cells while oestradiol-17 $\beta$  synthesis is stimulated by FSH through its induction of aromatizing enzymes in granulosa cells.

The relative contribution of thecal and granulosa cells to total oestradiol-17 $\beta$  secretion by the human follicle remains uncertain. The developing follicle secretes mainly androgen, the concentration of androgen in human ovarian venous blood throughout the follicular phase being 3-10-fold higher than that of oestradiol-17 $\beta$  (McNatty *et al.* 1976). This reflects the relative capacity of thecal tissue to synthesize androgen and oestradiol-17 $\beta$  (as indicated by *in-vitro* studies with thecal tissue), and it has been suggested that the androgen and oestradiol-17 $\beta$  found in ovarian venous blood (extrafollicular steroids) are secreted by thecal tissue under the influence of LH (Baird, 1977; Channing *et al.* 1978). In human follicles containing FSH in follicular fluid there is a highly significant positive corre-

lation between follicular fluid concentrations of oestradiol-17 $\beta$  (intrafollicular oestradiol-17 $\beta$ ) and the number of granulosa cells in the follicle, and it has been suggested that up to 90% of this intrafollicular oestradiol-17 $\beta$  originates from granulosa cells exposed to FSH (McNatty & Baird, 1978). The androgen precursor of this intrafollicular oestradiol-17 $\beta$  is most likely a combination of androgen synthesized *de novo* by the granulosa cells and thecal androgen which diffuses into the granulosa cells, though the relative contributions of these two sources remain highly controversial at present.

These interactions between LH and FSH together with the cyclical changes in plasma concentrations of LH and FSH occurring throughout the menstrual cycle provide an attractive mechanism to account for the regulation of follicular growth. Plasma concentrations of both LH and FSH rise sharply during the first 2-3 days of the menstrual cycle. While plasma LH concentrations remain elevated and relatively constant for the remainder of the follicular period, plasma FSH concentrations decline during the mid-late follicular periods because of negative feedback by oestradiol-17 $\beta$  secreted by the developing follicle (Midgley & Jaffe, 1968). At the start of a menstrual cycle small growing follicles will be exposed to the elevated concentrations of LH and FSH. Interaction of LH with thecal cells stimulates extrafollicular androgen and oestradiol-17 $\beta$  secretion, while FSH, by inducing aromatizing enzymes in the granulosa cells, stimulates intrafollicular production of oestradiol-17 $\beta$ . The combined actions of FSH and oestradiol-17 $\beta$  are responsible for the steady increase in size of the follicle as it develops throughout the follicular period, promoting both granulosa-cell proliferation and the accumulation of follicular fluid (Goldenberg *et al.* 1972).

Accumulation of follicular fluid is an essential component of follicular development, since this provides a means by which the avascular granulosa cells in the follicle can be exposed to an environment different from that of serum and neighbouring follicles. The growing follicle has the ability to accumulate FSH, since follicular fluid concentrations steadily increase as the follicle matures, despite the decreasing concentrations of plasma FSH during the mid-late follicular period (McNatty *et al.* 1975a). The developing follicle also accumulates steroids in follicular fluid, in particular oestradiol-17 $\beta$ , the concentration of which in pre-ovulatory follicles reaches as high as 3.5  $\mu$ g/ml (Baird & Fraser, 1975; McNatty *et al.* 1975a). It is this accumulation and retention of FSH and oestradiol-17 $\beta$  in follicular fluid that maintain the growth and maturation of the developing follicle during the mid-late follicular period when concentrations of plasma FSH decline. Other follicles emerging from pre-antral growth during this period of declining concentrations of plasma FSH are unable to accumulate FSH (McNatty *et al.* 1975a) and are "androgenic" in nature, high concentrations of androstenedione ( $\approx$ 700 ng/ml) but only low concentrations of oestradiol-17 $\beta$  ( $\approx$ 150 ng/ml) being found in their follicular fluid (McNatty *et al.* 1976; McNatty & Baird, 1978). Presumably the granulosa cells of those follicles exposed only to low concentrations of FSH fail to develop an active aromatizing enzyme system to aromatize androgens synthesized by the follicle in response to plasma LH, the concentrations of which remain elevated throughout the mid-late follicular period. This contrasts with those follicles destined to ovulate which having accumulated FSH are "oestrogenic" in nature and have follicular fluid rich in oestradiol-17 $\beta$  (up to 3.5  $\mu$ g/ml) with relatively low concentrations of androstene-

dione ( $\approx$ 250 ng/ml) (McNatty *et al.* 1976; McNatty & Baird, 1978).

Studies in laboratory animals indicate that androgens promote follicular atresia (Payne & Runser, 1958; Louvet *et al.* 1975) and it has been suggested that the accumulation of androgen in those follicles devoid of FSH causes their subsequent atresia. However, it can also be argued that the failure to accumulate oestradiol-17 $\beta$  in follicles devoid of FSH causes follicular atresia, with the accumulation of androgen aggravating the situation. It is likely that only those follicles emerging from pre-antral growth at the time of elevated concentrations of plasma FSH are likely to accumulate sufficient FSH to induce adequate aromatizing activity in the granulosa cells. This in turn would ensure that the ratio of intrafollicular oestradiol-17 $\beta$  to androgen is sufficiently high to prevent atresia and allow the follicles to attain ovulatory maturity. Plasma FSH concentrations are elevated only for a short period in the menstrual cycle, during the very late luteal and early follicular phases. As follicular growth is a continuous process, most follicles will emerge from pre-antral growth out of phase with elevated concentrations of plasma FSH and so will be destined to become atretic because of a low ratio of intrafollicular oestradiol-17 $\beta$  to androgen. During the early luteal phase there is also an increase in the number of developing antral follicles, presumably in response to the mid-cycle surge of gonadotrophins (Block, 1951). In contrast to follicles developing in response to the elevated concentrations of plasma FSH during the early follicular phase, early luteal follicles fail to secrete oestradiol-17 $\beta$  (Baird & Fraser, 1975) and degenerate by the mid-late luteal phase. This failure to mature probably occurs because, as well as being exposed to elevated concentrations of FSH, these follicles are also exposed to the peak concentrations of plasma LH of the mid-cycle ovulatory surge which arrests follicular growth (as in pre-ovulatory follicle). Thus only those follicles developing in phase with the cyclical changes in plasma gonadotrophins will be exposed to the correct gonadotrophic environment, elevated concentrations of FSH during the early follicular phase being required to prevent atresia and promote follicular growth and development, while the mid-cycle surge of LH arrests this growth and induces the follicle to ovulate.

## 2 Granulosa-Cell Maturation

The maintenance of human pregnancy for the first 6-8 weeks following a successful fertilization is dependent upon progesterone secreted by the corpus luteum (Csapo *et al.* 1972); it is only after this period that the placenta alone is capable of producing adequate quantities of progesterone to support the pregnancy through to term. The human corpus luteum is composed predominantly of cells formed by luteinization of the granulosa cells in the pre-ovulatory follicle in response to the ovulatory surge of LH. These cells are thought to be the principal source of progesterone production by the human corpus luteum, large quantities of progesterone being secreted by human granulosa cells luteinized *in vitro* (McNatty & Sawers, 1975). In order that the human corpus luteum can produce sufficient progesterone to sustain pregnancy it is necessary that the pre-ovulatory follicle contain an adequate number of granulosa cells and that these cells be capable of responding to LH by luteinizing and secreting progesterone.

The accumulation of FSH and oestradiol-17 $\beta$  by the developing follicle is essential to ensure maximum proliferation

of follicular granulosa cells (Goldenberg *et al.* 1972; McNatty & Sawers, 1975). A specific nuclear receptor for oestradiol-17 $\beta$  has been demonstrated in rat granulosa cells (Richards, 1975), and interaction with this receptor probably mediates the mitotic activity of oestradiol-17 $\beta$ . FSH is only weakly mitogenic compared with oestradiol-17 $\beta$  but enhances the latter's mitotic activity (Goldenberg *et al.* 1972). A prolonged exposure of several days' duration to FSH and oestradiol-17 $\beta$  is also required by human granulosa cells to develop their capacity to synthesize maximum amounts of progesterone in response to LH (McNatty & Sawers, 1975). This has also been demonstrated in other species, where it has been found that the prolonged exposure to FSH and oestradiol-17 $\beta$  is required to induce the formation of LH receptors on the granulosa cells (Zeleznik *et al.* 1974; Nimrod *et al.* 1977). The accumulation of FSH and oestradiol-17 $\beta$  by the developing follicle throughout the follicular phase results in a steady increase in the number of LH receptors on granulosa cells, these reaching a maximum in the pre-ovulatory follicle. In the absence of FSH and oestradiol-17 $\beta$ , few LH receptors appear on granulosa cells and consequently these cells cannot respond to the ovulatory surge of LH by luteinizing and secreting progesterone.

The mechanism by which FSH and oestradiol-17 $\beta$  act together to stimulate formation of granulosa-cell LH receptors has been most thoroughly studied in the rat. In common with other protein hormones, FSH on interacting with its specific plasma membrane receptors activates adenylate cyclase (Tsafiri *et al.* 1976). While neither the binding affinity nor the number of FSH receptors per granulosa cell increases during follicular development in the rat (Nimrod *et al.* 1976), oestradiol-17 $\beta$  enhances the ability of FSH to stimulate cyclic AMP production, and also increases the intracellular concentrations of cyclic-AMP-binding proteins in rat granulosa-cell cytosol (Richards, 1979). Thus, while FSH initially stimulates the synthesis of intrafollicular oestradiol-17 $\beta$  by inducing aromatizing enzymes in granulosa cells, oestradiol-17 $\beta$  in turn acts within the granulosa cells essentially to amplify the intracellular actions of FSH. It is this amplification of the actions of FSH by oestradiol-17 $\beta$  which, at least in the rat, appears necessary to promote the increase in granulosa-cell LH receptor formation. Additional studies in animal species indicate that FSH itself is capable of stimulating progesterone secretion by granulosa cells isolated from follicles at various stages of follicular maturity (Armstrong & Dorrington, 1976; Thanki & Channing, 1978). This effect is independent of LH receptor formation (Hillier *et al.* 1978) and suggests that FSH may have an additional, but as yet largely undefined, role in the induction of the progesterone-synthesizing capacity of granulosa cells.

While the accumulation of high concentrations of intrafollicular androgens may promote atresia, recent studies suggest that some androgen may be necessary for full development of the progesterone-secreting capacity of granulosa cells in their response to gonadotrophins. Studies with isolated rat (Armstrong & Dorrington, 1976; Hillier *et al.* 1977; Lucky *et al.* 1977) and porcine (Schomberg *et al.* 1976) granulosa cells indicate that androstenedione, testosterone and dihydrotestosterone can both stimulate and synergize with FSH stimulation of progesterone production in vitro. This stimulatory action of androgens appears to be independent of gonadotrophin receptor induction (S G Hillier, A J Zeleznik, R A Knazek and G T Ross, 1977<sup>1</sup>), but may act in part by enhancing the coupling of FSH receptors to adenylate cyclase (Nimrod & Lindner, 1976). The possible physiological relevance of these in-vitro findings

has recently been strengthened by the demonstration that intra-ovarian application of anti-androgen in vivo reduces the subsequent capacity of follicular granulosa cells to secrete progesterone in vitro (Schomberg *et al.* 1978). Androgen enhancement of progesterone secretion by human granulosa cells has not yet been demonstrated, and whether the relatively low concentrations of intrafollicular androgen found in developing follicles are required for the proper development of granulosa-cell progesterone-secreting capacity is not known.

### 3 Ovulation

The steadily increasing amounts of oestradiol-17 $\beta$  secreted by the developing follicle elicit a positive feedback effect on the hypothalamus and pituitary, resulting in the mid-cycle surges of LH and, to a much lesser extent, of FSH. The mid-cycle surge of LH initiates a series of events within the pre-ovulatory follicle, which culminates in ovulation and corpus luteum formation. One of the most striking events is the change in follicular steroidogenesis, the pre-ovulatory surge of LH stimulating progesterone production but inhibiting that of oestradiol-17 $\beta$  (Landgren *et al.* 1977). This switch in follicular steroidogenesis has also been demonstrated in other species, both in vivo and with isolated pre-ovulatory follicles in vitro, where it has been shown that the rise in progesterone production is accompanied by a fall in both androgen and oestradiol-17 $\beta$  production (Moor, 1974; Hillensjö *et al.* 1976).

Follicular fluid concentrations of steroid also reflect this change in both the rabbit (Bahr, 1978) and the rat (Goff & Henderson, 1979) in that, following an ovulatory surge of LH, progesterone concentrations rise while those of androgen and oestradiol-17 $\beta$  fall. The mechanism(s) of this inhibitory action of LH on androgen and oestradiol-17 $\beta$  production by the follicle just before ovulation is still obscure. It has been suggested that LH may stimulate production of a protein which inhibits the enzymes involved in the cleavage of the side chain of progesterone at C<sub>(17)</sub>-C<sub>(20)</sub> (Lieberman *et al.* 1975). Exposure to high concentrations of LH similar to those occurring at ovulation can cause desensitization or "down regulation" of LH receptors, in that they lose their capacity to respond to LH (Hunzicker-Dunn & Birnbaumer, 1976; Lindner *et al.* 1977). Desensitization of thecal LH receptors in response to the pre-ovulatory surge of LH could therefore also account for the fall in androgen production and subsequently in that of oestradiol-17 $\beta$  by the follicle. In addition, Katz & Armstrong (1976) have suggested that LH inhibits oestradiol-17 $\beta$  production by reducing ovarian androgen aromatizing activity and, consistent with this, Henderson & Moon (1979) have recently demonstrated that luteinization of granulosa cells is accompanied by a progressive loss in their aromatizing capacity. While their aromatizing activity may decline, luteinization of granulosa cells in response to the pre-ovulatory surge of LH stimulates production of progesterone. This stimulatory action of LH on ovarian steroidogenesis is well established and is understood to be mediated through cyclic AMP (Marsh, 1976; Channing & Tsafiri, 1977).

As well as inducing "functional" luteinization, i.e., stimulating progesterone production, LH on interacting with its plasma membrane receptors also induces "structural" luteinization of the granulosa cells, in that they undergo extensive hypertrophy and develop an agranular endoplasmic reticulum, diffuse lipo-

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proteins and pleomorphic mitochondria with a complex system of tubular internal cristae (Guraya, 1971)—all components essential for the cells to sustain their elevated levels of progesterone synthesis and secretion. In addition, luteinization is accompanied by a reduction in mitotic activity of the granulosa cells (Delforge *et al.* 1972). Thus the pre-ovulatory surge of LH in effect terminates the growth and maturational phase of granulosa-cell development and promotes their differentiation into luteinized granulosa cells whose progesterone synthesis and secretion can support the initial stages of pregnancy should a successful fertilization occur.

The mechanism(s) by which the pre-ovulatory LH surge induces follicular rupture remains uncertain. Proteolytic enzymes have been proposed (Espey, 1974; Rondell, 1974), together with the suggestion that their synthesis may be regulated by the changes in follicular steroids occurring just before ovulation—notably the fall in androgen and oestradiol-17 $\beta$  but rise in progesterone concentrations. In addition, recent studies indicate that rat granulosa cells under gonadotrophic stimulation secrete a plasminogen activator which can activate the inactive proteolytic enzyme, plasminogen. This enzyme, which is present in rat pre-ovulatory follicular fluid, could possibly be responsible for the observed follicular dissolution that occurs at ovulation (Beers *et al.* 1975). Prostaglandins also have an essential role in the mechanism by which LH causes follicular rupture. Systemic administration of indomethacin, a potent inhibitor of prostaglandin synthesis, blocks both spontaneous and LH-induced ovulation in rats and rabbits (Armstrong & Grinwich, 1972; Grinwich *et al.* 1972). Several studies indicate that this block is exerted at the level of the follicle (Tsafiri *et al.* 1972; Tsafiri *et al.* 1973; Armstrong *et al.* 1974). Organ culture of human (Plunkett *et al.* 1975) and rabbit (Marsh *et al.* 1974; Moon *et al.* 1974) follicles indicates that LH stimulates prostaglandin E (PGE) and prostaglandin F (PGF) synthesis, and both these prostaglandins have been implicated in the ovulatory process (Yang *et al.* 1974; LeMaire *et al.* 1975).

Recent studies suggest that this involvement of prostaglandins in follicular rupture may depend on their ability to influence smooth-muscle contractility. Examination by fluorescent microscopy of serial sections of rat follicles treated with specific antisera to smooth-muscle myosin or actin, and then treated with fluorescein-labelled anti- $\gamma$ -globulin, showed that the external theca contained several layers of elongated cells which reacted intensely with both the anti-actin and anti-myosin (Amsterdam *et al.* 1977). This, together with the fact that the thecal cells are rich in microfilaments (Coutinho & Maia, 1972), indicates that the theca externa cells have the properties of smooth-muscle cells. Moreover, the follicle wall is capable of a contractile response to prostaglandins which is consistent with this concept (Okamura *et al.* 1974). Thus thecal contractions, stimulated by prostaglandins synthesized in response to the ovulatory surge of LH, may contribute to ovum extrusion by providing the final mechanical disruption necessary to rupture the follicle wall after it has already been considerably weakened by the action of proteolytic enzymes.

### 4 Corpus Luteum Function

After ovulation has taken place, concentrations of gonadotrophins in blood fall to the lowest levels seen in the menstrual cycle, because of the negative feedback effect of steroids secreted by the corpus luteum (Midgley & Jaffe, 1968).

As a consequence follicular development is minimal, and it is not until the corpus luteum regresses that the concentration of gonadotrophins can rise to levels sufficient to promote the development and maturation of a fresh crop of graafian follicles. While the ovulatory surge of LH initiates corpus luteum formation, the continued secretion of progesterone for the duration of a normal luteal phase (14–16 days) requires the support of the low concentrations of LH present in blood during the luteal phase. In the absence of this support the corpus luteum regresses within seven days (Vande Wiele *et al.* 1970). If the recently shed ovum is not successfully fertilized it is imperative that a new cycle be initiated, and this necessitates the regression of the corpus luteum. In many non-primate species the natural luteolysin responsible for the regression of the corpus luteum during a non-fertile cycle is PGF<sub>2 $\alpha$</sub>  (Horton & Poyser, 1976). While the primate luteolysin has not yet been conclusively identified, there is evidence that it too may be PGF<sub>2 $\alpha$</sub> , possibly produced by the ovary itself in response to oestradiol-17 $\beta$ . This evidence is as follows: (i) PGF<sub>2 $\alpha$</sub>  is luteolytic in monkeys (Russell, 1975); (ii) a specific receptor for PGF<sub>2 $\alpha$</sub>  is present in the human corpus luteum (Powell *et al.* 1974); (iii) elevated concentrations of PGF<sub>2 $\alpha$</sub>  are associated with the morphological regression of the human corpus luteum (Shutt *et al.* 1976); (iv) PGF<sub>2 $\alpha$</sub>  inhibits progesterone production by human luteinized granulosa cells in tissue culture (Henderson & McNatty, 1975; McNatty *et al.* 1975b); (v) human corpora lutea have the enzymic capacity to synthesize substantial amounts of PGF<sub>2 $\alpha$</sub>  (Challis *et al.* 1976); and (vi) the luteolytic action of oestradiol-17 $\beta$  is probably mediated by stimulating intra-ovarian production of PGF<sub>2 $\alpha$</sub>  (Auletta *et al.* 1976; Auletta *et al.* 1978). In non-primate species, PGF<sub>2 $\alpha$</sub>  is thought to initiate luteal regression by inhibiting LH activation of adenylate cyclase, thereby reducing intracellular levels of cyclic AMP which in turn leads to reduced progesterone production (Henderson *et al.* 1977; Thomas *et al.* 1978). Studies in the monkey indicate that the corpus luteum progressively loses its capacity to produce progesterone in response to LH (Stouffer *et al.* 1977); this could be brought about by endogenous PGF<sub>2 $\alpha$</sub>  inhibiting LH-activated adenylate cyclase.

In a fertile cycle, the human corpus luteum does not regress but is maintained, almost certainly by the luteotrophic action of human chorionic gonadotrophin (hCG) secreted by the implanting blastocyst (Vaitukaitis *et al.* 1971). hCG mimics the action of LH in that it binds to LH receptors and stimulates ovarian steroidogenesis through the activation of adenylate cyclase. Recent studies suggest that hCG may prevent luteal regression by interacting with vacant luteal LH receptors, and by a "saw" type of interaction prevent PGF<sub>2 $\alpha$</sub>  from interacting with its luteal receptors. Any inhibition of progesterone production is thereby prevented (Henderson & McNatty, 1977).

### 5 Prolactin

While the foregoing has concentrated on the roles of LH and FSH in regulating ovarian activity (their actions being summarized in Table 1), there is now increasing evidence that a third pituitary gonadotrophin, prolactin, may also regulate human ovarian activity at the ovarian level. Unlike LH and FSH, prolactin concentrations in blood throughout the menstrual cycle show no consistent pattern (McNeilly & Chard, 1974). There is, however, a cyclical change in follicular fluid concentrations of prolactin, high concentrations being found in follicles during the early follicular phase, the concentrations then falling

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TABLE I. Summary of actions of gonadotrophins on the ovary

Follicular development	Ovulation	Luteal function
Elevated concentrations of plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) during early follicular phase promote development of follicle beyond primary stage	Pre-ovulatory LH surge inhibits follicular androgen and oestradiol-17 $\beta$ production	Low concentrations of plasma LH and FSH during luteal phase prevent development of follicles beyond primary stage. Low concentrations of plasma LH are sufficient, though, to maintain progesterone secretion by corpus luteum
LH binds to thecal cells and stimulates extrafollicular androgen and oestrogen secretion	Pre-ovulatory LH surge inhibits granulosa-cell proliferation and initiates the cells' functional and structural luteinization	During the pregnant cycle, human chorionic gonadotrophin secreted by the conceptus prevents luteal regression, possibly by blocking luteolytic action of prostaglandin F <sub>2a</sub>
FSH binds to granulosa cells and induces aromatizing activity, thereby preventing intrafollicular accumulation of androgens and atresia by promoting synthesis of oestradiol-17 $\beta$	Increased progesterone production by granulosa cells on luteinizing is accompanied by reduced aromatizing activity	
Accumulation of FSH and oestradiol-17 $\beta$ in follicular fluid of developing follicles promotes (i) further follicular fluid formation; (ii) granulosa-cell proliferation; (iii) maturation of granulosa-cell progesterone-synthesizing capacity; and (iv) development of LH receptors on granulosa cells	Pre-ovulatory LH surge stimulates follicular prostaglandin production which is essential for follicular rupture	
Prolactin is possibly involved in developing capacity of granulosa cells to synthesize and secrete progesterone in response to ovulatory LH surge	Pre-ovulatory LH surge may stimulate formation of proteolytic enzymes involved in follicular rupture	

progressively towards the late follicular phase and rising again during the luteal phase (McNatty *et al.* 1975a). Similar findings have also been made with sheep follicles during the oestrous cycle (McNatty, 1977). What function these fluctuating prolactin concentrations serve remains largely speculative. Specific receptors for prolactin have been demonstrated in the human ovary (Saito & Saxena, 1975), though their relative distribution amongst the various cell types is not known. Prolactin receptors have, however, been demonstrated in rat (Richards & Williams, 1976) and porcine (Rolland & Hammond, 1975; Rolland *et al.* 1976) granulosa cells and luteal tissue. Studies with porcine granulosa cells indicate that the number of available binding sites in each cell decreases during follicular maturation; this could result from increased occupancy by endogenous prolactin. If a similar situation existed in the human, then binding of the prolactin present in follicular fluid by receptors being formed on the granulosa cells could account for the falling prolactin concentrations seen in follicular fluid during the follicular phase. The possibility that prolactin may interact with granulosa cells to promote their maturation has been raised by the finding that prolactin is essential for maximum production of progesterone by human luteinized granulosa cells in tissue culture (McNatty *et al.* 1974). In addition bromocriptine treatment both of women with normal menstrual cycles (Schulz *et al.* 1976) and hyperprolactinaemic amenorrhoeic

patients (Mühlenstedt *et al.* 1977), in whom prolactin levels were suppressed below normal, was associated with corpus luteum insufficiency, which would be consistent with the granulosa cells failing to mature sufficiently before ovulation occurred. How prolactin might act is unclear, though studies in the rat indicate that prolactin is involved in the formation and maintenance of luteal LH receptors (Holt *et al.* 1976; Richards & Williams, 1976) and may influence the pool of steroid precursors available for progesterone synthesis (Armstrong *et al.* 1970; Behrman *et al.* 1970). Receptors for prolactin, like those for LH and FSH, appear to be located on the plasma membrane (Saito & Saxena, 1975) but, in contrast to LH and FSH, interaction of prolactin with its receptor does not stimulate adenylate cyclase (Mason *et al.* 1973) and no "second messenger" for prolactin has been found. Indeed, recently prolactin has been located inside rat lutein cells and it has been suggested that prolactin may have intracellular sites of action—the plasma membrane receptor acting only to facilitate its entry into the cell (Nolin, 1978).

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## Luteinization of bovine granulosa cells and corpus luteum formation associated with loss of androgen-aromatizing ability

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**Summary.** The relative aromatizing ability of bovine luteinizing granulosa cells and dispersed luteal cells in tissue culture was studied. Luteinization of granulosa cells, as indicated by steadily increasing progesterone production (from 50 to 300 ng/10<sup>5</sup> cells/day over 4–5 days), was accompanied by a dramatic reduction in their capacity to aromatize exogenous androgen; oestradiol-17 $\beta$  production falling from 200 to <10 ng/10<sup>5</sup> cells/day over 4–5 days. Luteal cells also had only a very limited capacity to aromatize exogenous androgen, maximum oestradiol-17 $\beta$  production being <600 pg/10<sup>5</sup> cells/day. The loss in aromatizing capacity of granulosa cells during luteinization was also reflected in the relative endogenous steroid content of non-luteinized granulosa cells and luteal tissue, the former containing high levels of oestradiol-17 $\beta$ ,  $\leq$ 28 ng/mg protein, while the latter, although containing substantial amounts of testosterone,  $\leq$ 5.7 ng/g tissue, contained very little oestradiol-17 $\beta$ ,  $\leq$ 0.35 ng/g tissue. These findings suggest that luteinization of bovine granulosa cells and subsequent corpus luteum formation is associated with a loss in androgen aromatase activity.

### Introduction

Studies in several species indicate that while follicular granulosa cells have only a very limited capacity to synthesize androgens (Bjersing & Carstensen, 1967; Channing, 1969; Lacroix, Eechaute & Leusen, 1974; Fortune & Armstrong, 1977; Fowler, Fox, Edwards, Walters & Steptoe, 1978), they can, under the influence of follicle-stimulating hormone (FSH), readily aromatize androgens to oestradiol-17 $\beta$  (Dorrington, Moon & Armstrong, 1975; Moor, 1977; Fortune & Armstrong, 1978). It has therefore been suggested that granulosa cells may contribute to follicular oestradiol-17 $\beta$  production through aromatization of androgens secreted by other ovarian compartments, in particular thecal tissue (see review by Armstrong & Dorrington, 1977). Little, however, is known of the way in which the aromatizing capacity of granulosa cells is affected by their luteinization during corpus luteum formation. The purpose of the present investigation was to study and compare androgen aromatization by bovine granulosa cells undergoing luteinization *in vitro*, and by dispersed bovine luteal cells *in vitro*.

### Materials and Methods

#### *Radioimmunoassay of culture medium*

The progesterone content of the culture medium was assayed directly by radioimmunoassay (RIA), utilizing the antibody and methods described previously by Orczyk, Hichens, Arth & Behrman (1974). The antiserum was prepared against 11 $\alpha$ -hydroxyprogesterone hemisuccinate

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conjugated to bovine serum albumin (BSA). The antiserum showed negligible cross-reactivity (<0.1%) with androgens and oestrogens. The validity of direct assay of culture medium was checked by comparing values obtained by this method with values for petroleum ether-extracted aliquots taken from the same samples. Regression analysis of the results gave the equation  $y = 0.90x - 13.37$ , the coefficient of linear correlation being 0.98 ( $n = 19$ ). The limit of sensitivity of the assay was 25 pg per tube. Oestradiol-17 $\beta$  and oestrone were measured in diethyl ether-extracted aliquots of culture media by RIA using antisera and methods described and validated previously by Dorrington & Armstrong (1975) and Dorrington, Fritz & Armstrong (1976) respectively. The oestradiol-17 $\beta$  antiserum was raised against oestradiol-17 $\beta$ -6-carboxymethyl-oxime-BSA and the oestrone antiserum against oestrone-6-(O-carboxymethyl) oxime-BSA. Both antisera had low cross-reactivity (<10%) with other phenolic steroids and negligible cross-reactivity (<0.1%) with neutral steroids. The limit of sensitivity of both oestrogen assays was 5 pg per tube. The intra- and inter-assay coefficients of variation of all the above assays were <10%.

#### *Culture of granulosa cells*

Bovine ovaries were obtained from cattle within 1 h of their slaughter at a local abattoir and transported to the laboratory in chilled Minimum Essential Medium (Modified) with Earle's Salts (EMEM) and supplemented with HEPES buffer (20 mM), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50  $\mu$ g/ml), amphotericin B (0.625  $\mu$ g/ml) and non-essential amino acids (all reagents obtained from Flow Laboratories, Mississauga, Ontario, Canada). Large antral follicles (7–15 mm in diameter) were dissected out, the follicle wall slit open and the granulosa cells gently scraped with a platinum loop into chilled EMEM, supplemented as above. The harvested cells were pooled, washed three times with the above medium and an aliquot was taken to determine total cell number using a haemocytometer, and cell viability using nigrosin dye. Each dish contained  $1.5\text{--}2.0 \times 10^5$  'live' cells which were cultured at 36°C in a humidified incubator on 15 mm-diameter round plastic coverslips (Thermanox: Lux Scientific Corp., California, U.S.A.) in 1 ml culture medium consisting of 10% (v/v) fetal bovine serum and 90% EMEM without HEPES buffer but otherwise supplemented with glutamine, antibiotics and non-essential amino acids as described above. The gas phase was 5% CO<sub>2</sub>:95% air. Exogenous testosterone or androstenedione (both from Sigma) was added daily throughout the culture period as 10 ng to 1  $\mu$ g in 10  $\mu$ l ethanol, control cultures receiving 10  $\mu$ l ethanol only. The culture medium was replaced daily and stored at -20°C until RIA of steroids. At the end of the culture period the cells were washed and stained with haematoxylin and eosin. The number of cells remaining was estimated by counting the number of cells within a 0.3 mm<sup>2</sup> area at 12 sampling points on the coverslip, the total number of cells being estimated by extrapolation. Luteinization of the granulosa cells was indicated by a sustained production of progesterone accompanied by cellular hyperplasia and hypertrophy with an increase in the cytoplasm:nucleus ratio.

#### *Cell and organ culture of luteal tissue*

*Cell culture.* Individual bovine corpora lutea (CL) were enucleated from ovaries obtained from cattle within 1 h of their slaughter at a local abattoir. The CL were freed of adherent connective tissue and chopped into pieces approximately 2 mm in diameter in Hanks' Balanced Salt Solution without magnesium and calcium (Flow Laboratories, Mississauga, Ontario, Canada), and supplemented with HEPES (20 mM), glutamine (2 mM), and antibiotics as described above (HBS-HGA). The chopped tissue was washed twice with chilled HBS-HGA and incubated for 20 min at 37°C with stirring in HBS-HGA containing 0.2% collagenase (Type II, Sigma). The medium was decanted and the released cells were collected by low-speed centrifugation and stored at 4°C in HBS-HGA. The remaining fragile tissue fragments were reincubated in 0.2% collagenase in HBS-HGA at 37°C and final dispersal of cells was achieved by drawing through a 2 ml syringe tip and a series of needles (18–22 gauge), the cells

being collected by centrifugation. All the released cells were pooled together, filtered through 2 layers of sterile gauze and washed 4 times to remove any traces of collagenase. After estimating cell number and cell viability as above, cultures of  $1.5\text{--}2.0 \times 10^5$  'live' cells (10–30  $\mu\text{m}$  in diameter) were set up exactly as described for the granulosa cells. At the end of the culture period, the number of cells remaining was estimated as described for the granulosa cells.

The age of the CL was estimated from their gross morphological appearance and by histological examination (Donaldson & Hansel, 1965).

*Organ culture.* Bovine luteal tissue fragments approximately 2 mm in diameter were prepared as above. Cultures were initiated by placing 3 or 4 of the tissue fragments on a 25 mm-diameter Millipore filter, pore size 0.45  $\mu\text{m}$ , floating in 1 ml culture medium contained in a Petri dish. The culture medium and incubation conditions were as described for the granulosa cells. At the end of the culture period the tissue fragments were taken up in 1 M-sodium hydroxide for subsequent protein determination by the method of Lowry, Rosebrough, Farr & Randall (1951).

#### *Extraction of steroids from granulosa cells and luteal tissue*

Granulosa cells were harvested from individual large antral follicles (7–15 mm diameter) as described above. An aliquot of the cells was taken up in 1 ml NaOH for subsequent protein determination (Lowry *et al.*, 1951). The remaining cells were homogenized in 1.5 ml redistilled ethanol using a glass/glass homogenizer and shaken for 4 h at room temperature ( $\sim 20^\circ\text{C}$ ). The cellular debris was separated by centrifugation, the supernatant removed and the remains reincubated in 1 ml redistilled ethanol for a further 1 h at room temperature with shaking, before being left to stand overnight at  $4^\circ\text{C}$ . The cellular debris was again separated, the supernatant removed, combined with the earlier one, the volume adjusted to 4 ml and stored at  $-20^\circ\text{C}$  until assayed. Individual CL dissected free of connective tissue were minced, and approximately 1.5 g amounts were homogenized as above in 10 ml redistilled ethanol. The remainder of the procedure was as described for the granulosa cells except that the second incubation volume of ethanol was 5 ml, and that after pooling the two supernatants, the final volume was adjusted to 20 ml.

Aliquots of the ethanolic extractions of the granulosa cells and CL were assayed for oestradiol-17 $\beta$  and testosterone by RIA after column chromatography on Sephadex LH-20 as described by Carr, Mikhail & Flickinger (1971). Testosterone was separated using the solvent system hexane:benzene:methanol (80:10:10 by vol.) while oestradiol-17 $\beta$  was separated using benzene:methanol (85:15 v/v). Recovery was estimated by the addition of 2000 c.p.m. [2,4,6,7,16,17- $^3\text{H}$ ]oestradiol-17 $\beta$  (sp. act. 151 Ci/mmol: New England Nuclear), or 2000 c.p.m. [1,2,6,7- $^3\text{H}$ ]testosterone (sp. act. 90 Ci/mmol: New England Nuclear) to the ethanolic aliquots before chromatography. The oestradiol-17 $\beta$  RIA was as described above. The testosterone RIA was carried out using an antiserum and methods previously described and validated by Auletta, Caldwell & Hamilton (1974). The antiserum was raised against testosterone-3-carboxymethyl-oxime-BSA and cross-reacted (testosterone 100%) with  $5\alpha$ -reduced androstanes as follows: 17 $\beta$ -hydroxy- $5\alpha$ -androstan-3-one (DHT; 53%),  $5\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (30%) and  $5\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (13%). The antiserum showed negligible cross-reactivity ( $<0.1\%$ ) with oestrogens and progesterone. The level of sensitivity of this assay was 12 pg/tube and the intra- and inter-assay coefficients of variations were each  $<12\%$ .

## Results

### *Morphological appearance of dispersed bovine luteal cells*

The typical histological appearance of the collagenase dispersed luteal cells is shown in Plate 1. At least two different cell types were present: (a) large spherical cells having a granular, highly eosinophilic cytoplasm, small prominent nuclei, and diameters in the range 10–30  $\mu\text{m}$ , and (b) small epithelial-type cells with no distinguishing morphological characteristics and  $<5 \mu\text{m}$  in diameter.

*Androgen aromatization by luteinizing granulosa cells*

Table 1 shows oestradiol-17 $\beta$  and progesterone production in response to various concentrations of testosterone during Day 1 and Day 4 of culture. Large amounts of oestradiol-17 $\beta$  were produced in a dose-dependent fashion in response to the added testosterone during Day 1 of culture. By Day 4 there was a dramatic, significant reduction in the ability of the cells to produce oestradiol-17 $\beta$  in response to all the concentrations of added testosterone ( $P < 0.01$ , Student's  $t$  test). In contrast to this fall in oestradiol-17 $\beta$  production, there was a significant ( $P < 0.01$ , Student's  $t$  test) 7-fold increase in progesterone production by the luteinizing granulosa cells, the levels rising from about 35 ng/10<sup>5</sup> cells on Day 1 to about 250 ng/10<sup>5</sup> cells on Day 4 of culture. Testosterone had no effect on progesterone production ( $P > 0.05$ , Student's  $t$  test). Similar results, from a separate experiment, are shown in Text-figs 1(a) and 1(b): the loss in aromatizing capacity was not due to a change in substrate specificity, there being a similar loss in the capacity of the luteinizing granulosa cells to aromatize both testosterone and androstenedione. Moreover, androstenedione was aromatized less effectively than testosterone, there being significantly less oestradiol-17 $\beta$  produced in response to androstenedione during Days 1 and 2 of culture than to the same concentration of testosterone ( $P < 0.02$ , Student's  $t$  test). The fall in oestradiol-17 $\beta$  production throughout the culture period could not be accounted for by any increase in oestrone production, the oestrone levels in the androgen-treated cultures at all times being only 7–11% of those of oestradiol-17 $\beta$ . No detectable oestrone was present in the control cultures. Neither could the loss in aromatizing capacity be explained by changes in cell numbers, since the number of cells remaining in the androgen-treated cultures at the end of the culture period was 69–77% of the starting number. This apparent decline in the cell number over the culture period may be due to the different methods of estimating cell numbers at the start and finish of the culture period. The daily addition of gonadotrophins (250 ng NIH-FSH-S11/ml and 250 ng NIH-LH-B8/ml) had no effect on progesterone or oestradiol-17 $\beta$  production by either the control or androgen-treated cultures. However, it is possible that endogenous gonadotrophins present in the culture medium derived from the calf serum, or the high concentrations of gonadotrophins found in bovine follicular fluid (ranges: 2.2–9.0 ng LH/ml,  $n = 80$ ; 34–200 ng FSH/ml,  $n = 70$ ; K. M. Henderson & A. S. McNeilly, unpublished observations), may have been sufficient to stimulate maximally steroid production by the cells.

**Table 1.** The effect of testosterone (added daily) on the production (mean  $\pm$  s.d. of 4 replicate cultures) of oestradiol-17 $\beta$  and progesterone by bovine granulosa cells during Days 1 and 4 of culture

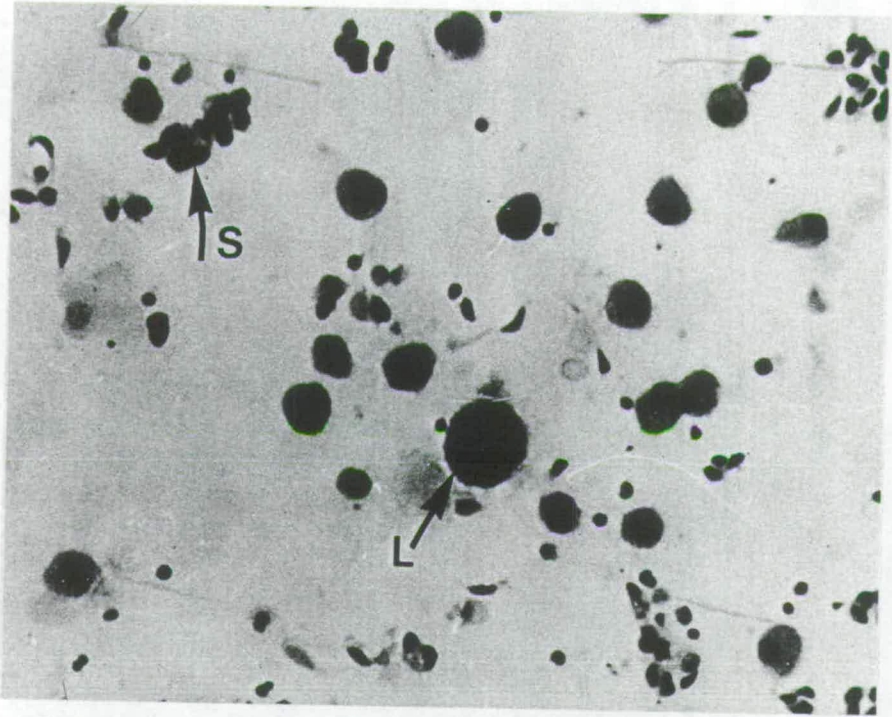
Testosterone conc. (ng/ml)	Oestradiol-17 $\beta$ (ng/10 <sup>5</sup> cells)		Progesterone (ng/10 <sup>5</sup> cells)	
	Day 1	Day 4	Day 1	Day 4
0	0.45 $\pm$ 0.05	N.D.	38 $\pm$ 5	248 $\pm$ 24
10	3.6 $\pm$ 0.4	0.14 $\pm$ 0.03	40 $\pm$ 4	251 $\pm$ 11
50	13.5 $\pm$ 2.2	0.26 $\pm$ 0.02	32 $\pm$ 7	240 $\pm$ 34
100	33 $\pm$ 4	0.32 $\pm$ 0.03	29 $\pm$ 6	260 $\pm$ 29
500	112 $\pm$ 9	0.57 $\pm$ 0.06	35 $\pm$ 5	251 $\pm$ 28
1000	131 $\pm$ 15	0.78 $\pm$ 0.04	34 $\pm$ 5	234 $\pm$ 36

N.D., not detectable.

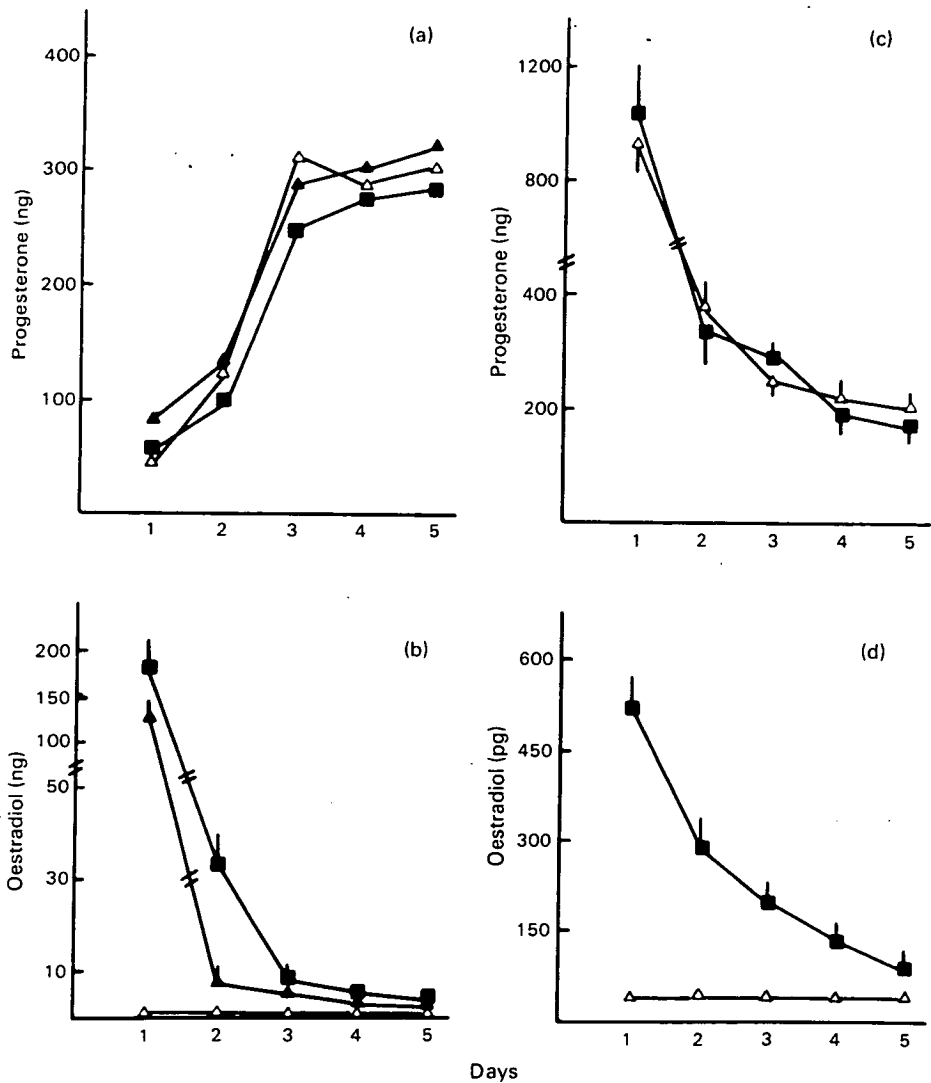
*Androgen aromatization by bovine luteal tissue*

**Luteal cell cultures.** Text-figures 1(c) and 1(d) show typical progesterone and oestradiol-17 $\beta$  production by control and testosterone (1  $\mu$ g/ml/day)-treated cultures of luteal cells prepared from CL judged to be less than about 5 days old on the basis of morphological and histological characteristics as described by Donaldson & Hansel (1965). Progesterone

PLATE 1



Haematoxylin-eosin stained smear of dispersed bovine luteal cells. L = large luteal cells, 10–30  $\mu\text{m}$  in diameter; S = small luteal cells,  $<5 \mu\text{m}$  in diameter.  $\times 350$ .



**Text-fig. 1.** The production (mean  $\pm$  s.d. for  $n = 5$ ) of progesterone (a, c) and oestradiol-17 $\beta$  (b, d) by bovine luteinizing granulosa cells (a, b) and dispersed luteal cells (c, d) in culture with different substrates added daily. All values are for  $10^5$  cells/day.  $\Delta$ , No addition;  $\blacksquare$ , testosterone, 1  $\mu$ g;  $\blacktriangle$ , androstenedione, 1  $\mu$ g. The s.d. lines have been omitted in (a) for clarity, but no significant differences between the groups were observed at any of the 5 days of culture ( $P > 0.05$ , Student's  $t$  test).

production by the luteal cells was maximal on Day 1 but declined thereafter. Examination of the cell types present throughout the culture period revealed that the large luteal cells did not survive well but died, as indicated by the presence of large luteal cells with broken plasma membranes, during the first 48–72 h of culture. In contrast, the small luteal cells underwent hyperplasia and hypertrophy. If the large luteal cells were the principal source of progesterone synthesis, which would be consistent with their morphological appearance, then the decline in progesterone would be compatible with their death.

Significant amounts of oestradiol-17 $\beta$  were produced by luteal cells only in the presence of added androgen. However, the maximum amount of oestradiol-17 $\beta$  produced was very low

(approx. 500 pg/10<sup>5</sup> cells) compared to the amounts produced by the granulosa cells before their luteinization (approx. 200 ng/10<sup>5</sup> cells). Like progesterone production, oestradiol-17 $\beta$  production by the luteal cells fell during the culture period. No detectable oestrone was produced by the luteal cells. While low amounts of oestradiol-17 $\beta$  were produced in response to added testosterone in cultures prepared from newly formed CL, i.e. those estimated to be less than about 5 days old, there was no measurable oestradiol-17 $\beta$  or oestrone produced in response to added testosterone (1  $\mu$ g/ml/day) by cultures prepared from older CL.

**Organ cultures.** To test the possibility that the low aromatizing capacity of the bovine luteal cells might be an artefact of the preparative procedure, androgen aromatization by bovine luteal tissue in organ culture was studied. Table 2 shows daily progesterone and oestradiol-17 $\beta$  production, in the presence and absence of testosterone, by tissue taken from a newly formed CL and cultured for 2 days. The findings are similar to those obtained with the luteal cells, in that oestradiol-17 $\beta$  was produced only in the presence of added androgen, and the amounts produced were very low, relative to progesterone production. As in the studies with luteal cells, steroid production was reduced during the 2nd day of culture. The daily addition of gonadotrophins had little effect on either progesterone or oestradiol-17 $\beta$  production. No detectable oestradiol-17 $\beta$  was produced in similar organ culture studies with CL estimated to be more than about 1 week old.

#### *Testosterone and oestradiol-17 $\beta$ content of granulosa cells and luteal tissue*

The loss in aromatizing capacity of granulosa cells during luteinization, observed in the culture studies, was also reflected in the endogenous concentrations of testosterone and oestradiol-17 $\beta$  found in the non-luteinized granulosa cells, measured at the time of collection, and in CL. While the granulosa cells contained substantial amounts (ng/mg protein) of both testosterone (0.17–8.6,  $n = 10$ ), and oestradiol-17 $\beta$  (2.0–28,  $n = 10$ ), reflecting an active aromatase system, the luteal tissue, although containing appreciable amounts (ng/g tissue) of testosterone (1.6–5.7,  $n = 8$ ), contained only extremely small amounts of oestradiol-17 $\beta$  (not detectable–0.35,  $n = 8$ ), reflecting a relatively inactive aromatase system.

**Table 2.** The production (mean  $\pm$  s.d. of 4 replicate cultures) of oestradiol-17 $\beta$  and progesterone by newly formed (<5 days) bovine CL in organ culture

Treatment (daily)	Day 1		Day 2	
	Progesterone (ng/mg protein)	Oestradiol-17 $\beta$ (pg/mg protein)	Progesterone (ng/mg protein)	Oestradiol-17 $\beta$ (pg/mg protein)
None	515 $\pm$ 45	N.D.	281 $\pm$ 40	N.D.
Testosterone*	525 $\pm$ 63	353 $\pm$ 28	222 $\pm$ 64	269 $\pm$ 26
LH $\dagger$ + testosterone*	597 $\pm$ 110	285 $\pm$ 57	272 $\pm$ 76	238 $\pm$ 49
FSH $\ddagger$ + testosterone*	551 $\pm$ 78	356 $\pm$ 30	301 $\pm$ 36	251 $\pm$ 56
LH $\dagger$ + FSH $\ddagger$ + testosterone*	678 $\pm$ 93	414 $\pm$ 70	372 $\pm$ 47	306 $\pm$ 37

N.D., not detectable.

\* 1  $\mu$ g/ml.

$\dagger$  0.25  $\mu$ g NIH-LH-B8/ml.

$\ddagger$  0.25  $\mu$ g NIH-FSH-S11/ml.

#### Discussion

While bovine non-luteinized follicular granulosa cells can readily aromatize exogenous androgen to oestradiol-17 $\beta$  (Lacroix *et al.*, 1974), the above studies with granulosa cells undergoing luteinization in tissue culture suggest that this capacity rapidly declines as the cells luteinize. This finding is unlikely to be an artefact of the particular culture system used, since bovine luteal tissue,



of which luteinized granulosa cells constitute a major portion, also had only a very limited capacity to aromatize exogenous androgen. Moreover, the relative amounts of oestradiol-17 $\beta$  found in non-luteinized follicular granulosa cells and luteal tissue are also consistent with a reduction in aromatase activity as the granulosa cells luteinize during corpus luteum formation. It could be argued, however, that the endogenous steroid content of the granulosa cells might not necessarily reflect steroidogenesis by the cells, some of the steroids possibly diffusing in from follicular theca cells. This possibility may account for the high levels of testosterone found in the granulosa cells, since bovine granulosa cells have only a very limited capacity to synthesize androgen, while the theca cells can readily synthesize testosterone (Lacroix *et al.*, 1974). It is likely, however, that the oestradiol-17 $\beta$  found in the granulosa cells originated from androgen aromatization in these cells, since theca cells have only a relatively weak aromatase enzyme system (Lacroix *et al.*, 1974). Bovine luteal tissue, although containing appreciable amounts of testosterone, contained extremely low amounts of oestradiol-17 $\beta$ , indicating very low aromatase activity relative to the granulosa cells, which is consistent with the findings of the culture studies. This is also compatible with the early findings of Savard & Telegdy (1965) who used short-term incubation studies of bovine luteal tissue with <sup>14</sup>C-labelled radioactive precursors and concluded that the bovine CL completely lacked any aromatase activity. Using more sensitive techniques of cell culture combined with RIA, however, it was possible, in the present study, to demonstrate limited aromatase activity providing the CL were newly formed, i.e. less than about 5 days old. Maximum progesterone production by the dispersed luteal cells during Day 1 of culture was 3 times greater than the maximum amount produced by the luteinized granulosa cells. This suggests that while the granulosa cells luteinized to a certain extent *in vitro*, luteinization was not complete. Incomplete luteinization might also explain why the luteinized granulosa cells, although having a much reduced aromatase activity than before luteinization, still had a greater aromatase activity than the luteal cells. Progesterone secretion by the luteal cells declined rapidly during culture, probably due to the death of the large cells which had the morphological characteristics of steroid-secreting cells. Why the large luteal cells failed to survive in culture is uncertain, although it is possible that being highly differentiated they were merely less able to adapt to the culture conditions than the less well differentiated granulosa cells. The role of the small luteal cells is also uncertain. While these cells survived well in culture they did not appear to contribute towards steroid secretion, as indicated by the declining levels of progesterone and oestradiol-17 $\beta$ .

The culture conditions seem to be important for demonstrating this loss in aromatizing activity of the granulosa cells during luteinization. In a previous study utilizing bovine granulosa cells from similarly sized follicles, this loss in aromatase activity was not observed (Henderson & Swanson, 1978). These former studies, however, utilized different culture conditions; the culture medium was Medium 199 supplemented with HEPES buffer and contained 20% donor calf serum with air as the gas phase. Under these conditions, the maximum amount of oestradiol-17 $\beta$  which could be produced in response to exogenous androgen was only 200–400 pg/10<sup>5</sup> cells/day. In view of the much greater levels found in the present study, both before and after luteinization, it is possible that the former culture conditions were less favourable for oestradiol-17 $\beta$  production, thereby masking any loss in aromatase activity.

In contrast to the bovine CL which seems to lose completely its capacity to synthesize oestradiol-17 $\beta$ , human and porcine CL do synthesize oestradiol-17 $\beta$  (Hammerstein, Rice & Savard, 1964; Watson & Leask, 1975). Preliminary findings, however, utilizing human and porcine ovarian cells in tissue culture, suggest that the aromatase activity of the granulosa cell of these species is much greater than that of luteal cells (K. M. Henderson, Y. S. Moon & B. K. Tsang, unpublished data). Thus, in these species also, luteinization of granulosa cells and subsequent corpus luteum formation may be associated with a reduction, if not a complete loss, in aromatase activity. The mechanism by which the aromatizing capacity of the bovine granulosa cells is lost during luteinization is uncertain. However, the bovine CL, although

containing substantial amounts of mitochondrial cytochrome P-450, contains relatively little microsomal cytochrome P-450 (McIntosh, Uzgiris, Alonso & Salhanick, 1971). Microsomal cytochrome P-450 is required for androgen aromatization (Savard, 1973). Thus, a loss in microsomal cytochrome P-450 together with a rise in mitochondrial cytochrome P-450 could account for the fall in aromatizing capacity but the rise in progesterone production occurring during luteinization and subsequent corpus luteum formation. This possibility is currently under investigation.

In conclusion, while functional luteinization of granulosa cells has generally been defined only in terms of increasing progesterone production, this study suggests that, at least for bovine granulosa cells, functional luteinization should perhaps also be defined in terms of decreasing androgen aromatizing capacity.

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## Interactions of Estrogen and Androgen with Gonadotropins on Ovarian Progesterone Production<sup>1</sup>

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### ABSTRACT

In hypophysectomized immature female rats, treatment with estradiol-17 $\beta$  (E<sub>2</sub>) *in vivo* for 3 days decreased the ovarian production of progesterone in response to luteinizing hormone (LH). Concomitant treatment with E<sub>2</sub> and purified follicle stimulating hormone (FSH) prevented this inhibitory action of E<sub>2</sub>. The possibility of a positive interaction between E<sub>2</sub> and FSH was further substantiated by similar observations *in vitro*. While E<sub>2</sub> inhibited progesterone accumulation by cultured ovarian cells, this inhibitory action was antagonized by the concomitant presence of FSH in the culture medium. By contrast, dihydrotestosterone (DHT) increased progesterone accumulation by the ovarian cells; concomitant treatment with DHT and FSH further enhanced this stimulatory effect. These results are consistent with the notion that steroid hormones exert local modulatory influences on ovarian responses to gonadotropic hormone stimulation.

### INTRODUCTION

It has long been known that estrogen stimulates ovarian growth (Williams, 1940) and enhances the ovarian response to gonadotropins (Pencharz, 1940; Payne and Runser, 1958). Estradiol-17 $\beta$  (E<sub>2</sub>) has also been shown to interact with follicle stimulating hormone (FSH) to regulate follicular growth (Goldenberg et al., 1972b) and to enhance the induction by FSH of ovarian receptors for FSH as well as for luteinizing hormone (LH) (Richards and Midgley, 1976; Ireland and Richards, 1978). In contrast to these stimulatory effects, we have recently demonstrated an inhibitory action of E<sub>2</sub> on ovarian responsiveness to gonadotropins *in vivo* (Leung et al., 1978). In these studies, E<sub>2</sub> inhibited LH-induced ovarian progesterone production in hypophysectomized, but not in intact immature female rats. The cause(s) for this discrepancy is not known. It has been shown, in the porcine ovary, that E<sub>2</sub> inhibits progesterone secretion by granulosa cells from small (Thanki and Channing, 1976) and medium sized follicles (Schomberg et al.,

1976), but not from large preovulatory follicles (Goldenberg et al., 1972a). Thus, it is possible that the effect of E<sub>2</sub> upon ovarian progesterone may differ depending upon the stage of follicular maturation. Since it is well known that ovaries from hypophysectomized animals fail to mature due to the lack of gonadotropins (Evans, 1939), the present study was undertaken to investigate further the difference in the effects of E<sub>2</sub> on hypophysectomized vs intact immature female rats. The hypothesis tested was that E<sub>2</sub> and FSH may interact to regulate progesterone production by the ovarian cells. For comparison, the effects of androgens (dihydrotestosterone) were also determined, since recent studies have shown that androgens augmented FSH-induced progesterone secretion by cultured rat granulosa cells (Armstrong and Dorrington, 1976; Nimrod and Lindner, 1976).

### MATERIALS AND METHODS

Luteinizing hormone (NIH-LH-B8) was donated by the Hormone Distribution Office, National Institutes of Health, Bethesda, MD. The purified preparation of ovine FSH (FSH-S, batch #S1528C2, with a potency of approximately 50 times that of NIH-FSH-S10 by the hCG augmentation test and an LH activity of less than 0.01 times that of NIH-LH-S19) was provided by Dr. M. R. Sairam, Clinical Research Institute of Montreal, Montreal, Quebec. Estradiol-17 $\beta$  and 17 $\beta$ -OH-5 $\alpha$ -androstane-3-one (DHT) were obtained from Steraloids and Searle, respectively.

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*Interaction of E<sub>2</sub> and FSH  
on Ovarian Progesterone  
Production in vivo*

Sprague-Dawley female rats, hypophysectomized at 21 days of age, were obtained from Hormone Assay Laboratories, Chicago, IL and maintained in light and temperature controlled quarters. They were given Purina laboratory chow *ad libitum* and a choice of water or 3% glucose. Beginning at 24 days of age, the animals were injected s.c., once daily for 3 days, with either sesame oil (0.2 ml) or with E<sub>2</sub> (1 mg in 0.2 ml sesame oil) and/or purified FSH (FSH-S, 2.5 µg in 0.2 ml saline). On the fourth day, either saline or LH (NIH-LH-B8, 10 µg) was injected i.v., according to a 2 × 2 × 2 factorial design. The animals were killed 1 h later by cervical dislocation and each ovary was removed, weighed and homogenized in 1.0 ml absolute ethanol for extraction of steroid hormones.

*Interaction of Steroids and  
Gonadotropins on Progesterone  
Accumulation in vitro*

Intact immature Sprague-Dawley female rats were obtained from Bio-Breeding Laboratories, Ottawa, Canada. The animals were sacrificed by cervical dislocation when 27 days old. The ovaries were removed, freed of adherent connective tissues, and cut into pieces of approximately 2 mm diameter in Hanks' balanced salt solution without magnesium and calcium and supplemented with HEPES (20 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) and amphotericin B (0.625 µg/ml) (HBS-HGA). The chopped tissue was washed twice with chilled HBS-HGA and incubated for 20 min at 37°C with stirring in HBS-HGA containing 0.2% collagenase (Type II, Sigma). The medium was decanted and the released cells collected by low speed centrifugation and stored at 4°C in HBS-HGA. The remaining fragile tissue fragments were reincubated in 0.2% collagenase in HBS-HGA at 37°C and final dispersal of cells was achieved by drawing the suspension through the tip of a 3 ml syringe and a series of needles (18–22 gauge). The cells were then collected by centrifugation for 5 min at 1,000 rpm in an International bench top clinical centrifuge. All the released cells were pooled, filtered through 2 layers of sterile gauze and washed 4 times to remove the collagenase. An aliquot was taken to determine total cell number using a hemacytometer and cell viability (70–80%) using nigrosin dye.

Aliquots of 7.5–10 × 10<sup>5</sup> "live" dissociated ovarian cells were transferred to 1 ml of Minimum Essential Medium (Modified) with Earle's salts and supplemented with glutamine, antibiotics and non-essential amino acids, as described previously (Armstrong and Dorrington, 1976). Exogenous steroids of varying concentrations, as described in Results, were added to the culture medium in 10 µl absolute ethanol, in the presence or absence of exogenous LH (NIH-LH-B8, 1 µg/ml) or purified FSH (FSH-S, 1 µg/ml). Control cultures received 10 µl ethanol. The cells were cultured in Linbro flatbottom plastic wells (17 × 16 mm) for 24 h (chronic treatment) at 37°C under an atmosphere of 5% CO<sub>2</sub>:95% air. In 1 experiment, an additional 4 h culture period (acute treatment) was carried out. At the end of the culture period, the culture medium was pipetted off and

stored at -20°C until radioimmunoassay (RIA) of steroid hormones was performed. The protein contents of the attached viable cells were determined by the method of Lowry et al. (1951), using bovine serum albumin as standards.

*Measurements of Progesterone*

The concentrations of progesterone in the ethanol extracts of the ovaries and in the culture medium were measured directly by RIA, as described by Fortune and Armstrong (1978).

*Statistical Analyses*

Analysis of variance and Duncan's New Multiple Range test were used to test for statistical significance of differences due to treatments. When there was evidence of nonhomogeneity of variance, as determined by Bartlett's test, statistical analyses were performed on logarithmically transformed data (Steel and Torrie, 1960).

## RESULTS

*Interaction of E<sub>2</sub> and FSH  
on Ovarian Progesterone  
Production in vivo*

The effect of *in vivo* treatment of hypophysectomized immature female rats with E<sub>2</sub> and FSH, separately and together for 3 days on their subsequent ovarian progesterone response to LH is shown in Fig. 1. Analysis of variance revealed a highly significant (P<0.01) interaction between FSH and LH. While ovarian progesterone concentrations were significantly elevated (P<0.01) 1 h after LH injection in all groups, pretreatment of the rats with FSH enhanced this acute response to LH. There was also a significant (P<0.01) interaction between E<sub>2</sub> and FSH. While treatment with E<sub>2</sub> alone reduced the LH-induced ovarian progesterone

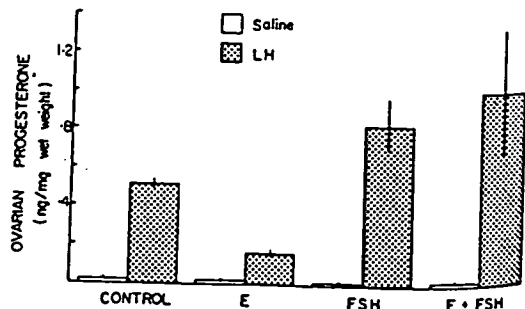


FIG. 1. Effects of treatment of hypophysectomized immature rats with either sesame oil (control), E<sub>2</sub> (1 mg), FSH (2.5 µg) or E<sub>2</sub> + FSH for 3 days on ovarian progesterone concentrations 1 h following the i.v. injection of LH (10 µg) or saline (mean ± SEM; n = 4).

levels, treatment with FSII alone enhanced it and  $E_2$  in the presence of FSII failed to exert any inhibitory effect.

#### *Effects of $E_2$ or DHT on Progesterone Accumulation in vitro*

Enzyme-dissociated ovarian cells from intact immature female rats were cultured for 24 h. The effects of increasing concentrations of  $E_2$  in the culture medium on progesterone are shown in Fig. 2. Analysis of variance revealed highly significant ( $P < 0.01$ ) dose (quadratic) and dose (linear) effects, indicating that when present at concentrations  $> 10$  ng/ml,  $E_2$  inhibited progesterone accumulation. By contrast, presence of exogenous DHT ( $1 \mu\text{g/ml}$ ) in the culture medium significantly ( $P < 0.01$ ) increased progesterone accumulation by the ovarian cells in the same experiment (Fig. 2).

#### *Effects of $E_2$ on LH-Induced Progesterone Accumulation in vitro*

Ovarian cells from intact immature rats were cultured for 24 h in medium containing LH alone,  $E_2$  ( $1 \mu\text{g/ml}$ ) alone, or  $E_2 + \text{LH}$  in a  $2 \times 2$  factorial experiment. As shown in Fig. 3, LH significantly ( $P < 0.01$ ) increased progesterone accumulation in the absence or presence of  $E_2$  and  $E_2$  exerted a significant inhibition ( $P < 0.01$ ) in the absence or presence of LH, while a highly significant ( $P < 0.01$ ) interaction indicated that  $E_2$  inhibited progesterone accumulation to a lesser extent in the presence than in the absence of LH.

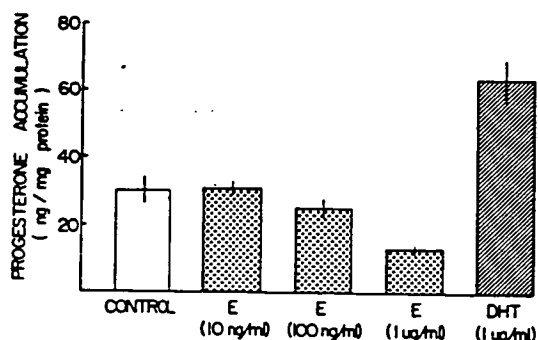


FIG. 2. Effects of culturing rat ovarian cells for 24 h with medium (M) alone (control), M +  $E_2$  (10 ng/ml -  $1 \mu\text{g/ml}$ ) or M + DHT ( $1 \mu\text{g/ml}$ ) on progesterone accumulation (mean  $\pm$  SEM;  $n = 4$ ).

#### *Interactions of Steroids and Gonadotropins on Progesterone Accumulation in vitro*

The effects of culturing dissociated ovarian cells with medium containing either  $E_2$  ( $1 \mu\text{g/ml}$ ) or DHT ( $1 \mu\text{g/ml}$ ) in the presence of either saline, LH or FSII for 24 h (chronic treatments) on the subsequent ability of these cells to respond to an acute exposure (4 h) to either saline, LH or FSII by the accumulation of progesterone were tested in a  $3 \times 3 \times 3$  factorial experiment. As shown in Fig. 4, there was a highly significant ( $P < 0.01$ ) interaction between  $E_2$  and chronic gonadotropin treatments. Although  $E_2$  by itself exerted an inhibitory effect ( $P < 0.01$ ), it attenuated progesterone accumulation to a lesser extent in the presence than in the absence of chronic FSH. By contrast, a highly significant ( $P < 0.01$ ) interaction between DHT and chronic gonadotropin treatments was brought about because, although DHT by itself exerted a stimulatory effect ( $P < 0.01$ ), it enhanced progesterone accumulation to a greater extent in the presence than in the absence of chronic FSH. In both the  $E_2$  and DHT cultures, there was also a significant ( $P < 0.01$ ) interaction between chronic and acute gonadotropin treatments, since chronic LH treatment obliterated the subsequent progesterone response of the cells to the acute treatment with either LH or FSH (Fig. 4). On the other hand, chronic treatment with FSH abolished the subsequent acute stimulation by FSH, but not by LH.

## DISCUSSION

We have recently demonstrated an inhibitory

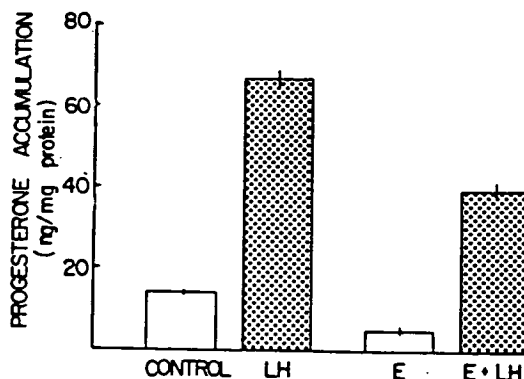


FIG. 3. Effects of culturing rat ovarian cells for 24 h with medium (M) alone (control), M +  $E_2$  ( $1 \mu\text{g/ml}$ ) and/or LH ( $1 \mu\text{g/ml}$ ) on progesterone accumulation (mean  $\pm$  SEM;  $n = 5$ ).

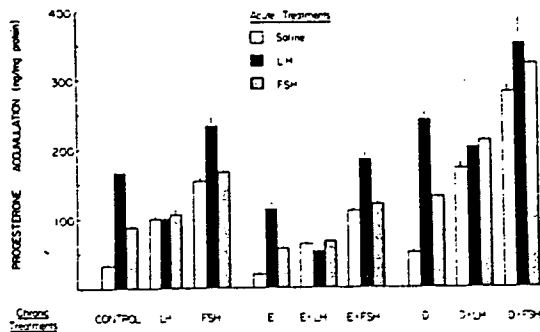


FIG. 4. Effects of culturing rat ovarian cells for 24 h (chronic culture) with medium (M), M + E<sub>2</sub> (1 μg/ml) or M + DHT (1 μg/ml), alone or in combination with either saline, LH (1 μg/ml) or FSH (1 μg/ml), on the ability of the cells to accumulate progesterone in response to either saline, LH (1 μg/ml) or FSH (1 μg/ml) during a further 4 h (acute) culture (mean ± SEM; n = 4).

action of E<sub>2</sub> on ovarian responsiveness to LH in the production of progesterone *in vivo*, in hypophysectomized, but not in intact immature female rats (Leung et al., 1978). It appears that some pituitary factor(s) may be lacking in the hypophysectomized animals, which is responsible for the maintenance of the ovarian progesterone response to LH. Results from the present study offer a possible explanation for this discrepancy, in that there may be an interaction between E<sub>2</sub> and FSH in the control of ovarian progesterone production. While pretreatment of hypophysectomized rats with E<sub>2</sub> reduced the progesterone response to LH *in vivo*, this inhibitory action of E<sub>2</sub> could be prevented by concomitant treatment with FSH (Fig. 1).

In rat granulosa cells, FSH has been shown to stimulate progesterone production (Armstrong and Dorrington, 1976; Nimrod and Linder, 1976), most likely via the stimulation of an adenylate cyclase-coupled FSH receptor system (Goff and Armstrong, 1977). Furthermore, in immature hypophysectomized rats, priming with E<sub>2</sub> *in vivo* has been shown to enhance the ability of FSH to induce receptors for both FSH and LH in granulosa cells (Richards and Midgley, 1976). Thus, the present observation that FSH priming of hypophysectomized rats *in vivo* increased the ovarian progesterone response to LH and that E<sub>2</sub> in the presence of FSH failed to exert its inhibitory action is consistent with the notion of a positive interaction between E<sub>2</sub> and FSH.

Secretion of endogenous FSH may also be responsible, in part at least, for preventing the

inhibitory action of E<sub>2</sub> in intact immature rats *in vivo*. This is supported by the observation that, while E<sub>2</sub> failed to lower ovarian progesterone levels in intact rats *in vivo* (Leung et al., 1978), at concentrations >10 ng/ml, E<sub>2</sub> significantly attenuated progesterone accumulation by ovarian cells from these rats *in vitro* (Fig. 2). However, while E<sub>2</sub> inhibited the responsiveness of these cells to LH stimulation (Fig. 3), this inhibitory effect was significantly attenuated in the concomitant presence of E<sub>2</sub> + FSH in the cultures (Fig. 4). Thus, similar to the effects observed in hypophysectomized rats *in vivo*, FSH appeared to antagonize the inhibitory action of E<sub>2</sub> *in vitro*.

The response(s) of the different cellular components of the ovary to the actions and/or interactions of E<sub>2</sub> and FSH has not been clearly established. Although E<sub>2</sub> *in vivo* has been shown to increase the ovarian uptake of tritiated FSH (Goldenberg et al., 1972b), this enhancement reflected the increase in the number of granulosa cells, rather than an increase in the number of receptors per cell (Louvet and Vaitukaitis, 1976). Thus, estrogen treatment alone does not appear to stimulate FSH or LH receptor production (Richards and Midgley, 1976). In fact, E<sub>2</sub> treatment of intact immature rats resulted in a significant decrease in granulosa cell response to gonadotropins in terms of cyclic AMP production (Goff and Armstrong, 1977). This reduction in granulosa cell responsiveness may be accounted for by either an indirect action via a negative feedback effect on pituitary LH/FSH release or by a direct action of E<sub>2</sub> in the ovary. The present observation of an estrogen inhibition of progesterone accumulation in dissociated ovarian cells *in vitro* supports a direct mode of action, assuming that granulosa cells are the target cells in question. The interaction of FSH and E<sub>2</sub> may thus be due to better maintenance of preformed LH/FSH receptors, induction of new gonadotropin binding sites or stimulation of steroidogenesis independent of hormone receptor induction (Hillier et al., 1978). The manner in which progesterone production by the other cellular compartments of the ovary is affected by E<sub>2</sub> and/or FSH remains to be examined.

Regardless of the mechanism, this interaction with E<sub>2</sub> appears to be specific for FSH. Dissociated ovarian cells cultured for 24 h in the presence of E<sub>2</sub> + LH resulted in a further inhibition of progesterone accumulation, as



compared to cultures in medium containing LH alone (Fig. 4). In all cases, chronic culture with LH resulted in a loss of responsiveness of the cells to a subsequent acute treatment with both LH and FSH, whereas chronic culture with FSH resulted in subsequent loss of responsiveness of FSH, but not to LH. These observations probably reflect the so-called "down receptor regulation" of the cells or other related desensitization phenomena (Hunzicker-Dunn and Birnbaumer, 1976; Lindner et al., 1977). Similar findings have been reported recently for isolated sheep follicular cells (Weiss and Armstrong, 1979).

While  $E_2$  inhibited progesterone accumulation by ovarian cells from immature rats in the present study, an ability of  $E_2$  to enhance FSH-induced progesterone secretion by cultured granulosa cells isolated from estrogen-treated immature rats has been reported previously (Armstrong and Dorrington, 1976). A direct stimulatory effect of  $E_2$  on progesterone secretion by granulosa cells isolated from mature follicles of rats primed with pregnant mare's serum gonadotropin has also been demonstrated (Bernard, 1975). The cause(s) of the discrepancy between the cellular responses to  $E_2$  in these studies is not clear. Since a nonhomogeneous cellular population was employed in the present *in vitro* study, the possibility can be raised that different cell types are involved. Alternatively, granulosa cells from different stages of follicular maturation may respond to  $E_2$  in a dissimilar manner, as suggested by observations in the pig (Goldenberg et al., 1972a; Schomberg et al., 1976; Thanki and Channing, 1976, 1978).

In contrast to the actions of  $E_2$ , exogenous DHT appeared to stimulate progesterone accumulation by dissociated ovarian cells *in vitro* (Fig. 2), as well as to act synergistically with FSH to increase the responsiveness of the cells to subsequent gonadotropic stimulation (Fig. 4). Similar actions of androgens have been described in granulosa cells *in vitro* (Armstrong and Dorrington, 1976; Nimrod and Lindner, 1976; Lucky et al., 1977; Hillier et al., 1977). Recently, it has been shown that intraovarian application of antiandrogens (flutamide or its hydroxylated metabolite) *in vivo* reduced subsequent progesterone secretion by porcine granulosa cells (Schomberg et al., 1978), thereby suggesting a role for androgens in granulosa cell steroidogenesis during follicular development. Whether or not progesterone

production by nongranulosa cells is similarly affected by androgens remains to be determined.

In summary, the above findings suggest that in immature female rats,  $E_2$  exerts a negative action on the ovary in the absence of FSH in that it inhibits LH-stimulated progesterone secretion. In the presence of FSH, however, the inhibitory action of  $E_2$  is overcome. On the other hand, androgens can act synergistically with FSH to enhance progesterone production. Thus, it is possible that a critical balance between the intragonadal levels of estrogens and androgens is important in the regulation of the steroidogenic responses to gonadotropins.

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**Authors:** K.M. Henderson, A.M.S. Gorban & G.S. Boyd

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## Effect of LH factors regulating ovarian cholesterol metabolism and progesterone synthesis in PMSG-primed immature rats

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**Summary.** Administration of an ovulatory dose of LH (10 µg, i.v.) to PMSG (4 i.u., s.c.)-primed immature rats increased ovarian pregnenolone levels 5-fold and ovarian progesterone levels 40-fold within 6 h and the levels, although fluctuating, remained elevated for 72 h. Serum progesterone levels mimicked those of the luteal phase in the normal cycle. The sustained increase in steroidogenesis was accompanied by a decrease in both basal and cAMP-stimulatable ovarian cholesterol ester hydrolase activity and a net increase in ovarian cholesterol ester content. Ovarian free cholesterol levels were essentially unchanged during the 72 h study. LH does not, therefore, chronically stimulate steroidogenesis by providing additional substrate for the steroidogenic enzymes through activating cholesterol ester hydrolase to bring about hydrolysis of cholesterol esters. Moreover, ovarian cholesterol esters are unlikely to be the primary source of cholesterol utilized to support luteal steroidogenesis. Studies with isolated mitochondria suggested that the mechanisms by which LH stimulated steroidogenesis were by (a) stimulating mitochondrial pregnenolone production probably by facilitating the intramitochondrial movement of cholesterol to the site of side-chain cleavage, and (b) promoting the metabolism of pregnenolone to progesterone.

### Introduction

While the stimulation of ovarian progesterone production by luteinizing hormone (LH) is understood to be mediated through adenosine-3',5'-monophosphate (cAMP) (Marsh, 1976; Sala, Dufau & Catt, 1979), the mechanism by which cAMP actually brings about and maintains increased progesterone production is poorly understood. Cholesterol is the obligatory precursor of progesterone and changes in either its cellular concentration or metabolism might, therefore, be expected to play a central role in regulating progesterone biosynthesis. The greatest increase in ovarian progesterone production occurs at the time of ovulation when the preovulatory LH surge initiates progesterone production by the corpus luteum. The purpose of this study was to attempt to correlate this chronic increase in ovarian progesterone production, induced by LH, with changes in ovarian cholesterol content and the biochemical conditions which might be expected to regulate the metabolism of cholesterol to progesterone.

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## Materials and Methods

### Animals

Immature Wistar strain female rats, 21–23 days old and weighing 30–45 g, were obtained from the Small Animal Breeding Station (University of Edinburgh). The animals were housed under constant temperature and lighting conditions and were allowed pelleted food and water *ad libitum*. At 23–25 days of age, follicular development was stimulated with a subcutaneous (s.c.) injection of 4 i.u. PMSG (Gestyl: Organon Laboratories Ltd, Crown House, Morden, Surrey, U.K.) in 0.2 ml saline (9 g NaCl/l). Follicular development initiated in this way mimics that of the mature animal in that, after an ovulatory stimulus, the induced ovulation closely resembles a spontaneous ovulation in terms of preovulatory serum oestradiol-17 $\beta$  and progesterone concentrations, number of follicles ovulated (5–7 per ovary) and post-ovulatory serum progesterone concentrations (Guillet & Rennels, 1964; Goff & Henderson, 1979). Ovulation was induced 2 days after PMSG priming by an intravenous (i.v.) injection through a tail vein of 10  $\mu$ g LH (NIH-LH-S18) in 0.2 ml saline. Animals were killed by decapitation at selected times after LH administration. Trunk blood was collected and stored overnight at 4°C and the serum was collected and stored at –20°C until assayed for progesterone. Ovaries were excised, trimmed free of fat, weighed and homogenized individually in 3 ml chloroform/methanol (2:1 v/v) for sterol and steroid extraction, or pooled and homogenized at 4°C in 10 ml 0.25 M-sucrose (pH 7.0) for subsequent preparation of mitochondria or the 105 000  $g_{av}$  supernatant used in the assay of cholesterol ester hydrolase.

### Preparation of mitochondria and mitochondrial incubations

Ovarian homogenates in 0.25 M-sucrose were centrifuged for 10 min at 650  $g_{av}$  to sediment nuclei and cell debris. The supernatant was centrifuged at 8500  $g_{av}$  for 15 min to sediment the mitochondrial fraction which was washed in 0.25 M-sucrose and centrifuged again. The final pellet was taken up in chloroform/methanol (2:1 v/v) for cholesterol and steroid extraction or in 10 mM-potassium phosphate buffer (pH 7.4) containing 5 mM-MgCl<sub>2</sub> and 100 mM-sucrose for measurement of cholesterol side-chain cleavage activity and cytochrome P-450 content. Mitochondrial protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Mitochondrial cholesterol side-chain cleavage activity was determined at 37°C in 1 ml potassium phosphate buffer which also contained 0.2 mM-NADP<sup>+</sup>. The reaction was initiated with 10 mM-DL-isocitrate after the mitochondria had been preincubated at 37°C for 15 min with 100  $\mu$ M-cholesterol or 100  $\mu$ M-24-hydroxycholesterol added in 10  $\mu$ l ethanol. Control incubations received 10  $\mu$ l ethanol only. Aliquots (0.2 ml) were pipetted into 2 ml chloroform/methanol (2:1 v/v) and vortexed briefly to stop the reaction at 2, 5, 10 and 20 min after the addition of isocitrate. Final extraction of steroids into the chloroform/methanol was achieved by vortexing the samples for 90 sec. The aqueous layer was removed and discarded, and the organic phase was stored at –20°C until assayed for pregnenolone and progesterone. The efficiency of extraction was monitored by the addition of tritiated steroid and was >95% for both pregnenolone and progesterone.

Mitochondrial cytochrome P-450 content was determined spectrophotometrically as described by Omura & Sato (1964) by recording the difference spectrum between reduced cytochrome P-450 and its carbon monoxide complex. The concentration of cytochrome P-450 was calculated by using an extinction coefficient of 91 mM<sup>-1</sup> cm<sup>-1</sup> for the absorbance change at 450–490 nm. The reducing agent was sodium dithionite.

### Cholesterol, cholesterol ester and cholesterol ester hydrolase determinations

Trace amounts of [<sup>14</sup>C]cholesterol and [<sup>14</sup>C]cholesteryl oleate were added to aliquots of the ovarian and mitochondrial chloroform/methanol extracts. Cholesterol and cholesterol ester were

separated by thin-layer chromatography on Silica Gel H-plates developed in a solvent system consisting of petroleum ether:diethyl ether:glacial acetic acid (75:24:1, by vol.). Cholesterol and cholesterol ester were located by using a Panax thin-layer radioactive scanner and were eluted from the silica gel with diethyl ether:acetone (1:1 v/v). The eluates were dried under  $N_2$  and the residues were taken up in ethanol.

Cholesterol was measured by the fluorescence technique described by Gamble, Vaughan, Kruth & Avigan (1978). In this method cholesterol is specifically oxidized with the enzyme cholesterol oxidase (B. D. H. Chemicals Ltd, Poole, Dorset, U.K.) and the  $H_2O_2$  generated further reacted with *p*-hydroxyphenylacetic acid in a reaction catalysed by horseradish peroxidase (Sigma Chemical Co., London, U.K.) to yield a stable fluorescent product. This technique is sensitive to as little as 100 ng cholesterol. Cholesterol ester was measured by liberating cholesterol during saponification in 10% potassium hydroxide in ethanol at 60°C and assaying the cholesterol by the method above. Recovery of cholesterol and cholesterol ester was >90% as monitored by added [ $^{14}C$ ]sterols.

Cholesterol ester hydrolase activity in the 105 000  $g_{av}$  supernatant of the ovarian homogenates was determined as described by Gorban & Boyd (1977), the method being based on the hydrolysis of [ $^{14}C$ ]cholesteryl oleate. The ability of cAMP (10  $\mu M$ ) to activate this hydrolase *in vitro* was also determined as described by Gorban & Boyd (1977). In these studies, usually only one ovary from each animal was used in the preparation of the 105 000  $g_{av}$  supernatant while the other ovary was homogenized in chloroform/methanol for subsequent progesterone determination.

#### Radioimmunoassays

Progesterone and pregnenolone were measured by the specific RIAs described and validated previously (Neal, Baker, McNatty & Scaramuzzi, 1975; Mason, Arthur & Boyd, 1978a). Progesterone was extracted from serum before assay with petroleum ether. The extraction efficiency was monitored by the addition of [ $^3H$ ]progesterone and the mean recovery was 84%. The progesterone antiserum was prepared against  $11\alpha$ -hydroxyprogesterone hemisuccinate conjugated to bovine serum albumin (BSA). The pregnenolone antiserum was generated against 20-(O-carboxymethyl)-oxime-BSA. Both antisera showed negligible cross-reactivity (<1%) with  $C_{27}$ -sterols. The progesterone antiserum cross-reacted <1% with pregnenolone and the pregnenolone antiserum cross-reacted <1% with progesterone. Cross-reactivity with other  $C_{21}$  steroids was <10% for both antisera. The limit of sensitivity of the progesterone and pregnenolone assays was 25 and 50 pg per tube respectively. The intra- and inter-assay variations of both assays were each <13%.

#### Statistical analysis

The Wilcoxon rank sum test was used to determine whether separate sets of observations differed significantly.

### Results

#### *Effect of LH on serum progesterone and ovarian steroid and sterol concentrations*

The results in Table 1 show that LH initiated a rapid and sustained increase in serum and ovarian progesterone concentrations although there were marked fluctuations in the extent of the increases during the 72 h period; the greatest increases were at 6, 12 and 48 h after LH. Ovarian pregnenolone concentrations were also increased by LH and remained elevated throughout the 72 h period. There was a slight, but significant ( $P < 0.05$ ), decrease in ovarian cholesterol

concentration 6 h after LH administration, but by 12 h the values were similar to those at 0 h and remained at this level for the remainder of the study. The ovarian cholesterol ester concentration was also slightly, but significantly ( $P < 0.05$ ) reduced 6 h after LH but rose steadily thereafter and by 72 h was double the 0 h value.

Table 1. Effect of LH on serum progesterone and ovarian progesterone, pregnenolone, cholesterol and cholesterol ester concentrations (mean  $\pm$  s.e.m. for at least 4 rats/group)

Time after LH (h)	Serum progesterone (ng/ml)	Ovarian progesterone (ng/mg)	Ovarian pregnenolone (ng/mg)	Ovarian cholesterol ( $\mu$ g/mg)	Ovarian cholesterol ester ( $\mu$ g/mg)
0	3.2 $\pm$ 0.5 <sup>a</sup>	0.2 $\pm$ 0.04 <sup>a</sup>	0.2 $\pm$ 0.05 <sup>a</sup>	2.0 $\pm$ 0.2 <sup>a,c</sup>	6.4 $\pm$ 0.6 <sup>a</sup>
6	52 $\pm$ 9 <sup>b</sup>	9.0 $\pm$ 1.5 <sup>b</sup>	1.4 $\pm$ 0.1 <sup>b</sup>	1.2 $\pm$ 0.1 <sup>b</sup>	4.1 $\pm$ 0.7 <sup>b</sup>
12	43 $\pm$ 7 <sup>b</sup>	6.9 $\pm$ 0.4 <sup>b</sup>	1.1 $\pm$ 0.1 <sup>b,c</sup>	1.9 $\pm$ 0.1 <sup>a</sup>	4.7 $\pm$ 0.9 <sup>a,b</sup>
24	6.8 $\pm$ 0.8 <sup>c</sup>	3.0 $\pm$ 0.1 <sup>c</sup>	0.9 $\pm$ 0.09 <sup>c</sup>	2.3 $\pm$ 0.1 <sup>c</sup>	9.4 $\pm$ 0.8 <sup>c</sup>
48	34 $\pm$ 6 <sup>b</sup>	6.6 $\pm$ 0.7 <sup>b</sup>	0.4 $\pm$ 0.06 <sup>d</sup>	2.5 $\pm$ 0.1 <sup>c</sup>	9.9 $\pm$ 1.1 <sup>c</sup>
72	18 $\pm$ 4 <sup>d</sup>	2.8 $\pm$ 1.0 <sup>c</sup>	0.5 $\pm$ 0.10 <sup>d</sup>	2.7 $\pm$ 0.3 <sup>c</sup>	13.3 $\pm$ 1.4 <sup>d</sup>

Differences between means with different superscripts in the same column are significant ( $P < 0.05$ ).

#### Effect of LH on cholesterol ester hydrolase activity

In Exps I and II, cholesterol ester hydrolase activity was determined at 3 and 6 h after LH treatment (Table 2) when ovarian cholesterol ester concentration falls and ovarian progesterone concentrations are at their highest (Table 1). At both times there was a reduction in the basal activity of the enzyme and its capacity to be activated by cAMP. Similar results were obtained in Exp. III in which the basal activity of the enzyme and its sensitivity to cAMP were still suppressed 48 h after LH treatment (Table 2) when ovarian cholesterol ester concentration was rising and ovarian progesterone concentration was again at its highest (Table 1). In all 3 experiments the inhibitory effects of LH on the hydrolase activity were accompanied by increases in the ovarian progesterone concentrations.

Table 2. Effect of LH on basal and cAMP-activated ovarian cholesterol ester hydrolase activity

Exp.	Treatment	Time of autopsy (h)	Cholesterol ester hydrolase activity (pmol oleic acid released/mg protein/min)		Ovarian progesterone (ng/mg)
			Basal	cAMP activated	
I	Saline (5)	3	139 $\pm$ 3	163 $\pm$ 13	0.11 $\pm$ 0.04
	LH (5)	3	94 $\pm$ 7 <sup>**</sup>	98 $\pm$ 7 <sup>**</sup>	13 $\pm$ 0.6 <sup>**</sup>
II	Saline (5)	6	209 $\pm$ 12	573 $\pm$ 11	0.12 $\pm$ 0.05
	LH (5)	6	169 $\pm$ 15 <sup>*</sup>	429 $\pm$ 7 <sup>**</sup>	6.3 $\pm$ 1.8 <sup>**</sup>
III	LH (12)	0	116 $\pm$ 12	170 $\pm$ 9	0.33 $\pm$ 0.06
	LH (12)	6	73 $\pm$ 10 <sup>*</sup>	150 $\pm$ 26	3.7 $\pm$ 0.2 <sup>**</sup>
	LH (12)	48	65 $\pm$ 6 <sup>**</sup>	86 $\pm$ 6 <sup>**</sup>	4.7 $\pm$ 0.7 <sup>**</sup>

Values are mean  $\pm$  s.e.m. for the no. of replicate determinations indicated in parentheses.

Values significantly different from the corresponding saline (Exps I and II) or 0 h (Exp. III) values:

\* $P < 0.05$ ; \*\* $P < 0.01$ .

#### Effect of LH on ovarian mitochondrial cytochrome P-450, pregnenolone, progesterone and cholesterol concentrations

The mitochondrial concentrations of pregnenolone and progesterone were elevated 6 h and 48 h after LH (Table 3). Cytochrome P-450 concentrations were unchanged after LH, as were the cholesterol concentrations. The mean mitochondrial cholesterol values, however, followed a pattern similar to that for ovarian cholesterol (Table 1) in that there was a fall after 6 h and a return towards the 0 h values by 48 h.

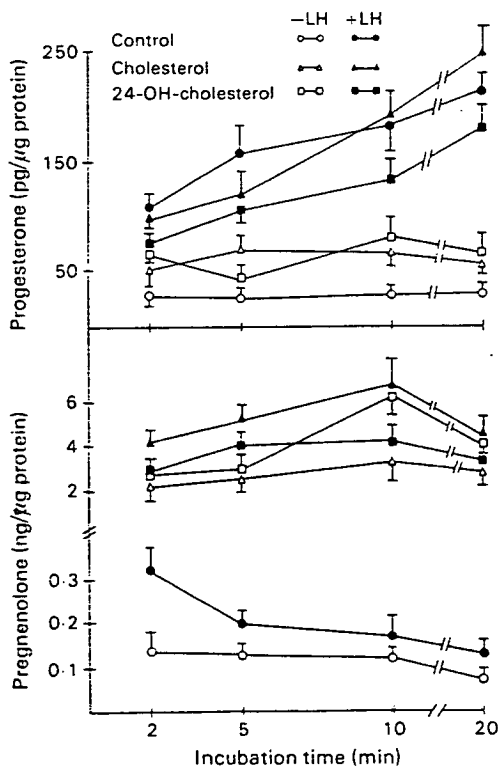
**Table 3.** Effect of LH on ovarian mitochondrial cytochrome P-450, pregnenolone, progesterone and cholesterol concentrations (mean  $\pm$  s.e.m. for 6 replicates)

	Time after LH administration		
	0 h	6 h	48 h
Cytochrome P-450 (nmol/mg protein)	0.87 $\pm$ 0.09 <sup>a</sup>	0.97 $\pm$ 0.12 <sup>a</sup>	0.99 $\pm$ 0.04 <sup>a</sup>
Pregnenolone (ng/mg protein)	16 $\pm$ 2 <sup>a</sup>	64 $\pm$ 12 <sup>b</sup>	26 $\pm$ 3 <sup>c</sup>
Progesterone (ng/mg protein)	57 $\pm$ 6 <sup>a</sup>	286 $\pm$ 25 <sup>b</sup>	161 $\pm$ 17 <sup>c</sup>
Cholesterol ( $\mu$ g/mg protein)	71 $\pm$ 12 <sup>a</sup>	47 $\pm$ 7 <sup>a</sup>	58 $\pm$ 8 <sup>a</sup>

Values in the same row with different superscript letters are significantly different ( $P < 0.05$ ).

#### Steroid production by ovarian mitochondria *in vitro*

Ovarian mitochondria were prepared from PMSG-primed rats injected with LH or saline and killed 6 h later. Steroid production in response to endogenous substrate and exogenous cholesterol or 24-hydroxycholesterol was compared (Text-fig. 1). The activity of the cholesterol side-chain cleavage enzyme with endogenous substrate, as estimated by the relative amount of pregnenolone plus progesterone produced during the incubation period was significantly greater ( $P < 0.01$ ) in mitochondria obtained from the LH treated rats than in those from control rats. Basal pregnenolone production by mitochondria from LH-treated rats fell during the incubation period, perhaps reflecting increased conversion to progesterone, the levels of which increased 2.5-fold between 2 and 20 min of incubation. Addition of cholesterol to mitochondrial



**Text-fig. 1.** Pregnenolone and progesterone formation *in vitro* by ovarian mitochondria prepared 6 h after treatment of PMSG-primed immature rats with LH or saline (mean  $\pm$  s.e.m. for 6 replicates).



incubations from control rats stimulated pregnenolone production by about 20-fold. There was a further 2–3-fold increase ( $P < 0.05$ ) in pregnenolone production when mitochondria from LH treated rats were incubated with cholesterol. Addition of 24-hydroxycholesterol to mitochondria from control rats similarly increased pregnenolone production 20–30-fold relative to basal values, but there was no further increase in pregnenolone production when mitochondria from LH treated rats were incubated with 24-hydroxycholesterol. Similar findings were obtained with 25- and 26-hydroxycholesterol (data not shown).

While pregnenolone production was stimulated 20–30-fold after addition of cholesterol or 24-hydroxycholesterol to mitochondria from control rats, there was no increase in progesterone production by these mitochondria. Only mitochondria from LH treated rats had the capacity to metabolize pregnenolone to progesterone, as indicated by the steady increase in progesterone during the incubation period.

### Discussion

Previous studies of the ovary and adrenal (Behrman & Armstrong, 1969; Behrman, Armstrong & Greep, 1970; Boyd, Arthur, Beckett, Mason & Trzeciak, 1975) have demonstrated that acute stimulation of steroidogenesis by LH and ACTH respectively is accompanied by a depletion of cellular cholesterol ester and a concomitant increase in cholesterol ester hydrolase activity. Together these findings suggest that cellular cholesterol ester may be an important source of the free cholesterol necessary to sustain increased steroidogenesis. There was little evidence for this in the present study, however, which was designed to investigate more chronic stimulation of steroidogenesis. After administration of an ovulatory dose of LH to PMSG-primed immature rats, ovarian pregnenolone and progesterone levels rose rapidly, and although fluctuating, remained elevated, relative to 0 h values, over the entire 72 h period while serum progesterone concentrations mimicked those of the luteal phase in mature cyclic rats (Smith, Freeman & Neill, 1975). While there was a slight, but significant, drop in ovarian cholesterol ester concentration 6 h after LH, the values rose rapidly thereafter and by 72 h were twice the 0 h value. Sustained increased steroidogenesis was therefore associated with an accumulation rather than a depletion of ovarian cholesterol ester, as found by Schuler, Scavo, Kirsch, Flickinger & Strauss (1979) in studies with rats induced to superovulate. The transient decline in cholesterol ester seen 6 h after LH is most probably due to a reduction in synthesis rather than increased hydrolysis since both basal and cAMP-stimulated hydrolase activity was suppressed during the first 6 h (Table 2). The initial reduction in free cholesterol concentration would also be compatible with cholesterol ester synthesis being inhibited by lack of substrate. The activity of the cholesterol ester hydrolase was still suppressed 48 h after LH treatment and this sustained, reduced activity may have contributed towards the gradual increase in ovarian cholesterol ester concentration seen between 6 and 72 h after LH. As found in other studies (Bisgaier, Treadwell & Vahouny, 1979), cAMP activated cholesterol ester hydrolase *in vitro*. Although LH is thought to mediate its actions through cAMP (Marsh, 1976; Sala *et al.*, 1979) administration of LH *in vivo* depressed both the basal activity of the hydrolase and its capacity to be activated by cAMP *in vitro* (Table 2). The reason for this apparent disparity is unknown, but highlights the fact that the behaviour of an enzyme *in vitro* may not necessarily reflect its activity *in vivo* where several factors may be acting to influence its activity. For example, one or more of the proteins/peptides induced by LH (Landfield, Campbell & Midgley, 1979) may also be capable of influencing cholesterol ester hydrolase activity. These findings do, however, indicate that activation of the hydrolase and hydrolysis of cholesterol ester is not obligatory in the mechanism by which LH chronically stimulates ovarian steroidogenesis. Moreover, ovarian cholesterol ester is unlikely to be the primary source of cholesterol utilized to maintain the increased steroidogenesis. Studies by Christie, Strauss & Flickinger (1979) indicate that cholesterol derived from plasma lipoproteins is the principal source of cholesterol used to sustain ovarian

progesterone production. Studies on the mechanism of action of ACTH in the adrenal suggest that ACTH may similarly be capable of stimulating steroidogenesis without activating cholesterol ester hydrolase and inducing hydrolysis of cholesterol ester (Pedersen & Brownie, 1979).

Cytochrome P-450 is an essential component of the mitochondrial enzymes involved in cholesterol side-chain cleavage (Simpson, 1979). The finding that LH treatment did not change mitochondrial cytochrome P-450 concentration indicates that the increased steroidogenesis was not a consequence of LH causing an increase in the concentration of cholesterol side-chain cleavage enzyme. Previous studies have suggested that cAMP induced by LH stimulates steroidogenesis through activating a mitochondrial protein kinase which in turn activates the cholesterol side-chain cleavage enzymes (Marsh, 1976; Downing & Dimino, 1979). In the present studies, it was found that addition of cholesterol to mitochondrial incubations from control animals increased pregnenolone formation 20-fold and that this was only increased a further 2-3-fold when mitochondria from LH-treated animals were used (Text-fig. 1). The greatest increase in pregnenolone formation therefore occurs in response to increasing the cholesterol environment of the mitochondria, which suggests that while LH may activate the side-chain cleavage enzymes to some extent, the major limitation to pregnenolone formation is the availability of cholesterol and not the activity of the cleavage enzymes. Steroidogenesis stimulated by LH *in vivo* was not, however, accompanied by any noticeable increase in mitochondrial cholesterol concentration (Table 3). Indeed, there was a tendency towards a reduction in the cholesterol concentration which might reflect some utilization of the mitochondrial cholesterol for the increased steroidogenesis. Rather than promoting a net increase in mitochondrial cholesterol concentration, LH may act by facilitating the movement of cholesterol to the sites of steroidogenesis within the mitochondria. Evidence for this is provided by the finding that while LH pretreatment stimulated mitochondrial pregnenolone production *in vitro* in response to exogenous cholesterol, it had no stimulatory effect on pregnenolone formation in response to exogenous 24-hydroxycholesterol (Text-fig. 1). Being more polar, hydroxysterols are likely to traverse mitochondrial membranes with greater ease than cholesterol (Mason *et al.*, 1978b) and so more readily reach the site of side-chain cleavage which is thought to be located in the inner mitochondrial membrane (Yago *et al.*, 1970). Intramitochondrial translocation of cholesterol to the steroidogenic sites in the adrenal is slow and probably rate-limiting, and ACTH may act primarily to facilitate this translocation (Mason *et al.*, 1978a). The results of the present study and those of Toaff, Strauss, Flickinger & Shattil (1979) indicate that a similar situation may occur in the ovary and that the primary action of LH may be to stimulate intramitochondrial movement of cholesterol to the steroidogenic sites. By this mechanism LH does not stimulate pregnenolone formation in response to 24-hydroxycholesterol because the intramitochondrial movement of 24-hydroxycholesterol is not rate-limiting in the steroidogenic process.

While mitochondria from control and LH-treated animals could metabolize exogenous cholesterol and 24-hydroxycholesterol to pregnenolone, it was only mitochondria from LH treated animals that could metabolize this pregnenolone to progesterone (Text-fig. 1). The initiation of luteal progesterone production in response to LH therefore requires not only an increase in pregnenolone formation but is dependent also on LH acting to promote the metabolism of pregnenolone to progesterone.

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## **Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size**

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**Summary.** The concentrations of LH, FSH and prolactin, and oestradiol-17 $\beta$ , androstenedione, testosterone and progesterone were measured in follicular fluid from small, medium and large bovine follicles. As follicle size increased, there was a significant increase in median fluid concentrations of prolactin (2-fold) and oestradiol-17 $\beta$  (14-fold) and a significant decrease in concentrations of LH (to 73%), androstenedione (to 30%) and testosterone (to 10%). There was no relationship between follicle size and fluid concentrations of FSH or progesterone, or between fluid concentrations of FSH and the relative concentrations of androgen and oestradiol-17 $\beta$ . As follicle size increased there was a significant increase in the proportion of follicles in which follicular fluid concentrations of oestradiol-17 $\beta$  exceeded those of androgen. There was a significant relationship between follicular fluid concentrations of prolactin and progesterone; as fluid prolactin concentrations increased, the maximum concentration of progesterone observed decreased.

### **Introduction**

While the concentrations of ovarian steroids and gonadotrophins present in blood throughout the oestrous cycle of the cow have been well documented (Kanchev, Dobson, Ward & Fitzpatrick, 1976; Schams, Schallenberger, Hoffmann & Karg, 1977; Ireland, Coulson & Murphree, 1979; Rahe, Owens, Fleeger, Newton & Harms, 1980), no information is available regarding the concentrations of gonadotrophins present in follicular fluid or their relationship to steroids present there. Studies in other species indicate that the relative follicular fluid concentrations of steroids and gonadotrophins vary widely between follicles, and that the intrafollicular environment of steroids and gonadotrophins may be an important regulator of follicular development (Moor, Hay, Dott & Cran, 1978; McNatty, 1978; McNatty, Gibb, Dobson, Thurley & Findlay, 1981). The purpose of this study was to determine what interrelationships existed between steroids and gonadotrophins present in bovine follicular fluid.

### **Materials and Methods**

#### *Collection of follicular fluid*

Ovaries were obtained from 53 adult, non-pregnant cows within 1 h of their slaughter during 16 separate visits to a local Edinburgh abattoir between the months of October and February. The ovaries were transported to the laboratory in ice-chilled Eagle's Minimum Essential Medium

(Modified) with Earle's Salts (Flow Laboratories, Irvine, Scotland). Follicular fluid from all antral follicles protruding at the surface of the ovary was aspirated individually using a syringe (1 or 2 ml) and needle (20–26 gauge). Fluid from follicles containing  $\leq 0.1$  ml fluid was pooled for follicles from the same ovary or each pair of ovaries. All other fluids were analysed separately. Follicles containing  $< 0.3$  ml fluid were classified as small. This group consisted of 31 separate pools of follicular fluid from follicles containing  $\leq 0.1$  ml fluid and 15 fluids from individual follicles with  $> 0.1$  ml to  $< 0.3$  ml fluid. Follicles containing  $\geq 0.3$  ml to  $< 0.8$  ml fluid and  $\geq 0.8$  ml fluid were classified as medium ( $n = 35$ ) and large ( $n = 37$ ) respectively. The median volume collected from large follicles was 1.0 ml and the greatest volume was 2.2 ml. All fluids were frozen immediately after aspiration until assayed for gonadotrophin and steroids by radioimmunoassay. The time between collecting the ovaries and freezing the aspirated follicular fluid was  $< 75$  min. Regression analysis on 23 individual follicles containing 0.05–1.3 ml follicular fluid indicated that follicular volume ( $V$ ;  $\mu\text{l}$ ) could be related to follicular diameter ( $D$ ; mm) by the equation  $V = 0.3 \times D^3$ .

### Radioimmunoassays

**Gonadotrophins.** The concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin in follicular fluid were measured using the assays described previously by Scaramuzzi, Caldwell & Moor (1970), McNeilly, McNeilly, Walton & Cunningham (1976) and McNeilly & Andrews (1974) respectively. While these assays were originally developed to measure ovine gonadotrophins, the LH and FSH assays have been validated for use in the cow (Bass, McNeilly & Moreton, 1979). The prolactin assay was specific for bovine prolactin (NIH-P-B12), showing negligible cross-reaction ( $< 0.5\%$  w/w at 50% B/Bo) with other bovine pituitary hormones: LH (NIH-LH-B8), FSH (CH-1-76 potency  $164 \times$  NIH-FSH-B1), TSH (30 i.u./mg, Pierce) and GH (NIH-GH-B15). It has been established previously that no serum interference occurred in the prolactin radioimmunoassay (McNeilly & Land, 1979). Dilution curves of plasma from cows were parallel to the bovine prolactin standard and recovery (mean  $\pm$  s.e.m.) of added hormone to plasma was  $97 \pm 3\%$  ( $n = 30$ ). All the follicular fluid samples were assayed in one assay for each gonadotrophin and the intra-assay coefficient of variation was  $\leq 6\%$  in each instance. The detection limits of the assays were 0.16 ng LH/ml, 16 ng FSH/ml and 0.5 ng prolactin/ml. Results are expressed in terms of NIH-LH-B8, NIH-FSH-B1 and NIH-P-B12 for LH, FSH and prolactin respectively.

**Steroids.** Concentrations of steroids in follicular fluid were determined using specific assays utilizing antisera and methods described previously for progesterone (Neal, Baker, McNatty & Scaramuzzi, 1975), oestradiol-17 $\beta$  (Van Look, Hunter, Corker & Baird, 1977), testosterone (Corker & Davidson, 1978) and androstenedione (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). Steroids were extracted from follicular fluid before assay with petroleum ether (progesterone), diethyl ether (oestradiol-17 $\beta$  and androstenedione) or hexane:diethyl ether (4:1 v/v) (testosterone). Recovery of steroids from follicular fluid was monitored by the addition of trace amounts of tritiated steroid ( $\sim 1500$  c.p.m.) to the samples before their extraction. The mean procedural recoveries from fluid were: progesterone 72%, oestradiol-17 $\beta$  85%, androstenedione 81% and testosterone 73%. Steroids showing  $> 1\%$  cross-reactivity with the antisera used were: 11 $\alpha$ -hydroxyprogesterone (35%); 11 $\beta$ -hydroxyprogesterone (12%); 5 $\alpha$ -pregnane-3,20-dione (17%); and 17-hydroxyprogesterone (1.2%) with the progesterone antiserum; oestrone (5%) and oestradiol-17 $\alpha$  (2%) with the oestradiol-17 $\beta$  antiserum; 5 $\alpha$ -dihydrotestosterone (24%) with the testosterone antiserum; 11 $\beta$ -hydroxyandrostenedione (36%) and androsterone (43%) with the androstenedione antiserum. The limits of sensitivity of the assays were: 1.5 ng progesterone/ml, 0.2 ng oestradiol-17 $\beta$ /ml and 0.5 ng testosterone/ml and 0.5 ng androstenedione/ml. The intra- and inter-assay coefficients of variation were each  $< 10\%$  and  $< 16\%$  respectively for all of the assays.

*Statistics*

Non-parametric statistics were used to avoid making assumptions about the distribution of the values in the populations from which the sample data were drawn. Accordingly, sample medians are given together with 95% confidence limits calculated as described by Campbell (1967) using Nair's table. Unless otherwise stated, significant differences were determined using the Kruskal-Wallis test (Hollander & Wolfe, 1973) and comparisons of rank sums as described by Dunn (1964).

**Results***Gonadotrophin concentrations in follicular fluid in relation to follicle size*

Table 1 shows that, as follicle size increased, FSH concentrations in follicular fluid remained unchanged, LH concentrations declined significantly and prolactin concentrations increased significantly.

**Table 1.** Concentration of gonadotrophins (ng/ml) in bovine follicular fluid in relation to follicle size

	Follicular size (ml antral fluid/follicle)		
	Small ( $<0.3$ ) *(36, 42, 41)	Medium ( $\geq 0.3$ to $<0.8$ ) *(28, 33, 33)	Large ( $\geq 0.8$ ) *(36, 36, 37)
FSH	70 <sup>a</sup> (60-90)	71 <sup>a</sup> (62-82)	65 <sup>a</sup> (60-74)
LH	4.5 <sup>a</sup> (4.2-4.7)	3.7 <sup>b</sup> (3.4-4.0)	3.3 <sup>bc</sup> (3.0-3.5)
Prolactin	14 <sup>a</sup> (10-17)	22 <sup>ac</sup> (17-27)	30 <sup>c</sup> (24-45)

\* No. of samples assayed for FSH, LH and prolactin respectively.

Values are medians with 95% confidence limits in parentheses.

Values with different superscripts in the same row are significantly different:

<sup>ab</sup>  $P < 0.05$ ; <sup>ac</sup>  $P < 0.01$ .

*Steroid concentrations in follicular fluid in relation to follicle size*

Table 2 shows that, as follicle size increased, follicular fluid concentrations of oestradiol-17 $\beta$  rose significantly, testosterone and androstenedione concentrations fell significantly and progesterone concentrations remained unchanged. The range of concentrations of steroids in follicular fluid within each follicle size class was very large. Oestradiol-17 $\beta$  concentrations (ng/ml) ranged from  $<0.2$  to 65 in small follicles, from  $<0.2$  to 890 in medium follicles and from  $<0.2$  to 920 in large follicles. Testosterone and androstenedione concentrations (ng/ml) ranged from 1.6 to 138 and 2.0 to 166 respectively in small follicles, 0.5 to 240 and  $<0.5$  to 313 respectively in medium follicles and 0.7 to 121 and  $<0.5$  to 760 respectively in large follicles. Progesterone concentrations (ng/ml) ranged from 7 to 115 in small follicles, 10 to 164 in medium follicles and 10 to 390 in large follicles.

**Table 2.** Concentration of steroids (ng/ml) in bovine follicular fluid in relation to follicle size

	Follicle size (ml antral fluid/follicle)		
	Small ( $<0.3$ ) *(46)	Medium ( $\geq 0.3$ to $<0.8$ ) *(35)	Large ( $\geq 0.8$ ) *(36, 37, 35, 34)
Oestradiol-17 $\beta$	5 <sup>a</sup> (1-8)	23 <sup>c</sup> (15-97)	69 <sup>c</sup> (37-142)
Androstenedione	32 <sup>a</sup> (25-41)	18 <sup>ab</sup> (6-30)	10 <sup>b</sup> (5-15)
Testosterone	30 <sup>a</sup> (19-49)	5 <sup>c</sup> (2-14)	3 <sup>c</sup> (2-6)
Progesterone	35 <sup>a</sup> (24-53)	37 <sup>a</sup> (21-49)	39 <sup>a</sup> (25-58)

\* No. of samples assayed for oestradiol-17 $\beta$ , androstenedione, testosterone and progesterone respectively.

Values are medians with 95% confidence limits in parentheses.

Values with different superscripts in the same row are significantly different: <sup>ab</sup>  $P < 0.05$ ; <sup>ac</sup>  $P < 0.01$ .

#### *Relationship between follicle size and the ratio of oestradiol-17 $\beta$ to androgen concentrations in follicular fluid*

As follicle size increased, there was a significant increase ( $P < 0.001$ ; contingency table analysis) in the proportion of follicular fluids in which the concentration of oestradiol-17 $\beta$  (E) in follicular fluid exceeded that of androgen (androstenedione + testosterone; A + T). The proportion of fluids with an E:A + T ratio  $> 1$  was 3/46 for small follicles (1/31 for the pools of fluid and 2/15 for the fluids from individual follicles), 18/35 for fluids from medium sized follicles and 30/36 for large follicles.

#### *Relationship between FSH, oestradiol-17 $\beta$ and androgen concentrations in follicular fluid*

Table 3 shows that the relative concentrations of oestradiol-17 $\beta$  and androgen (testosterone + androstenedione) in follicular fluid were not related to those of FSH ( $P > 0.05$ ; Kruskal-Wallis).

**Table 3.** Relationship between bovine follicular fluid concentrations of FSH (ng/ml) and the ratio of fluid concentrations of oestradiol-17 $\beta$  and androgen (androstenedione + testosterone)

	Ratio of oestradiol-17 $\beta$ to androgen				
	$<10^{-2}$	$\geq 10^{-2}$ to $<10^{-1}$	$\geq 10^{-1}$ to $<10^0$	$\geq 10^0$ to $<10$	$\geq 10$
No. of samples	8	24	20	33	15
FSH	81 (52-185)	66 (56-90)	71 (60-96)	64 (60-74)	68 (60-100)

Values are medians with 95% confidence limits of the median in parentheses.



*Relationship between prolactin and progesterone concentrations in follicular fluid*

There was a significant relationship between progesterone and prolactin concentrations in follicular fluid (Table 4): 70% of fluids examined had prolactin concentrations of  $\leq 30$  ng/ml and progesterone concentrations in these fluids ranged from 7 to 390 ng/ml, although only 20% of these fluids had progesterone concentrations  $> 80$  ng/ml. As prolactin concentrations increased above 30 ng/ml there was a significant progressive decrease in the proportion of follicular fluids with progesterone concentrations  $> 35$  ng/ml ( $P < 0.02$ , contingency table analysis).

**Table 4.** Contingency table showing the distribution of 106 follicular fluid samples with respect to follicular fluid prolactin and progesterone concentrations

Follicular fluid prolactin conc. (ng/ml)	Follicular fluid progesterone conc. (ng/ml)		
	$\leq 35$	$> 35$ to $\leq 80$	$> 80$
$\leq 30$	30	30	15
$> 30$ to $\leq 65$	12	10	0
$> 65$	8	1	0

There was a significant relationship between follicular fluid concentrations of prolactin and those of progesterone ( $P < 0.02$ ; contingency table analysis).

### Discussion

The interpretation of the results of the present study in terms of possible physiological events is limited by the fact that the ovaries were obtained from animals passing through an abattoir. Consequently, no information was available on the reproductive history of the animals or of the stage of their oestrous cycle at the time of slaughter. Nevertheless, the data do demonstrate that there were significant relationships between follicle size and the relative concentrations of gonadotrophins and steroids in bovine follicular fluid.

As follicle size increased, the steroid environment of follicular fluid changed from being predominantly androgenic to predominantly oestrogenic. In only 7% of small follicles examined was the oestradiol-17 $\beta$  concentration in follicular fluid greater than that of the androgens while in large follicles this figure was 83%. In the cow, granulosa cells are the principal site of follicular androgen aromatization with oestradiol-17 $\beta$  being the major product (Lacroix, Eechaute & Leusen, 1974). An increase in granulosa cell numbers and/or aromatase activity could account for the fall in fluid concentrations of androgen and rise of oestradiol-17 $\beta$  concentration associated with increasing follicle size (Table 2). The sharper fall in testosterone concentrations than in those of androstenedione is consistent with previous findings that bovine granulosa cells aromatize testosterone in preference to androstenedione (Henderson & Swanston, 1978; Henderson & Moon, 1979). FSH stimulates granulosa cell aromatase activity and in this way stimulates follicular oestradiol-17 $\beta$  production (Hillier, 1981). In women (McNatty, 1978) and sheep (McNatty *et al.*, 1981) FSH concentrations in follicular fluid are related to the relative concentrations of oestradiol-17 $\beta$  and androgen present there—high FSH concentrations are associated with oestradiol-17 $\beta$ :androgen ratios  $> 1$  and low FSH concentrations to oestradiol-17 $\beta$ :androgen ratios of  $< 1$ . No such relationship was evident in the present study (Table 3). Therefore, while FSH present in bovine follicular fluid may influence the aromatase activity of the follicle, it is unlikely to be the limiting factor regulating the aromatase activity

expressed by the follicle, as reflected in the relative concentrations of oestradiol-17 $\beta$  and androgen in follicular fluid.

While there was no significant change in follicular fluid concentrations of FSH in relation to follicle size, fluid concentrations of LH and prolactin did change significantly (Table 1). LH concentrations fell slightly, but significantly, as follicle size increased. This may reflect some utilization of the fluid LH by follicular cells, particularly granulosa cells which are bathed in the follicular fluid. In contrast to LH, prolactin concentrations in follicular fluid rose as follicle size increased, which is consistent with findings for sheep follicles (McNatty *et al.*, 1981). Fluid concentrations of prolactin appeared to limit those of progesterone. Although there was no relationship between fluid progesterone concentrations and follicle size (Table 2), analysis of individual follicles revealed that high fluid progesterone concentrations (>80 ng/ml) only occurred when those of prolactin were low  $\leq$ 30 ng/ml (Table 4). The high prolactin concentrations in follicular fluid could suppress granulosa cell progesterone production; prolactin inhibits progesterone production by human (McNatty, Sawers & McNeilly, 1974) and porcine (Veldhuis, Klase & Hammond, 1980) granulosa cells *in vitro*.

While there were significant relationships between follicle size and the hormonal environment of follicular fluid, there was considerable variation, particularly in the steroid concentrations, between individual follicles of the same size. Thus, while one can generalize on how one population of follicles of a particular size is likely to differ from another, it is not possible to predict the follicular fluid environment of any individual follicle drawn from populations of follicles of defined size.

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## [<sup>125</sup>I]hCG binding to bovine thecal tissue from healthy and atretic antral follicles

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### Summary

[<sup>125</sup>I]hCG binding to thecal tissue from healthy bovine follicles was examined and compared to [<sup>125</sup>I]hCG binding to other bovine ovarian tissues. [<sup>125</sup>I]hCG bound specifically to theca interna but not to theca externa. Binding to theca interna was a time- and temperature-dependent process, the rate of association obeying second-order kinetics with calculated rate constants of  $1.97 \pm 0.13 \times 10^5$  and  $0.85 \pm 0.04 \times 10^5$  l M<sup>-1</sup> sec<sup>-1</sup> at 37 and 22°C, respectively. The dissociation of [<sup>125</sup>I]hCG from theca interna was a slow biphasic process with only 40% of specifically bound [<sup>125</sup>I]hCG being liberated after 8 h at 37°C. Unlabelled hCG and LH, but not FSH, prolactin, GH, TSH or GnRH, inhibited [<sup>125</sup>I]hCG binding to theca interna. The specific binding of [<sup>125</sup>I]hCG to theca interna was saturable and equilibrium binding data produced a linear plot when fitted to the Woolf equation. The equilibrium dissociation constant ( $K_d$ ) and maximum binding capacity ( $B_{max}$ ) calculated from Woolf plots were  $0.21 \pm 0.02$  nM (mean  $\pm$  SEM) and  $34 \pm 4$  fmoles/mg protein, respectively. Constants for [<sup>125</sup>I]hCG binding to granulosa cells and luteal tissue, respectively, were  $0.29 \pm 0.02$  and  $0.31 \pm 0.04$  nM for the  $K_d$  values and  $32 \pm 6$  and  $116 \pm 13$  fmoles/mg protein for the  $B_{max}$  values. [<sup>125</sup>I]hCG binding constants for small (< 8 mm dia.) and large ( $\geq 8$  mm dia.) follicles (healthy or atretic) were not significantly different. In addition, there was no difference in the [<sup>125</sup>I]hCG binding constants of healthy and atretic follicles (large or small). Theca interna from large healthy follicles, but not large atretic follicles, responded steroidogenically to LH, when perfused in vitro, by increased production of androstenedione. (LH and hCG were equipotent in stimulating androstenedione production by perfused theca from healthy follicles.)

These studies show that the binding characteristics of bovine theca interna LH/hCG receptors are similar to those of the LH/hCG receptors present in granulosa cells and luteal tissue. These studies also show that lack of available receptors for LH/hCG is unlikely to be the cause of the failure of large atretic follicles to produce androgen in response to LH.

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Binding to a specific plasma membrane receptor is thought to be the first event in the action of luteinizing hormone (LH) on its target cells in the ovary (Catt and Dufau, 1977; Catt et al., 1980). Studies using radiolabelled LH or human chorionic gonadotrophin (hCG), which shares a common

receptor with LH (Lee and Ryan, 1973; Dufau and Catt, 1978), have demonstrated the presence of such receptors for LH/hCG in follicular theca, granulosa cells and the corpus luteum of several species (Channing and Kammerman, 1974; Channing and Tsafri, 1977; Dufau and Catt, 1978).

While the characteristics of these receptors have been extensively investigated in granulosa cells and the corpus luteum, little comparable information is available for the thecal LH/hCG receptor. Such studies are warranted in view of the important role that LH-stimulated thecal androgen production has in the regulation of follicle development (Hillier, 1981).

The purpose of this study was to examine the characteristics of [<sup>125</sup>I]hCG binding to thecal tissue from healthy and atretic bovine follicles and to compare these with binding to other bovine ovarian tissues.

## Materials and methods

### *hCG iodination*

Human chorionic gonadotrophin (hCG, CR121, 13 450 IU/mg) was iodinated to a specific activity of 30–50  $\mu\text{Ci}/\mu\text{g}$  using lactoperoxidase/ $\text{H}_2\text{O}_2$  (Miyachi et al., 1972). Routinely, 5  $\mu\text{g}$  hCG was iodinated using 0.5 mCi  $\text{Na}^{125}\text{I}$  (Amersham, U.K.) and the products purified on a column of Sephadex G-75 ( $20 \times 1$  cm).

### *Bovine ovarian tissues*

The characteristics of [<sup>125</sup>I]hCG binding to bovine theca were determined using tissue obtained from bovine ovaries collected from a local abattoir. The breeds of these cows and their reproductive status were unknown. The effect of follicular atresia on [<sup>125</sup>I]hCG binding was examined using theca from ovaries recovered from parous Angus cows, 2 to 7 years of age, at known stages of the oestrous cycle.

### *Preparation of theca*

Theca was collected from bovine follicles as described in detail previously (McNatty et al., 1983a). Briefly, individual follicles were dissected from ovaries and trimmed free of adhering tissues. The diameter of each follicle was recorded and then each was slit open to release the intrafollicular contents. The follicular fluid was aspirated and the follicle wall washed gently and repeatedly to remove the granulosa cells with medium (Medium A) consisting of Medium 199 with Earle's salts, L-glutamine (2 mM), gentamicin (50 mg/l), HEPES buffer (20 mM) (Gibco, Grand Island, NY,

U.S.A.), sodium heparin (50 IU/ml) and 0.1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO, U.S.A.). At this stage the oocyte was located and assessed subjectively as being healthy or degenerate (McNatty et al., 1983b). The follicle wall was washed several more times and the total number of granulosa cells in all the media washings quantitated using a haemocytometer. In those follicles where the internal face of the follicle wall was pink to dark red in colour (at 10–40  $\times$  magnification), the internal layer of tissue, designated theca interna, could be separated relatively easily from the external layer, designated theca externa, using finely pointed, curved watchmaker forceps. After separating, the theca externa and interna were each washed with several changes of Medium A. Theca interna prepared from such follicles was generally  $\sim 70\%$  pure with contamination by stroma or theca externa and slight contamination by granulosa cells (5–100 cells/ $7 \mu\text{m}$  section) (McNatty et al., 1983a). Theca externa was always devoid of theca interna and granulosa cells, and granulosa cells were always devoid of thecal tissue fragments.

Theca interna prepared from several follicles were pooled together (to provide sufficient tissue for replicate experiments), homogenized by hand at 4°C with a glass/glass Potter-Elvehjem homogenizer in 0.01 M phosphate-buffered saline (pH 7.4) containing 100 mM sucrose and 5 mM  $\text{MgCl}_2$  (PBS). The homogenate was filtered through several layers of sterile gauze and aliquots of the filtrate stored frozen at  $-20^\circ\text{C}$  until used for binding studies, usually within 1 week of preparation. In some instances, homogenates of pools of granulosa cells, theca externa and ovarian stroma were prepared as above for binding studies. The stroma used in these instances was ovarian tissue devoid of cortical tissue, antral follicles ( $\geq 0.5$  mm diameter), luteal tissue and corpora albicantia. Homogenates of pools of luteal tissue, prepared as above, were centrifuged at  $450 \times g$  for 10 min, the supernatants further centrifuged at  $20\,000 \times g$  for 1 h, and aliquots of the pellets, resuspended in PBS, stored frozen until used in the binding studies. Aliquots of each preparation kept for binding studies were assayed for protein by the method of Lowry et al. (1951).

### *Classification of follicles*

The characteristics of [ $^{125}$ I]hCG binding to theca were studied using bovine theca obtained from healthy, preovulatory-sized follicles. These follicles were  $\geq 10$  mm in diameter, had a good thecal vasculature, and the theca interna was pink to red in colour. In addition, these follicles contained a healthy-looking oocyte, the follicular fluid was free of debris and the follicle contained  $\geq 50\%$  of the maximum number of recoverable granulosa cells for a follicle of a given size (McNatty et al., 1982). In the study of the effect of follicular atresia on [ $^{125}$ I]hCG binding, healthy follicles had the properties described above, except that follicle diameters ranged from 4 to 14.5 mm. Follicles considered to be atretic had debris in the follicular fluid, and/or an absent thecal vasculature, and/or a pale to white-coloured theca interna. In addition, such follicles contained  $< 50\%$  of the maximum number of recoverable granulosa cells (McNatty et al., 1982). The diameters of the atretic follicles ranged from 2 to 15 mm.

### *[ $^{125}$ I]hCG binding studies*

Aliquots of tissue homogenates (100 to 1000  $\mu$ g protein) were incubated in disposable plastic tubes with [ $^{125}$ I]hCG (0.5 to 50 ng) in a final volume of 1 ml PBS containing 0.5% egg albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.). Incubations were performed at 22 or 37°C for up to 40 h. To separate receptor-bound from free [ $^{125}$ I]hCG, the tubes were centrifuged at  $4000 \times g$  for 30 min at 4°C. The supernatants were removed by aspiration under vacuum. The pellets were washed with 1 ml ice-cold incubation buffer and the radioactivity in the pellets following a second centrifugation at  $4000 \times g$  was determined using a Packard auto-gamma 500 counter. Non-specific binding was determined by co-incubating tissue homogenates with 10 IU/ml of unlabelled gonadotrophin (gonadotrophin LH, Paines & Byrne Ltd., Greenford, U.K.). The amount of specifically bound [ $^{125}$ I]hCG was calculated by subtracting non-specific binding from the total amount of [ $^{125}$ I]hCG bound. Owing to the limited amounts of theca interna which could be obtained at any one time, incubations were performed in duplicate or triplicate. The coefficient of variation between replicate incubations was  $< 8\%$  at all times.

### *Thecal perfusions*

To determine the effect of LH and hCG on thecal steroidogenesis, samples of bovine theca interna were perfused *in vitro* as described in detail previously (McNatty et al., 1983a). Briefly, 20–50 mg of theca interna was placed into glass columns ( $4 \times 0.7$  cm, Econo-column, Bio-Rad Laboratories, CA, U.S.A.) containing 50 mg of Sephadex G-25, and pre-washed for 1 h with perfusion medium consisting of sterile Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin (w/v) which was gassed continuously with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. An aliquot of the theca was homogenized in 1 ml ethanol to determine its endogenous steroid content. The theca on the columns was perfused at 37°C for 3 h with the above perfusion medium at a flow rate of 1.4 ml/min with 12 min fractions being collected. LH (NIH-LH-B10) or hCG (CR121) was introduced into the perfusion medium for 20 min after the theca had been perfused for 1 h. At the end of the 3 h period, the tissue was fixed for histological examination and the medium from each tube frozen until assayed for androstenedione by a specific radioimmunoassay (McNatty et al., 1981). Androstenedione is the major androgen produced by bovine theca interna under these conditions (McNatty et al., 1983a).

## **Results**

### *Characteristics of [ $^{125}$ I]hCG binding to theca interna*

Specific [ $^{125}$ I]hCG binding to theca interna varied with the concentration of [ $^{125}$ I]hCG and the amount of thecal tissue in the incubations (Fig. 1). Specific [ $^{125}$ I]hCG binding was also a time- and temperature-dependent process (Fig. 2). At 37°C specific binding to theca interna increased rapidly for the first 4 h, but thereafter proceeded more slowly to reach a maximum by 20 h. After 40 h incubation at 37°C, binding had fallen by 16% compared to the maximum observed after 20 h. Specific binding at 22°C proceeded more slowly with the maximum binding obtained being 34% less than at 37°C. No reduction in maximum binding, attained after 20 h, was observed when the incubations were continued for 40 h.

Analysis of the initial binding indicated that

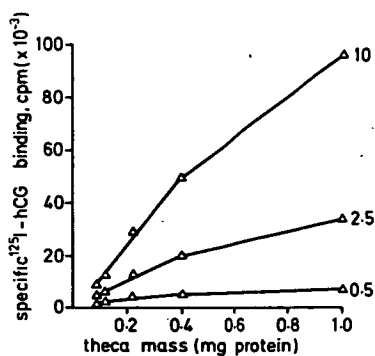


Fig. 1. Specific binding of 0.5, 2.5 and 10 ng [ $^{125}$ I]hCG to increasing amounts of bovine theca interna. Incubations were performed for 20 h at 22°C.

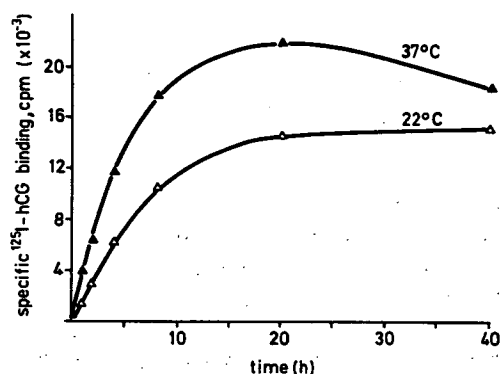


Fig. 2. Time-dependence of specific [ $^{125}$ I]hCG (5 ng/tube) binding to bovine theca interna (0.3 mg protein/tube) at 22 and 37°C.

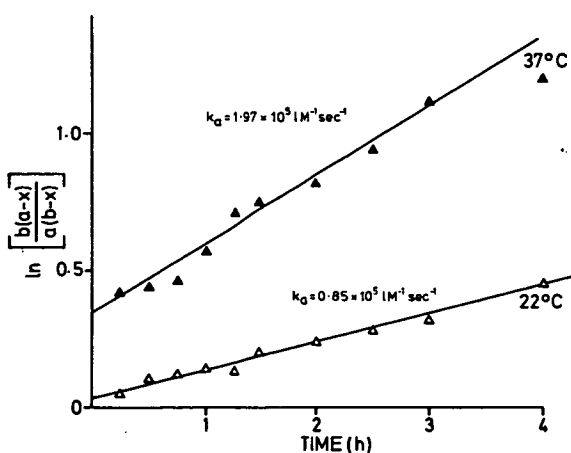


Fig. 3. Kinetics of specific [ $^{125}$ I]hCG (5 ng/tube) binding to bovine theca interna (0.3 mg protein/tube) at 22° and 37°C. The initial hormone concentration, receptor concentration and concentration of hormone-receptor complexes at each time are represented by  $a$ ,  $b$  and  $x$ , respectively. The slopes of the lines are equivalent to the association rate constant ( $k_a$ ).

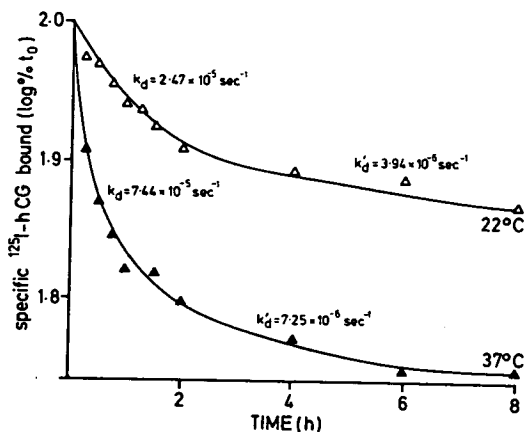


Fig. 4. Kinetics of dissociation of [ $^{125}$ I]hCG from bovine theca interna at 37 and 22°C. Theca interna (0.3 mg protein) was preincubated with [ $^{125}$ I]hCG (5 ng) for 20 h at 22°C. Bound [ $^{125}$ I]hCG was pelleted by centrifugation as described in Materials and Methods. The washed pellet was resuspended and reincubated in 1 ml buffer at 37 or 22°C for up to 8 h and the remaining specifically bound [ $^{125}$ I]hCG determined at each time point.  $t_0$  is specific binding at time 0.

specific binding of [ $^{125}$ I]hCG to bovine theca followed second-order kinetics, with calculated association rate constants ( $k_a \pm \text{SD}$ ) of  $1.97 \pm 0.13 \times 10^5$  and  $0.85 \pm 0.04 \times 10^5 \text{ l M}^{-1} \text{ sec}^{-1}$  at 37 and 22°C, respectively (Fig. 3). The dissociation of specifically bound [ $^{125}$ I]hCG from bovine theca was a slow process with only 44% (at 37°C) and 26% (at 22°C) of bound radioactivity being released within 8 h of incubation in 1 ml buffer. Plotting this data as a first-order rate reaction indicated that [ $^{125}$ I]hCG dissociation was biphasic (Fig. 4). The dissociation rate constants for the fast phase were ( $k_d \pm \text{SD}$ )  $7.44 \pm 0.63 \times 10^{-5} \text{ sec}^{-1}$  and  $2.47 \pm 0.14 \times 10^{-5} \text{ sec}^{-1}$  at 37 and 22°C, respectively, and for the slow phase ( $k'_d \pm \text{SD}$ )  $7.25 \pm 1.15 \times 10^{-6} \text{ sec}^{-1}$  and  $3.94 \pm 0.51 \times 10^{-6} \text{ sec}^{-1}$  at 37 and 22°C, respectively.

#### Specificity studies

Fig. 5 shows that binding of [ $^{125}$ I]hCG (1 ng) to bovine theca interna was progressively displaced by the addition of increasing amounts of unlabelled hCG. Ovine LH (NIH-LH-S19) and bovine LH (NIH-LH-B10) also displaced [ $^{125}$ I]hCG binding though less effectively than hCG: it required  $\sim 2 \times 10^{-9} \text{ M}$  ovine or bovine



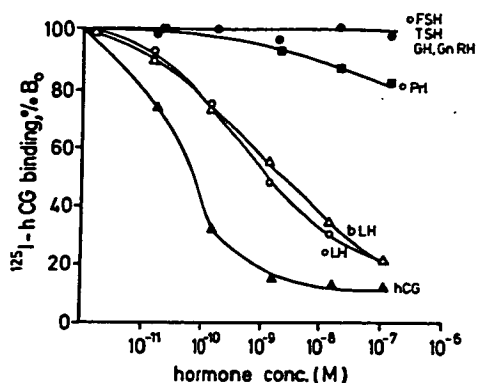


Fig. 5. Displacement of [ $^{125}$ I]hCG binding by increasing amounts of unlabelled hormones. Binding in the absence of unlabelled hormone ( $B_0$ ) was regarded as 100%.

LH, compared to  $\sim 7 \times 10^{-11}$  M hCG, to displace [ $^{125}$ I]hCG binding by 50%. Bovine prolactin (NIH-Prl-B4) was only very slightly effective in displacing [ $^{125}$ I]hCG binding, a concentration of  $10^{-7}$  M being required to reduce binding by 20%. Ovine follicle-stimulating hormone (NIH-FSH-S11), thyroid-stimulating hormone (NIH-TSH-S8), growth hormone (NIH-GH-0986C) and

gonadotrophin-releasing hormone (porcine LH-RH, Beckman, Palo Alto, CA, U.S.A.) were each ineffective in displacing [ $^{125}$ I]hCG binding at concentrations up to  $10^{-7}$  M. Because of the difference in effectiveness of LH and hCG in displacing bound [ $^{125}$ I]hCG from theca (Fig. 5), the relative potency of bovine LH and hCG in stimulating androstenedione production by bovine theca interna in vitro was examined. The results in Fig. 6 show that both gonadotrophins at each dose, except for 0.2 nM bLH, significantly increased androstenedione output compared to that observed in the controls ( $P < 0.05$ , Friedman analysis in conjunction with multiple comparisons of rank sums). While the stimulatory effects of both bLH and hCG were dose-dependent, there was no significant difference between the effectiveness of bLH and hCG at each of the doses tested ( $P > 0.05$ ).

#### Equilibrium binding studies

Binding to bovine theca interna as a function of [ $^{125}$ I]hCG concentration is shown in Fig. 7(a). Incubations were performed at 22°C for 20 h to achieve equilibrium. A linear plot was obtained

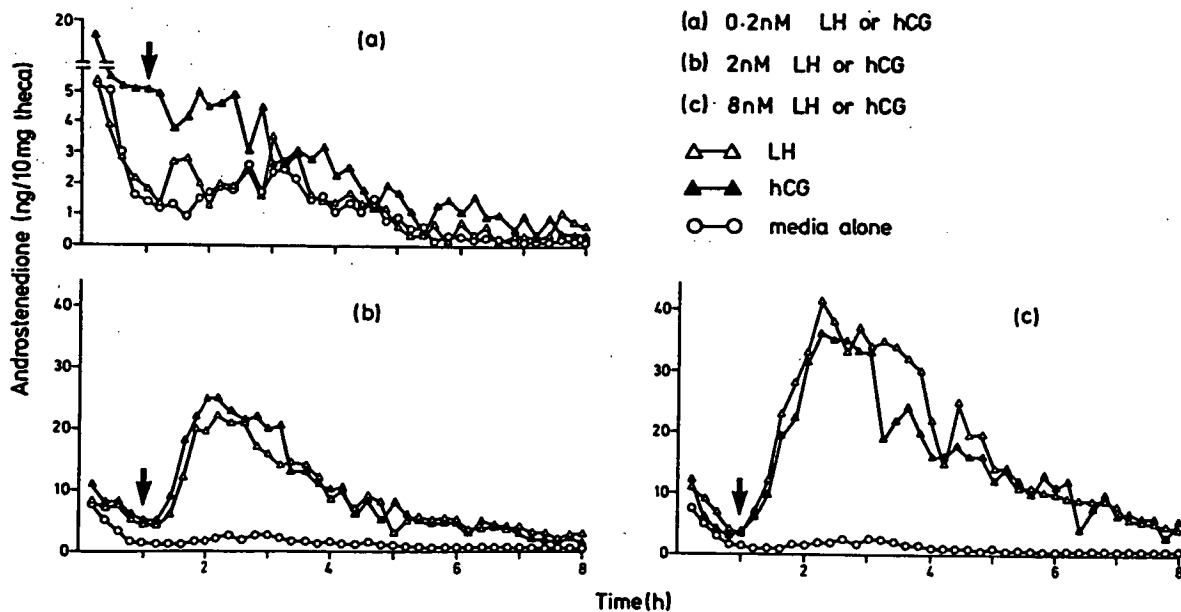


Fig. 6. Output of androstenedione by bovine theca interna every 12 min before, during and after 20 min exposure to 0, 0.2, 2 and 8 nM LH (NIH-LH-B10) or hCG (CR121). LH and hCG were added 1 h after the start of the perfusion ( $\downarrow$ ). The endogenous thecal concentration of androstenedione at the start of the infusion was 1.1 ng/mg theca.

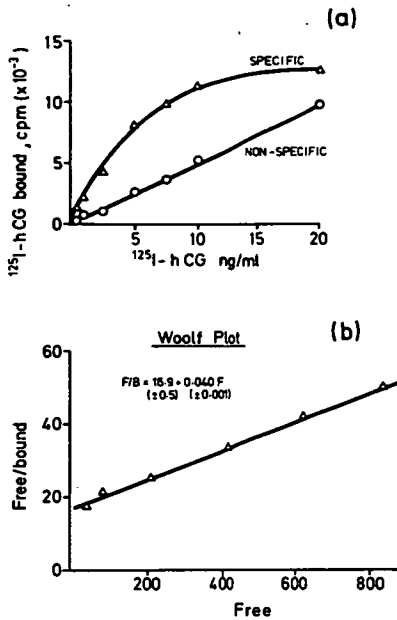


Fig. 7. (a) Specific ( $\Delta$ — $\Delta$ ) and non-specific ( $\circ$ — $\circ$ ) binding of [ $^{125}\text{I}$ ]hCG to theca interna as a function of [ $^{125}\text{I}$ ]hCG concentration. (b) Woolf plot of data for specific [ $^{125}\text{I}$ ]hCG binding to theca interna. Values in parentheses show  $\pm$  S.D. of intercept and gradient values.

when the specific binding data were fitted to the Woolf equation (Haldane, 1957; Cressie and Keightley, 1981) (Fig. 7(b)). Analysis of the intercept and gradient values gave a value for the apparent equilibrium dissociation constant ( $K_d$ )

TABLE 1

BINDING CONSTANTS FOR SPECIFIC [ $^{125}\text{I}$ ]hCG BINDING TO BOVINE OVARIAN TISSUES

Tissue	$K_d$ (nM)	$B_{max}$ (fmoles/ mg protein)
Theca interna	$0.21 \pm 0.02$	$34 \pm 4$
Theca externa	no specific binding	
Granulosa cells	$0.29 \pm 0.02$	$32 \pm 6$
Corpus luteum	$0.31 \pm 0.04$	$116 \pm 13$
Stroma	no specific binding	

Values are mean  $\pm$  SEM for 5 different pools of tissue. All binding constants were calculated after tissues were incubated for 20 h at 22°C to achieve equilibrium.

of 0.14 nM and for the binding capacity ( $B_{max}$ ) of 25 fmoles/mg protein.

The mean calculated equilibrium dissociation constant ( $K_d$ ) and binding capacity ( $B_{max}$ ) from Woolf plots of specific [ $^{125}\text{I}$ ]hCG binding to different bovine ovarian tissues are shown in Table 1. No specific [ $^{125}\text{I}$ ]hCG binding to homogenates of theca externa or ovarian stroma was observed. The dissociation constant for [ $^{125}\text{I}$ ]hCG binding to theca interna, granulosa cells and luteal tissue were each similar ( $\sim 0.2$ – $0.3$  nM). Although the binding capacity of theca interna and granulosa cells was similar ( $\sim 30$  fmoles/mg protein), that for luteal tissue was approximately 4-fold higher ( $\sim 120$  fmoles/mg protein).

TABLE 2

[ $^{125}\text{I}$ ]hCG BINDING CONSTANTS AND STEROIDOGENIC RESPONSE TO LH OF BOVINE THECA INTERNA FROM HEALTHY AND ATRETIC FOLLICLES

	Follicular diameter				Androstenedione (ng/10 mg theca/3 h)	
	< 8 mm		$\geq 8$ mm		- LH	+ LH
	$K_d$ (nM)	$B_{max}$ (fmoles/mg protein)	$K_d$ (nM)	$B_{max}$ (fmoles/mg protein)		
Healthy	$0.10 \pm 0.01$ (8)	$11 \pm 1$ (8)	$0.11 \pm 0.01$ (11)	$16 \pm 3$ (11)	$89 \pm 15$ (10)	$501 \pm 44$ (10)
Atretic	$0.10 \pm 0.01$ (10)	$13 \pm 2$ (10)	$0.10 \pm 0.02$ (8)	$13 \pm 1$ (8)	$80 \pm 12$ (5)	$70 \pm 17$ (5)

Values are mean  $\pm$  SEM of ( $n$ ) determinations. Binding constants were calculated from Woolf plots of specific [ $^{125}\text{I}$ ]hCG binding to thecal homogenates from small (< 8 mm) and large ( $\geq 8$  mm) diameter follicles. Perfusions were performed in vitro using thecal tissue from large follicles ( $\geq 8$  mm diameter), the theca being exposed to 200 ng/ml bovine LH (NIH-LH-B10) for 20 min. The endogenous thecal concentrations of androstenedione at the start of the experiment were < 20 ng/10 mg tissue.

*[<sup>125</sup>I]hCG binding constants and steroidogenic capacity of theca interna from healthy and atretic follicles*

The data in Table 2 show that there was no significant difference ( $P > 0.05$ , analysis of variance) in either the dissociation constants or the binding capacities for [<sup>125</sup>I]hCG binding to theca interna between small (< 8 mm) or large (> 8 mm) diameter follicles (healthy or atretic) or between healthy or atretic follicles (small or large). Theca interna from large healthy follicles responded steroidogenically to LH, when perfused in vitro for 3 h, and produced significantly more androstenedione ( $P < 0.001$ , Student's paired *t*-test) than theca perfused in the absence of LH (Table 2). LH did not stimulate androstenedione production by theca interna from large atretic follicles ( $P > 0.05$ , Student's paired *t*-test) (Table 2). The steroidogenic responsiveness to LH was not examined in small follicles because sufficient amounts of theca interna for perfusion could not be obtained from small atretic follicles.

### Discussion

The present study demonstrates that specific receptors for [<sup>125</sup>I]hCG in the bovine follicle wall are located exclusively in the theca interna. The binding characteristics of the receptor sites were described using iodinated hCG rather than bovine LH, because of the greater stability and binding activity of the former. Previous studies indicate that hCG and LH share common receptors in both the ovary and testis (Dufau and Catt, 1978). Consistent with this view was the finding that bovine LH and hCG were equipotent in stimulating androstenedione production by theca interna perfused in vitro (Fig. 6). The theca interna LH/hCG receptor showed a greater affinity for hCG than for LH as it required approximately 30-fold more ovine or bovine LH than hCG to displace 50% of [<sup>125</sup>I]hCG bound to theca interna (Fig. 5). Presumably this reflects an ability of the thecal receptor to distinguish between the physicochemical properties of the primate and non-primate gonadotrophins. Similar observations have previously been made in studies using luteal tissue (Cameron and Stouffer, 1981).

Binding of hCG to theca interna was time- and

temperature-dependent. Examination of the kinetics of binding indicated that they obeyed a second-order rate equation which is consistent with the kinetics of [<sup>125</sup>I]hCG and [<sup>125</sup>I]LH binding to other ovarian preparations (Lee and Ryan, 1973; Gospodarowicz, 1973; Rao, 1974; Stouffer et al., 1976; Cameron and Stouffer, 1982). Binding of [<sup>125</sup>I]hCG to theca interna was not readily reversible, but was slow and biphasic comparable to that observed for [<sup>125</sup>I]hCG and [<sup>125</sup>I]LH dissociation from granulosa cells (Stouffer et al., 1976) and luteal tissue (Gospodarowicz, 1973; Rao, 1974; Cameron and Stouffer, 1982).

Calculation of the equilibrium dissociation constant ( $K_d$ ) for [<sup>125</sup>I]hCG binding to theca interna from the rate constants for association ( $k_a = 0.85 \times 10^5 \text{ l M}^{-1} \text{ sec}^{-1}$ ) and dissociation ( $k_d = 2.47 \times 10^{-5} \text{ sec}^{-1}$ ) at 22°C gives a value of 0.29 nM which is quite comparable to those calculated from Woolf plots of the binding of [<sup>125</sup>I]hCG to theca interna at equilibrium (Fig. 7(b), Table 1). Comparison of the calculated constants for [<sup>125</sup>I]hCG binding to bovine theca interna, granulosa cells and luteal tissue from Woolf plots indicated that the values of the equilibrium dissociation constant were quite comparable, in the order of 0.2–0.3 nM. Thus, the LH/hCG receptor may be similar in each of these ovarian tissues. The binding capacity of luteal tissue, however, was approximately 4-fold higher than that for theca interna and granulosa cells which had similar values of approximately 30 fmoles/mg protein. The values of  $K_d$  and  $B_{\text{max}}$  found in this study are comparable to those found previously for [<sup>125</sup>I]hCG binding to porcine granulosa cells (Lee, 1976; Stouffer et al., 1976) and bovine luteal tissue (Rao, 1974; Papaionannou and Gospodarowicz, 1975).

Autoradiographic studies of bovine (Merz et al., 1981), rat (Uilenbroek et al., 1980), hamster (Oxberry and Greenwald, 1982) and monkey (Zeleznik, 1982) follicles have demonstrated that [<sup>125</sup>I]hCG binds to theca of almost all antral follicles, small or large, healthy or atretic. The present study demonstrates that the actual values of the binding constants for [<sup>125</sup>I]hCG binding to bovine theca interna are also independent of follicle size or health (Table 2). Recent studies have shown that LH responsiveness of theca per unit mass of tissue is not influenced by follicle size

(McNatty et al., 1983a), which is consistent with the present finding that the hCG binding constants are independent of follicle size (Table 2). Although hCG binding constants were independent of follicle health (Table 2), when theca interna from large healthy and atretic follicles were compared with regard to their capacity to produce androgen in response to LH, only theca interna from healthy follicles produced increased amounts of androstenedione (Table 2). Thus, lack of available receptors for LH/hCG would not seem to be the reason for the failure of LH to stimulate androgen production by large atretic follicles. More likely causes are that the receptors are uncoupled from adenylate cyclase and/or steroidogenesis or that the activity of one or more of the enzymes involved in androgen biosynthesis is impaired. Studies to distinguish between these possibilities are currently in progress.

In conclusion, the present studies demonstrate that the bovine LH/hCG receptor in the follicle wall is located exclusively in the theca interna and that the binding characteristics of this receptor are similar to those of the LH/hCG receptor in granulosa cells and luteal tissue, the other target cells for LH in the ovary. In addition, lack of available receptors for LH/hCG is unlikely to be the cause of the failure of large atretic follicles to produce androstenedione in response to LH.

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**Authors:** K.P. McNatty, D.A. Heath, S. Lun, J.M. Fannin, J.M. McDiarmid & K.M. Henderson

**Title:** Steroidogenesis by bovine theca interna in an *in vitro* perfusion system.

**Reference:** *Biology of Reproduction* (1984) 30, 159-170

## Steroidogenesis by Bovine Theca Interna in an In Vitro Perfusion System

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### ABSTRACT

The aims of these studies were: to examine the steroidogenic responses of perfused bovine theca interna to varying flow rates of media and varying amounts of luteinizing hormone (LH), and to compare the steroidogenic outputs of theca interna from follicles of differing size and health with those of other ovarian tissues. The results showed that the outputs of androstenedione by thecae interna from healthy but not atretic follicles, with or without stimulation by LH, were amplified by the flow rate of media. Steroidogenesis by perfused theca interna was also influenced by the mass and concentration of LH as well as by the duration of exposure to LH.

When expressed on a per unit mass basis, the outputs of androstenedione from LH-primed thecae interna from small (2-5.5 mm diameter), medium (6-9.5 mm diameter) and large (>10 mm diameter) healthy follicles were comparable. But when the above data were expressed per total mass of theca interna, the androstenedione output increased significantly with increasing follicular diameter ( $P < 0.01$ ).

Under the experimental conditions employed, the fraction of androstenedione produced by thecal tissue as a percentage of the total output of progesterone, androstenedione, testosterone and estradiol was 82%, whereas the progesterone, testosterone and estradiol fractions were 1%, 15% and 2%, respectively. By contrast, the granulosa cell output of progesterone, androstenedione, testosterone and estradiol were 79%, 0%, 0% and 21%, respectively. When this cell type was supplied with saturating amounts of androstenedione, it contributed >90% of the total quantity of estradiol by the two cell types in isolation.

### INTRODUCTION

It is generally accepted that thecal and/or stromal tissues are important intraovarian sources of androgen (see Peters and McNatty, 1980, for review). In the cow, as in other species, it is considered that the synthesis of ovarian androgen is regulated, at least in part, by the circulating concentrations of luteinizing hormone (LH) (Baird and McNeilly, 1981; Merz et al., 1981). In many mammals, the ovarian theca consists of two distinct layers of cells, namely the theca interna (i.e., the layer closest to the granulosa cells) and the theca externa (Marion et al., 1968; O'Shea, 1970, 1971; Peters and McNatty, 1980). Due to the heterogeneity of cell types in the follicle wall, there is only limited information on the steroidogenic function of purified theca interna and/or externa cells (Stoklosowa et al., 1978).

In the bovine ovary, the theca interna contains an extensive blood supply. Although the rate of blood flow through the theca is unknown, it is reasonable to speculate that the endocrine milieu at the level of the theca interna is a rapidly changing one and that the steroids synthesized in this region would be cleared rapidly into the peripheral circulation and to a lesser extent into the follicular antrum (McNatty et al., 1981a). However, to our knowledge, the dynamic aspects of ovarian thecal function have not been investigated in vitro.

The aims of the present studies were: to examine the steroidogenic responses of perfused bovine theca interna to varying flow rates of media and varying amounts of LH, and to compare the steroidogenic outputs of theca interna from follicles of differing size and health with those of other ovarian tissues.

### MATERIALS AND METHODS

#### *Ovarian Tissue*

For most studies bovine ovaries were collected from the local abattoir and transported to the labora-

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tory in a plastic bag immersed in ice water. The breeds of cow and reproductive status were unknown. In some studies (where specified) the ovaries were recovered from parous Angus cows (2–7 years of age) at known days of the estrous cycle.

Individual follicles were dissected from the ovaries which were immersed in Medium A at room temperature. Medium A consisted of sterile Medium 199 with Earle's salts, L-glutamine, gentamycin (50 mg/l), Hepe's buffer (20 mM; Gibco, Grand Island, NY) sodium heparin (50 IU/ml) and 0.1% (wt/vol) bovine serum albumin (BSA; fraction V; Sigma Chemical Co., St. Louis, MO). The inclusion of heparin was to minimize cell clumping (Metcalf, 1982).

#### *Recovery of Thecal Tissue, Stroma and/or Granulosa Cells*

After being cleanly dissected from extraneous ovarian tissue, the diameters of the individual follicles were measured. Each follicle was then transferred to a separate petri dish (35 × 10 mm, Falcon Plastics, Los Angeles), slit open to release its intrafollicular contents and, at 10–40X magnification (under the dissecting microscope), the follicular fluid was aspirated through a fine-bore capillary tube while taking care not to remove clumps (or sheets) of granulosa cells. Subsequently, the follicle wall was washed gently and repeatedly with ~2 ml of Medium A and the released clumps (or sheets) of granulosa cells were dispersed by pipetting several times with a finely drawn, orally controlled Pasteur pipette. At this stage, the oocyte was located and assessed subjectively as being healthy or degenerate as previously described (McNatty et al., 1983a). Thereafter, the follicle wall was removed to a new petri dish containing Medium A, scraped gently with a blunt spatula and washed several times more. All washings of the follicle wall were then combined and the total population of granulosa cells quantitated by hemocytometer. After being freed of most granulosa cells, the follicular fluid and the oocyte, an attempt was made to separate the theca interna from the externa. This was done by first identifying (at 10X magnification) the blood vessels on the internal face of the follicle wall. If, in the absence of medium, this internal layer of tissue was pale pink to dark red in color, it could be separated relatively easily from the remainder of the follicle wall using finely pointed, curved watchmaker's forceps.

The above vascular-enriched tissue constituted about 5 to 35% of the follicle wall. Once isolated, this tissue was washed several times more, weighed and then a portion was removed for histology. At this point of the procedure the vascularized tissue was tentatively referred to as theca interna and the remaining less vascular remnants of the follicle wall as theca externa. Normally, the theca externa varied in color between flat white and pale yellow. As with the interna, the theca externa was weighed and a portion fixed in 10% aqueous formalin for histology.

Unless stated otherwise, the thecae interna or externa from several follicles of identical characteristics (see follicle classification below) were pooled so that sufficient tissue was available for replicate experiments.

The stroma used for these studies was ovarian tissue that was devoid of cortical tissue, antral follicles (>0.5-mm diameter), luteal tissue and corpora albicantia.

The granulosa cells used in the steroid production-rate studies were washed in excess Medium A before use and then resuspended in Medium A without sodium heparin and quantitated once more by hemocytometer. The viability of the granulosa cells was >95% as assessed by the trypan blue dye exclusion test.

#### *Classification of Ovarian Follicles*

To assess the state of health of the various follicles from which the theca or granulosa cells were recovered, the following factors were considered: the presence or absence of thecal blood capillaries when the intact (but cleanly dissected) follicles were observed at 10X magnification; the presence or absence of debris in follicular fluid; the status of the oocyte; the total number of granulosa cells expressed as a percentage of the maximum number of cells observed in a follicle of that size; and the color of the theca interna (i.e., red, pink or white). In bovine follicles ranging in diameter from 1, 4, 6, 8, 10, 12, 14, 16 or 18 mm the respective maximum numbers of granulosa cells that have been recovered are 1, 4, 7, 10, 14, 18, 24, 36 and 40 million. Unless stated otherwise, all the thecae interna in this study were from follicles >10 mm in diameter, vascular (i.e., at 10–40X magnification) and pink to red in color. Moreover, the thecae also came from follicles that contained a healthy-looking oocyte, a follicular fluid free of debris, and >50% of the maximum number of recoverable granulosa cells for a follicle of a given size (McNatty et al., 1979, 1983a; McNatty, unpublished data). For the purpose of this study, these specific follicles were defined as healthy, preovulatory follicles.

#### *Tissue Perfusion System*

The theca (interna/externa) or stroma were stripped into fine sheets of tissue using forceps and then added to a glass column (4 × 0.7 cm, Econo-column, Bio-Rad Labs., Richmond, CA) containing a polypropylene disc (capable of retaining particles >35 μm) at its base, supporting 50 mg of Sephadex G-25 (fine; Pharmacia Fine Chemicals, Uppsala, Sweden). The purpose of the Sephadex support was to prevent the ovarian tissues from blocking the flow of medium through the polypropylene disc. Before being used for any of the studies, the entire column system was prewashed for 1 h with Medium B which consisted of sterile Krebs-Ringer bicarbonate solution and 1% BSA (wt/vol); this prewash with a 1% BSA solution was to minimize the potential adsorption of steroids, etc., to the Sephadex and effluent tubing. The medium was fed to the column from an elevated reservoir which was gassed continuously with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. The rate of medium flow through the column was regulated by an autoanalyzer pump (Technicon Instr., Tarrytown, NY) connected to the column outlet, and the column effluents were collected into glass test tubes in a fraction collector modified to collect effluents from 10 separate columns simultaneously. With individual columns, the flow rate was always held constant throughout the experiment. Thus, in studies where the flow rate was varied, the variation was between different columns. The dead space of the system between the column outlet and the collection tubes was 1.5 ml. At flow rates of 0.1 to 0.4 ml/min where media was being collected at 12-min intervals, the

collection times were adjusted to take into account the dead space. All the perfusion experiments were performed in a thermostatically controlled room heated to 37°C. With a 50 mg Sephadex G-25 support, only minimal retention of steroid occurred after the columns had been prewashed. At a perfusion rate of 1.4 ml/min and with tube changes every 12 min, 75% of radiolabeled androstenedione appeared in the first tube following addition of the label, and after tube number 3 (i.e., after 36 min) >95% of the label had been recovered. A similar pattern of elution was noted for progesterone, testosterone and estradiol. After prewashing the column, the ovarian tissues were added to the top of the Sephadex G-25 and perfused with Krebs-Ringer bicarbonate + 0.1% BSA (Medium C; after a prewash with a 1% BSA solution it was found that the BSA concentration could be reduced without increasing the adsorption of steroids, etc.). A smaller fraction of the tissue to be studied by perfusion was placed in 1 ml of ethanol, homogenized and stored at -20°C until assayed for steroids. After being placed in the columns, the tissues were perfused for 1 h before any studies concerning steroid production rates commenced. The fraction collector was timed so that the test tubes collected effluent for 12 min and, unless stated otherwise, the production rate studies continued for 3 h. At the end of the study, the tissues were fixed for histology and the medium from each tube frozen to -20°C until analyzed for steroids by radioimmunoassay (RIA).

The LH used in these studies was NIAMDD-bLH-4. The LH in Medium C was introduced to the top of the column via a 3-way stopcock. In some experiments, the theca interna was exposed to LH for variable lengths of time. When the LH exposure time was 0.5 or 1 min, the LH was delivered in a fixed volume at the predetermined flow rate. When the exposure time was 5 min or longer, the duration of exposure was determined by stopwatch.

#### *Granulosa Cell Cultures*

The washed granulosa cells resuspended in sterile Medium D (i.e., Medium 199, Earle's salts, L-glutamine + 0.1% BSA) were aliquoted (0.5 ml) into 10 × 75 mm stoppered plastic tubes (1–5 × 10<sup>5</sup> cells/tube), containing 0.5 ml of 2000 ng/ml of testosterone in Media D or Media D alone, gassed with 5% CO<sub>2</sub> in air and then incubated for 3 h in a shaking water bath (at 37°C). Preliminary studies revealed that, when granulosa cells from healthy, preovulatory-sized follicles were incubated under the above conditions, the rate of metabolism of testosterone to estradiol was linear for the first 3 h. Moreover, it was found that the production rate of estradiol under the above conditions was the maximum attainable and not enhanced if the cells were perfused at a rate of 0.1 to 0.4 ml/min.

#### *Hormone Assays*

Aliquots of the medium samples (0.1 ml) from the column perfusions and granulosa cell incubations were assayed directly, without extraction, for progesterone, androstenedione, testosterone or estradiol using antisera and assay methodologies reported elsewhere (McNatty et al., 1981b, 1982). All standard curves and internal standards were prepared in the appropriate Medium (C or D). Estradiol was also

measured in diluted (10- to 100-fold) aliquots of follicular fluid, the diluent being phosphate-buffered saline (PBS; 0.10 M, pH 7.4).

For the progesterone antiserum (WA-26), the major cross-reacting steroids were 11 $\alpha$ -hydroxyprogesterone, 120%; 11 $\beta$ -hydroxyprogesterone, 25%; 20 $\alpha$ -hydroxyprogesterone, 3.5%; and androstenedione, 0.45%. The minimal detectable quantity of progesterone was 20 pg per assay tube.

The major cross-reacting steroids with androstenedione antiserum (WA-965) were 4-androsten-3,11,17-trione, 40%; 11 $\beta$ -hydroxyandrostenedione, 31%; and testosterone, 0.4%. The detection limit for androstenedione was 8 pg per assay tube.

The major cross-reacting steroids with the testosterone antiserum (WA-36) were 5 $\alpha$ -dihydrotestosterone, 75%; 5 $\beta$ -dihydrotestosterone, 75%; 5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol, 37.5%; 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -biol, 0.1%; progesterone, 3%; androstenedione, 0.1%. The detection limit for testosterone was 5 pg per assay tube.

The major cross-reacting steroids with the estradiol antiserum (WA-27) were estrone, 7.3%; estriol, 1.4%; and estradiol-17 $\alpha$ , 1.4%. The detection limit for estradiol was 5 pg per assay tube.

The inter- and intraassay coefficients of variation for all the above steroid assays were <10%.

Some media fractions from column effluents were analyzed for bovine LH by a specific radioimmunoassay similar to that described elsewhere (McNatty et al., 1981b). The only differences to the published assay procedure were that the bovine LH preparation LER 1716-2 was used as the iodination standard and NIAMDD-bLH-4 for generating the standard curves. All samples were measured in one assay. The mean within assay coefficient of variation was 6% and the detection limit 0.2 ng/ml.

#### *Statistical Treatment*

Differences between different treatment groups were determined by analysis of variance. Where the data indicated heterogeneity of variance, the data were log transformed.

## RESULTS

### *Purification of Isolated Theca and Granulosa Preparations*

The identification and separation of a vascular-enriched theca interna from a less vascular externa was routinely possible in follicles adjudged to be healthy (Fig. 1A). In follicles with a low number of granulosa cells (i.e., <25% of the maximum number expected in a follicle of a given size), it was not possible to identify a vascular theca interna or a pink to red lining of tissue on the inner face of the follicle wall (Fig. 1B). On some occasions, the theca interna was found to be relatively free of granulosa cells (i.e., <5 recognizable cells per 7- $\mu$ m section) and theca externa tissue (Figs. 1C and D). However, in most instances some granulosa cells (i.e., between 5 and 100 cells per



7- $\mu$ m section) could be found attached to the theca interna both before and after perfusion of the tissue. Moreover, most thecae interna were contaminated with some (<40%) stromal or theca externa tissue (Figs. 1E and F). On average, the theca interna was assessed to be about 70% pure. The wet weight of purified

theca interna was linearly related to the amount of measurable protein as assessed by the method of Lowry et al. (1951): for thecal wet weights of 5, 10, 20, 30 and 40 mg the amounts of measurable protein were 0.25, 0.5, 1.0, 1.4 and 1.9 mg, respectively. The theca externa was always devoid of theca interna and granulosa

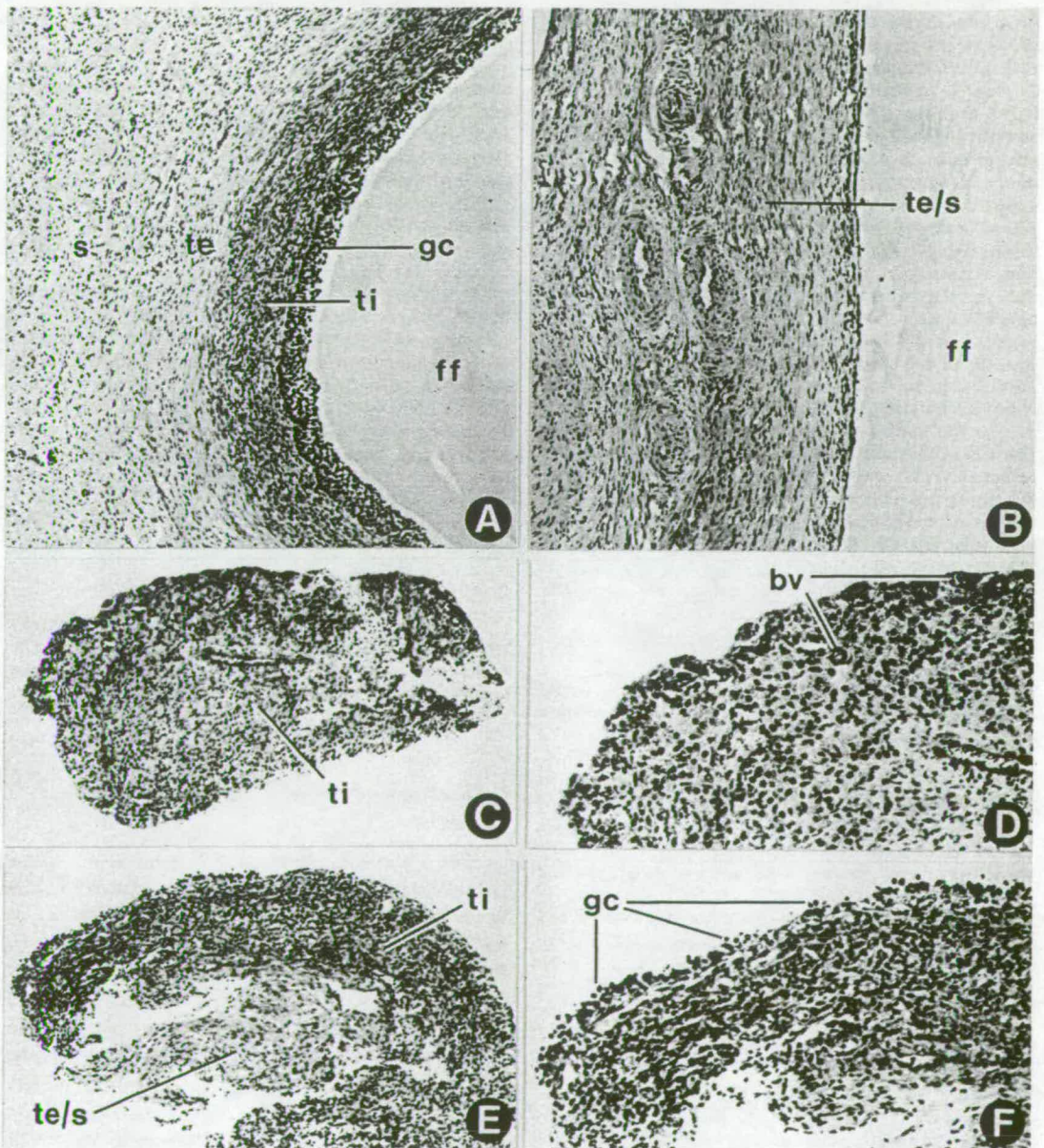


FIG. 1. Cross-sectional (7- $\mu$ m) views of various bovine ovarian follicular tissues. A) A healthy bovine follicle.  $\times 189$ . B) An atretic bovine follicle devoid of granulosa cells.  $\times 189$ . C) a cleanly dissected theca interna (i.e., free of granulosa cells and theca externa).  $\times 139$ . D) A higher magnification of C.  $\times 432$ . E) A more typical sample of the purity of theca interna that was perfused.  $\times 139$ . F) A higher magnification of E.  $\times 432$ . ff=follicular fluid; gc=granulosa cells; ti=theca interna; te=theca externa; s=stroma; bv=blood vessel.

cells. Granulosa cells were always devoid of thecal tissue fragments.

Perfusion of thecal or stromal tissues for 3-6 h had no noticeable effect on tissue viability, as assessed by histology.

#### Medium Flow Rate, LH Exposure and Androstenedione Output by Theca Interna

At the start of the experiments, the mean (and range) of androstenedione concentrations in theca interna was 16 (1-46) ng/10 mg tissue. The effects of perfusing thecal tissue at 0.1, 0.4, 0.7, 1.4 and 2.75 ml per min with Medium C are shown in Fig. 2. The amounts of androstenedione represent the cumulative amounts generated over a 3-h interval. The cumulative amounts of androstenedione, after being perfused at 0.7 or 1.4 ml/min, were ~7.5-fold higher than the amount produced after a perfusion rate of 0.1 ml/min ( $P < 0.01$ ) and were significantly higher than at Time 0 ( $P < 0.01$ ).

The effects of flow rate on the cumulative amounts of androstenedione produced by purified theca interna for a 3-h period during and after exposure to bovine LH (200 ng/ml) for 20 min are shown in Fig. 2. At all flow rates other than 0.1 ml/min, LH stimulated a significant increase in androstenedione output relative to the controls ( $P < 0.01$ ). The peak androstenedione response to LH was achieved at a flow rate of 1.4 ml/min.

To discriminate between flow rate and the total amount of LH presented to the tissue, a further set of experiments were performed where the total mass of LH delivered to purified thecae interna over a 20-min period was held constant (i.e., 5600 ng LH) while the flow rate of perfusion medium was varied from 0.1 to 2.75 ml/min. The effects of these treatments on the cumulative amounts of androstenedione produced by theca interna for a 3-h period during and after LH exposure are shown in Fig. 3. As the flow rates increased from 0.1 ml/min to 1.4 ml/min there was a progressively greater output of androstenedione even though the total mass of LH delivered to the respective tissues was held constant. The peak androstenedione output to 5600 ng of LH was achieved at a flow rate of 1.4 ml/min.

#### Effect of Duration of LH Exposure on Androstenedione Output by Theca Interna

These data are summarized in Fig. 4. Over a

time frame of 204 min and at a flow rate of 1.4 ml/min, the half-maximum androstenedione output was achieved following 45 sec of LH exposure and the peak output after 20 min LH exposure. Prolonged exposure of the tissue to LH beyond 20 min did not lead to an increase in androstenedione production.

#### Effect of LH Concentration on Androstenedione Output by Theca Interna

These data are summarized in Figs. 5 and 6.

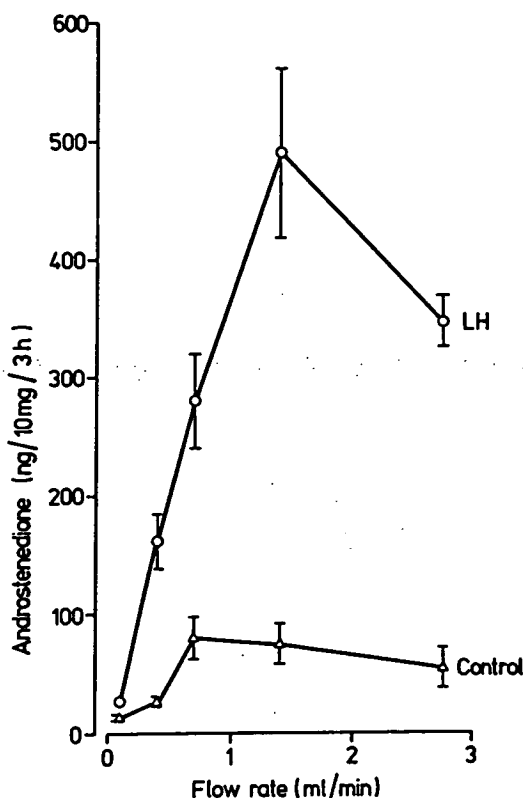


FIG. 2. The relationship between medium (Krebs-Ringer bicarbonate + 0.1% BSA) flow rate across theca interna and the cumulative (3-h) production of androstenedione with and without exposure to LH. Results are means  $\pm$  SEM. The thecae interna were from healthy preovulatory-sized bovine follicles ( $> 10$  mm diam). In these experiments, LH (at 200 ng/ml) was added for 20 min and the cumulative (3-h) production of steroid refers to the amounts produced during, as well as after, LH stimulation. Each point was determined from the results of 5-16 separate experiments. At 0.4, 0.7, 1.4 and 2.75 ml/min, the androstenedione output by LH-primed thecae was significantly greater than that of the controls (all  $P < 0.01$ ). For the control tissue, the outputs at flow rates of 0.7, 1.4 and 2.75 ml were significantly greater than at 0.4 or 0.1 ml/min ( $P < 0.01$ ).

Figure 5 refers to the cumulative 3-h output of androstenedione during and after 20 min exposure to different concentrations of LH at a media flow rate of 1.4 ml/min. The peak output of androstenedione was achieved at an LH concentration of 200 ng/ml and the half-maximum output with an LH concentration of 9 ng/ml. The output of androstenedione following exposure of the tissue to the lowest LH concentration (i.e., 1 ng/ml) was significantly greater ( $P < 0.01$ ) than that by the control tissue.

An example of the androstenedione output at 12-min intervals before, during and after 20

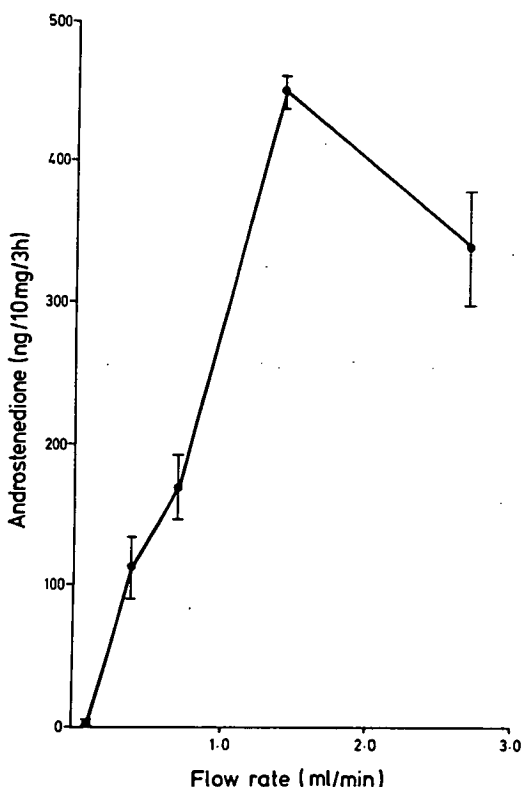


FIG. 3. The relationship between medium (Krebs-Ringer bicarbonate + 0.1% BSA) flow rate across thecae interna and the cumulative production of androstenedione during and after exposing the thecae to a constant-integrated amount of LH (5600 ng). The thecae interna were perfused with Krebs-Ringer bicarbonate + 0.1% BSA at 1.4 ml/min at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. Results are means  $\pm$  SEM of 4 experiments. The thecae interna were from healthy preovulatory-sized bovine follicles ( $>10$  mm diam). At each flow rate the LH was added for 20 min and the cumulative production refers to the 3-h output during and after LH stimulation.

min LH exposure (at 0, 5 and 200 ng/ml of LH) is shown in Fig. 6. During the first hour (i.e., the wash phase), the androstenedione output declined in all tissue preparations. In the tissues exposed to LH, an increased output of androstenedione (above basal values) was observed 12-24 min after the addition of LH; peak steroid outputs were observed about 60 min after LH had been added. At an LH concentra-

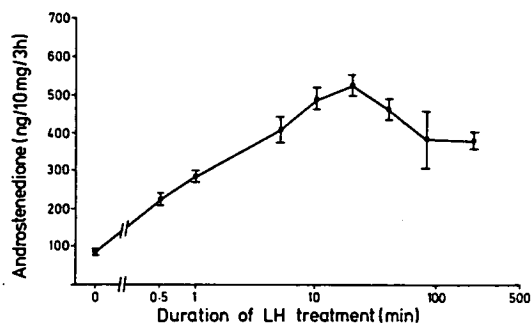


FIG. 4. The relationship between duration of LH treatment of thecae interna and the cumulative (3-h) production of androstenedione at a media flow rate of 1.4 ml/min. The LH concentration was 200 ng/ml. The thecae interna were perfused with Krebs-Ringer bicarbonate + 0.1% BSA at 1.4 ml/min at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. The thecae interna were from healthy preovulatory-sized bovine follicles ( $>10$  mm diam). Each point represents the mean ( $\pm$ SEM) from 6 separate experiments.

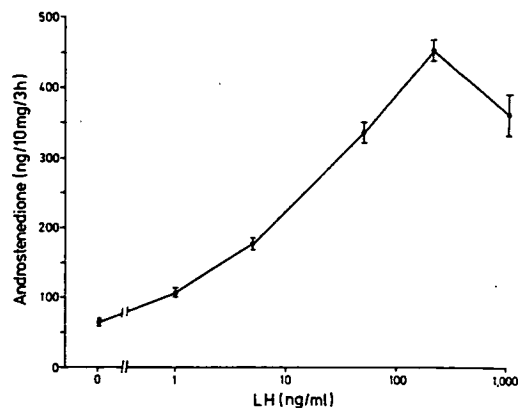


FIG. 5. The cumulative (3-h) outputs of androstenedione by thecae interna from preovulatory-sized healthy follicles ( $>10$  mm diam) exposed to different concentrations of bovine LH. The thecae interna were perfused with Krebs-Ringer bicarbonate + 0.1% BSA at 1.4 ml/min at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. The results are means  $\pm$  SEM from 7 experiments.

tion of 5 ng/ml, peak steroid output lasted for less than 30 min, whereas at an LH concentration of 200 ng/ml, peak output was sustained for about 3 h.

For the data shown in Fig. 6, LH was not detectable in any of the media samples once LH was no longer being added to the columns (i.e., from 24 min after LH was first added to the columns). However, in some other instances LH was present in the first 12-min fraction after LH addition had stopped; the concentration in this tube was always <7% of the original LH concentration that was added. However LH was always undetectable in the second or subsequent 12-min fractions after LH addition had stopped (i.e., <0.2 ng/ml). Thus, nonspecific retention of LH by the tissue, Sephadex and/or tubing was minimal.

#### Effect of LH Content on Androstenedione Output by Theca Interna

The effect of differing masses of LH to

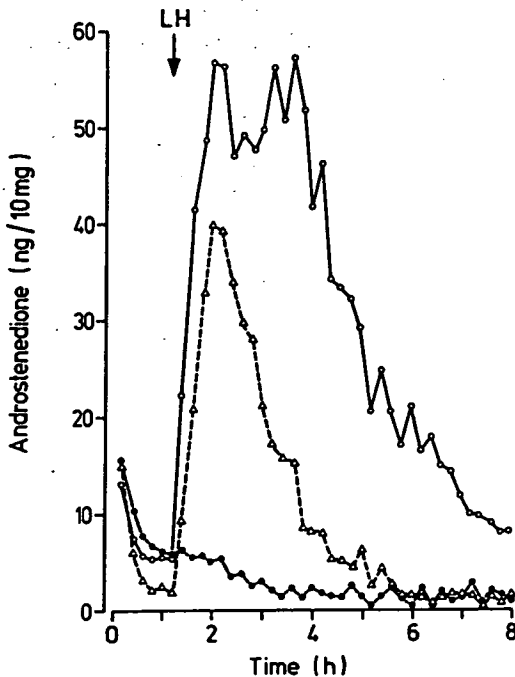


FIG. 6. The output of androstenedione by theca interna every 12 min before, during and after exposure to 0 ng LH (●—●), 5 ng/ml LH (△—△) and 200 ng/ml LH (○—○) for 20 min, at a medium flow rate of 1.4 ml/min. The medium used was Krebs-Ringer bicarbonate + 0.1% BSA gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. The data were from a single experiment.

stimulate androstenedione output was studied at a media flow rate of LH of 1.4 ml/min, during and after the tissue has been exposed to LH for 30 sec; these data are summarized in Fig. 7. The peak output of androstenedione was achieved with an LH content of about 20,000 ng and the half-maximum output with an LH content of 20 ng.

#### Effect of Mass of Theca Interna on Androstenedione Output

The effect of mass of theca interna on steroid output was studied at a media flow rate of 1.4 ml/min, during and after the tissue had been exposed to 200 ng/ml of LH for 20 min; these data are summarized in Fig. 8. The cumulative output of androstenedione was significantly influenced by the mass of theca interna which was being perfused. Peak rates of androstenedione output were obtained when the theca weights were between 20 and 39 mg. Over this range of weights, the cumulative outputs were not significantly greater than those from tissue weighing <20 mg but they were significantly greater than those from tissues weighing 50 mg or more ( $P < 0.01$ ).

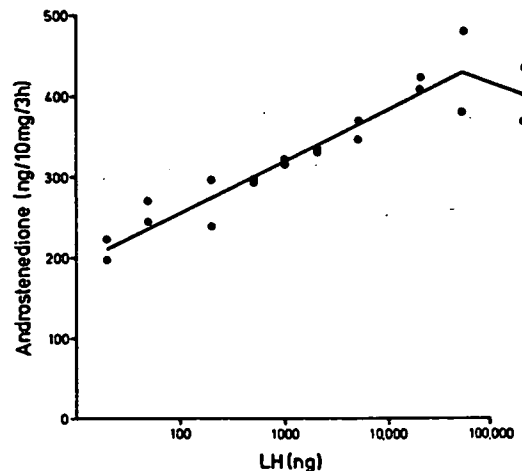


FIG. 7. The cumulative (3-h) output of androstenedione by thecae interna from preovulatory-sized healthy follicles ( $>10$  mm diam) exposed to different masses of bovine LH for 30 sec. The thecae interna were perfused with Krebs-Ringer bicarbonate + 0.1% BSA at 1.4 ml/min at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. The line represents the mean of two experiments; the individual points are indicated.

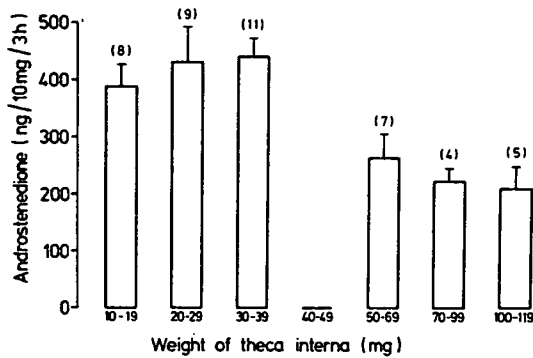


FIG. 8. Effect of mass of theca interna on cumulative (3-h) androstenedione output by LH (200 ng/ml for 20 min)-primed theca interna perfused at 1.4 ml/min with Krebs-Ringer bicarbonate + 0.1% BSA. The results (mean  $\pm$  SEM) for the thecal weight ranges of 20–29 and 30–39 mg were each significantly higher ( $P < 0.01$ ) than those for theca weight ranges 50–69 mg, 70–99 mg and 100–119 mg. In all experiments the theca interna were recovered from healthy follicles  $> 10$  mm diam. The number in parenthesis above each histogram refers to the number of separate experiments.

#### Effect of Follicular Diameter on Androstenedione Output by Theca Interna

These data are summarized in Table 1. For the data showing a cumulative output of androstenedione expressed per 10 mg of tissue, there was a significant treatment effect (i.e., LH

vs. control,  $P < 0.01$ ), but no effect of follicular diameter. Overall, LH treatment caused a 3- to 5-fold increase in androstenedione output. For the data showing a cumulative output of androstenedione, expressed per total mass of theca interna, there was a significant treatment effect (i.e., LH vs. control,  $P < 0.01$ ) as well as a significant effect of follicular diameter ( $P < 0.01$ ).

#### Comparison of Androstenedione Outputs from Theca Interna, Theca Externa and Stroma

For this study, the thecae interna and externa were recovered from the largest healthy follicles (10, 11.5 and 11.5 mm diameter) which were in three cows on either Day -1 ( $n = 2$  cows) or Day 0 of the estrous cycle (estrus = Day 0): these estimated days of the cycle were calculated from the mean cycle lengths of 6–11 previous and consecutive estrous cycles.

Data for the 3-h cumulative production rates of androstenedione for the theca interna, theca externa and stroma (adjacent to the previously mentioned follicle) with or without 20 min exposure to LH are shown in Fig. 9. The cumulative output from the theca interna was  $\geq 3.4$  times greater than that in the externa and stroma in the absence of LH and  $> 19$  times greater with LH stimulation. The effects of LH

TABLE 1. Relationship between follicular diameter and androstenedione output by thecae interna (values are means  $\pm$  SEM).

Cumulative (3-h) output per unit mass (10 mg) or total mass (TM) of theca interna	Follicular diameter (mm)			Treatment*
	2–5.5	6–9.5	$\geq 10$ mm	
ng/10 mg (n)	118 (2)	141 $\pm$ 19 <sup>a</sup> (9)	92 $\pm$ 15 <sup>c</sup> (8)	None
ng/10 mg (n)	373 $\pm$ 21 (6)	371 $\pm$ 73 <sup>b</sup> (9)	436 $\pm$ 71 <sup>d</sup> (8)	LH
ng/TM	228 (2)	262 $\pm$ 53 <sup>c</sup> (9)	343 $\pm$ 66 <sup>e</sup> (8)	None
ng/TM	410 $\pm$ 100 <sup>i</sup> (6)	889 $\pm$ 175 <sup>fj</sup> (9)	1483 $\pm$ 186 <sup>hk</sup> (8)	LH

\*No treatment: tissues perfused at 37°C with Krebs-Ringer bicarbonate + 0.1% BSA. LH treatment: tissues exposed to bovine LH (200 ng/ml) for 20 min at the start of the 3-h experimental period. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. All thecae interna were from healthy follicles (see *Materials and Methods*). In all experiments the thecae interna ranged in weight between 10 and 39 mg.

<sup>a-k</sup> vs. b, c vs. d, e vs. f, g vs. h, i vs. j, j vs. k: significantly different  $P < 0.01$ .

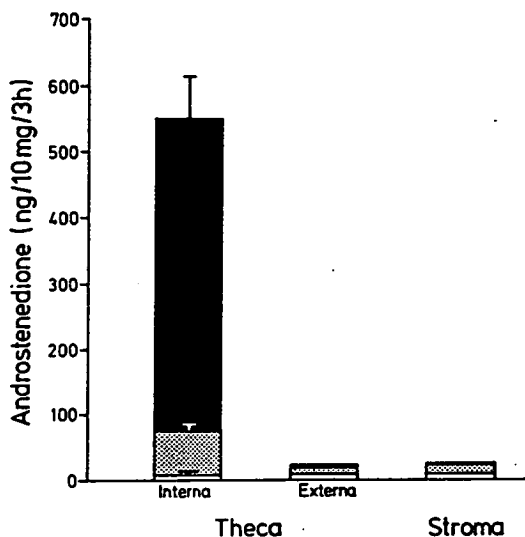


FIG. 9. Cumulative (3-h) production (mean  $\pm$  SEM) of androstenedione by theca interna, theca externa, and stroma exposed to LH (200 ng/ml) for 20 min (black histograms) or media alone (stippled histograms) as well as the respective amounts present in tissue at Time 0 (white histograms). For the thecae externa and stroma, the SEM for all histograms were  $<1$  ng/10 mg per 3 h. The thecae were recovered from healthy follicles 10–11.5 mm diam from 3 cows on Day -1 (n=2) or 0 (n=1) of the estrous cycle (Day 0=day of estrus). The stromal tissues were recovered from the regions adjacent to the above follicles. The medium was Krebs-Ringer bicarbonate + 0.1% BSA gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub> at 37°C.

on androstenedione output from the theca externa and stroma were negligible.

#### *Androstenedione Output from Theca Interna of Healthy or Atretic Follicles*

Thecae interna were recovered from 5 atretic follicles ( $>10$  mm diameter). They were classified as atretic because, although the thecae interna were vascularized and pink to red in color, the tissues were recovered from follicles that were deficient in granulosa cells and contained a considerable amount of debris in follicular fluid. The LH-stimulated outputs of androstenedione by the theca interna from these follicles were each compared to those from 5 healthy follicles ( $>10$  mm diameter) from the same ovaries. The mean ( $\pm$  SEM) outputs (i.e., 3-h cumulative outputs) during and after 20 min LH exposure

(200 ng/ml, flow rate of medium 1.4 ml/min) were 431 ( $\pm 80$ ) ng/10 mg per 3 h for the healthy follicles and 47 ( $\pm 10$ ) ng/10 mg per 3 h for the atretic follicles. The LH-stimulated theca interna from the atretic follicles produced the same amount of androstenedione as did their respective controls (i.e., 53  $\pm$  7 ng/10 mg per 3 h, healthy follicles; 49  $\pm$  6 ng/10 mg per 3 h, atretic follicles).

#### *Comparison of Steroids Secreted by Theca and Granulosa Cells*

For this study, the ovaries of three Angus beef cows on Day 0 of the estrous cycle (i.e., the estimated day of estrus based on the mean cycle length which was determined from 7–11 previous and consecutive estrous cycles) were dissected and the largest healthy follicles ( $>10$  mm diameter) recovered; one healthy follicle of this size range was recovered per pair of ovaries from each cow. Just before ovariectomy, the concentrations of estradiol in the ovarian vein draining the ovaries containing these large healthy follicles ranged from 3.7–4.8 ng/ml. In the veins draining the contralateral ovary of each cow, the estradiol concentrations ranged from 0.4–1.1 ng/ml. In all cows the LH concentration in plasma was  $<10$  ng/ml, suggesting that the ovaries were being removed before the preovulatory LH surge. In the follicular fluid of the previously mentioned large healthy follicles, the estradiol concentrations were, respectively, 350 ng/ml (follicle 1; 10.5 mm diameter), 300 ng/ml (follicle 2; 11.5 mm diameter) and 2000 ng/ml (follicle 3; 16 mm diameter): these concentrations were 1.5- to 1000-fold higher than those in all other follicles ( $>4$  mm diameter) from each cow, indicating that these follicles were the dominant estrogen-secreting structures.

Histologically, the theca interna from the above three follicles was estimated to be  $\sim 90\%$  pure. In follicles 1–3, the extent of granulosa cell contamination ranged, respectively, between 0–85, 0 and 0–15 cells per 7- $\mu$ m section of thecal tissue. In all cases,  $<10\%$  of the theca interna were contaminated with externa or stroma. For follicles 1–3, the respective numbers of granulosa cells that were recovered were 14, 16 and 34 million and the respective weights of theca interna were 34, 32 and 111 mg.

The cumulative (3-h) amounts of progesterone, androstenedione, testosterone and estradiol

produced by theca interna from follicles 1–3 during and after 20 min of LH (200 ng/ml) stimulation, at a perfusion rate of 1.4 ml/min, are summarized in Fig. 10A. Of the four steroids measured, androstenedione represented 82% of the total amount produced, and testosterone represented 15%; whereas estradiol and progesterone represented 2% and 1%, respectively. From the theca interna of follicle 2, which was devoid of granulosa cells, the output of estradiol was 99 ng per theca; whereas from the theca contaminated with granulosa cells the average was 97 ng per theca.

The cumulative (3-h) amounts of the above four steroids produced by granulosa cells are shown in Fig. 10B. The major steroid was progesterone; this steroid constituted 79.0% of the total. Estradiol was the second major steroid, representing 21% of the total; the amounts of androstenedione and testosterone were negligible. In the presence of androstenedione as a substrate for estradiol biosynthesis, the total estradiol output from granulosa cells increased 27-fold compared to cells without added substrate. Moreover, this level of estradiol production was 10-fold greater than that generated by perfused theca.

#### DISCUSSION

These data demonstrate that the level of steroidogenesis by theca interna in vitro is influenced by the flow of medium, the health of the follicle, the mass and concentration of LH, the duration of exposure to LH, as well as the mass of theca interna being perfused. The present study also supports the notion that the theca interna is the major source of follicular androstenedione. The importance of a perfusion methodology to optimize steroid production by theca interna in vitro suggests that the regulation of thecal blood flow in vivo may be a factor of some significance with respect to follicular androgen production and thus the level of follicular maturation (McNatty et al., 1983b for review). It could be argued that by enhancing blood flow through the thecal capillaries, the level of LH-induced steroidogenesis might be amplified considerably as was shown in the present in vitro experiments.

In estrous cycling cows, the basal level of LH is determined from the frequency and amplitude of LH release from the pituitary gland. In peripheral plasma these episodic secretions of LH may vary in amplitude from <1 ng/ml to 7

ng/ml and their frequency may range from 1 per h to 1 per 3 h (Rahe et al., 1980; McNatty, unpublished data). Moreover, each LH secretory episode normally lasts for 20–60 min. The present studies suggest that an LH pulse of 9 ng/ml amplitude and of 20–60 min duration can stimulate a half-maximum production rate of androstenedione for about 3 h. By extrapolation, it could be speculated that if the LH pulse frequency is increased from one per 3 h to one per h, the androstenedione production rate might be sustained at about half-maximum for as long as the substrate for androstenedione synthesis was available, and

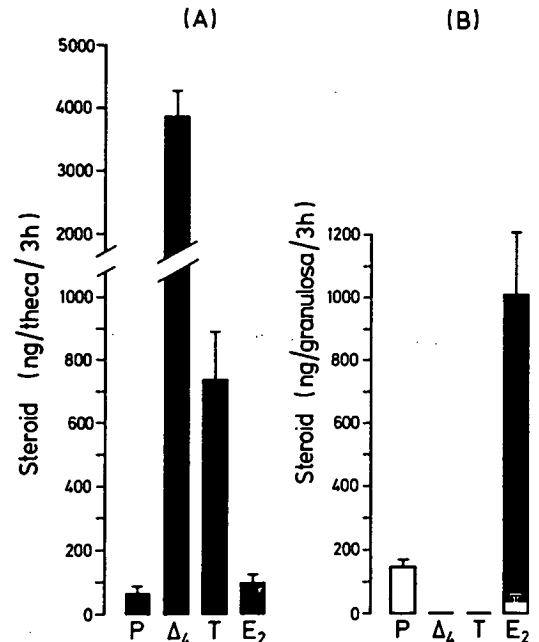


FIG. 10A. The cumulative (3-h) mean ( $\pm$  SEM) production of progesterone (P), androstenedione ( $\Delta_4$ ), testosterone (T) and estradiol ( $E_2$ ) by LH-primed (200 ng/ml LH for 20 min) theca interna perfused with Krebs-Ringer bicarbonate + 0.1% BSA at 1.4 mg/min at 37°C. Thecae interna were recovered from three healthy preovulatory follicles ( $>10$  mm diam). The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>.

FIG. 10B. The mean ( $\pm$  SEM) production of progesterone, androstenedione, testosterone and estradiol (white histograms) by granulosa cells incubated for 3 h in Medium 199, Earle's salts, L-glutamine and 0.1% BSA at 37°C. The medium was gassed with 5% CO<sub>2</sub>, in air. The production of estradiol (black histograms) by granulosa cells ( $2 \times 10^5$ ) exposed to excess androstenedione 2000 ng/ml is also shown. The granulosa cells for this study were recovered from the three preovulatory follicles ( $>10$  mm diam) mentioned in Fig. 10A.

provided the total quantity of LH traversing the theca was submaximal.

The finding that as little as a 45-sec exposure of theca interna to 200 ng/ml LH was sufficient to generate a half-maximum production rate of androstenedione, and that the androstenedione output in response to LH was on the increase within 24 min, suggests that the normally vascularized tissue is acutely sensitive to abrupt changes in LH secretion *in vivo*. Moreover, the finding that an LH concentration of 1 ng/ml (for 20 min) was sufficient to cause a significant increase in androstenedione over a 3-h period suggests that the theca interna is also acutely sensitive to a low amplitude LH pulse in plasma.

Since the theca interna was perfused with a medium free of steroid substrate (i.e., Medium C), the tissue must itself contain substantial amounts of precursors for androstenedione biosynthesis. At a media flow rate of 1.4 ml/min, LH-primed (200 ng/ml for 20 min), thecae interna from healthy preovulatory-sized follicles (>10 mm diameter) were capable of generating between 1 and 10  $\mu$ g of androstenedione in 3 h (i.e., when the data are adjusted for the total mass of theca interna per follicle). These *in vitro* production rates are comparable to the calculated production rates from ovaries containing preovulatory-sized follicles during a 3-h secretory phase *in vivo*. For example, in the veins draining bovine ovaries containing large healthy follicles (>10 mm diameter), the mean ( $\pm$  SEM) concentration of androstenedione was 1.5 ( $\pm$  0.3) ng/ml (n=7 animals; Henderson and McNatty, unpublished data). The rate of ovarian venous blood flow is reported to vary from 0.5 to 40 ml/min (Ford and Chenault, 1981; Wise et al., 1982). Taking these data together, it can be calculated that the 3-h secretion rate of androstenedione *in vivo* from the ovary with a dominant follicle ranges between 0.2 and 15  $\mu$ g (i.e., assuming a hematocrit value of 30%).

Under the experimental conditions employed, the theca interna of healthy but not atretic follicles was identified as the major follicular source of androstenedione; negligible amounts were produced by theca externa, stroma or granulosa cells. These findings are consistent with the ultrastructural evidence of Priedkalns and Weber (1968). About 15% of the total sum of steroid that was measured was testosterone. But in view of the high cross-reactivity (75%) of the testosterone anti-

body with dihydrotestosterone, it cannot be concluded that all of this material was exclusively testosterone. Of interest was the finding that the theca interna was a source of estradiol although the quantities produced relative to androstenedione were small (i.e., 2% vs. 82%). The amount of estradiol produced by theca interna devoid of granulosa cells was similar to that produced by theca contaminated with up to 85 cells per 7- $\mu$ m section of tissue. But, even if it is assumed that all the above estradiol originated from the theca interna, the amount was small (<10%) compared to that generated by granulosa cells when supplied with androstenedione substrate (see also Lacroix et al., 1974).

During antral follicular development, the mass of theca interna increases from <20 mg in 4-mm follicles to about 110 mg in 16-mm diameter follicles. Whether this increase in cell mass is associated with an increase in cell size or number, or both, is not known. Nevertheless, the present studies suggest that the LH-responsiveness of theca per unit mass of tissue probably remains constant during most of antral follicular development, and that the increased output of androstenedione that occurs during follicular maturation may be due in part to an accumulating mass of theca interna. Determination of whether there is also an increased thecal sensitivity to LH, independent of blood flow, as the follicle starts to secrete substantial estradiol, must await a careful sequential study of thecal steroidogenic function in preovulatory follicles around the time of estrus.

In conclusion, these studies have highlighted some of the advantages of a pulsatile LH signal to ovarian tissue. Moreover, the perfusion system described herein may be a suitable model for studying certain dynamic aspects of ovarian function.

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**Title:** Changes in gonadotrophin secretion and ovarian antral follicular activity in seasonally breeding sheep throughout the year.

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## Changes in gonadotrophin secretion and ovarian antral follicular activity in seasonally breeding sheep throughout the year

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**Summary.** Overall, significantly more antral follicles  $\geq 1$  mm diameter were present in Romney ewes during anoestrus than in the breeding season (anoestrus,  $35 \pm 3$  (mean  $\pm$  s.e.m.) follicles per ewe, 23 sheep; Day 9–10 of oestrous cycle,  $24 \pm 1$  follicles per ewe, 22 sheep;  $P < 0.01$ ), although the mean numbers of preovulatory-sized follicles ( $\geq 5$  mm diam.) were similar (anoestrus,  $1.3 \pm 0.2$  per ewe; oestrous cycle,  $1.0 \pm 0.1$  per ewe). The ability of ovarian follicles to synthesize oestradiol did not differ between anoestrus and the breeding season as assessed from the levels of extant aromatase enzyme activity in granulosa cells and steroid concentrations in follicular fluid. Although the mean plasma concentration of LH did not differ between anoestrus and the luteal phase of the breeding season, the pattern of LH secretion differed markedly; on Day 9–10 of the oestrous cycle there were significantly more ( $P < 0.001$ ) high-amplitude LH peaks (i.e.  $\geq 1$  ng/ml) in plasma and significantly fewer ( $P < 0.001$ ) low amplitude peaks ( $< 1$  ng/ml) than in anoestrous ewes. Moreover, the mean concentrations of FSH and prolactin were significantly lower during the luteal phase of the cycle than during anoestrus (FSH,  $P < 0.05$ , prolactin,  $P < 0.001$ ).

It is concluded that, in Romney ewes, the levels of antral follicular activity change throughout the year in synchrony with the circannual patterns of prolactin and day-length. Also, these data support the notion that anovulation during seasonal anoestrus is due to a reduced frequency of high-amplitude LH discharges from the pituitary gland.

### Introduction

In temperate-zone latitudes, most breeds of sheep are anovulatory and anoestrous during the spring and summer. Although limited, the data suggest that the ovaries of anoestrous ewes are relatively active with respect to follicular development and steroidogenesis. For example, the antral follicle population is reported to be similar to or lower than that found during the breeding season (Cole & Miller, 1935; Kammlade, Welch, Nalbandov & Norton, 1952; Cahill, 1979), and the ovaries of anoestrous ewes secrete androstenedione and oestradiol when stimulated with luteinizing hormone (LH) (Martensz, Baird, Scaramuzzi & Van Look, 1976; Scaramuzzi & Baird, 1977). It is possible that a reduced level of LH secretion may be the major cause of anovulation during anoestrus (Legan, Karsch & Foster, 1977; McNatty, Gibb, Dobson & Thurley, 1981b). The long-term administration of regular intravenous (i.v.) pulses of LH or gonadotrophin-releasing hormone

(GnRH) (10 µg ovine LH or 500 ng GnRH every 2 h) stimulated cyclic luteal activity comparable to that seen during the breeding season (McNatty, Ball, Gibb, Hudson & Thurley, 1982a; McNatty *et al.*, 1983b); in these studies the resulting LH pulse frequencies in plasma were similar to those observed during the luteal phase of the oestrous cycle (McNatty *et al.*, 1981b). Presumably, ovulation does not occur during the luteal phase, when there is an LH pulse frequency of 1 per 2 h, because of the combined negative feedback effects of oestradiol from the developing follicle and progesterone from the corpus luteum (Baird, Baker, McNatty & Neal, 1975). Although some data are available on seasonal changes in LH, follicle-stimulating hormone (FSH) and prolactin in sheep (Walton, McNeilly, McNeilly & Cunningham, 1977; Jackson & Davis, 1979; Munro, McNatty & Renshaw, 1980), little or no attempt has been made to examine how these changes in gonadotrophin secretory activity relate to seasonal changes in ovarian follicular and steroidogenic activity.

The objectives of this study of Romney ewes were (1) to determine the degree of antral follicle development throughout the year, and (2) to ascertain the circannual patterns of LH, FSH and prolactin secretion and to relate these changes to the degree of follicular activity within the ovary.

## Materials and Methods

### *Animals and experimental design*

Parous Romney ewes (40–64 kg) aged between 2·2 and 3·2 years were used. Throughout the year, the animals were grazed on pasture and were run with sexually active vasectomized rams fitted with marking harnesses. Oestrous behaviour was not observed in any ewe from September to February (inclusive). During the breeding season (March–August), ovarian dissections were performed on Day 9 or 10 of the oestrous cycle (on 3–7 animals per month), or on Day 9–10 but 10–48 h after ewes were treated with cloprostenol (125 µg s.c.), a synthetic prostaglandin F-2α derivative (ICI Tasman Ltd, Upper Hutt, NZ). In the ewes treated with cloprostenol, ovariectomy was performed at 10, 24, 36 and 48 h after cloprostenol injection (3 ewes at each time). Some animals (4–18 each month) that were not treated with cloprostenol were blood sampled every 10 min for 9 h (for LH and FSH determinations) on Day 9 or 10 of the oestrous cycle. On the day before intensive blood sampling, the animals were penned indoors and each was fitted with an intra-jugular cannula. While indoors, the animals were fed meadow hay, lucerne pellets and water *ad libitum*. Animals were selected at random from a flock of 350 ewes; individual animals were not sampled in successive months. One group of 8 ewes was blood sampled every 3rd day throughout the year for prolactin determinations; these ewes were grazed in a separate paddock from the main flock. Before this study began, the animals in the prolactin study were blood sampled 3 times weekly for 6 weeks to accustom them to handling and blood sampling procedures. The 8 ewes were anoestrous from September to February and showed oestrus at regular intervals from March until August.

### *Blood sampling*

From the sheep in the prolactin study, 10 ml blood were collected on each occasion. From the other sheep 2–4 ml blood were taken at each occasion into a heparinized tube. All blood samples were centrifuged (4000 g at 18–20°C for 20 min) within 15 min of collection, the plasma samples were then frozen to –20°C until assayed.

### *Ovarian studies*

Excised ovaries were weighed and their gross morphology recorded. All individual antral follicles (≥ 1mm diameter) were dissected free of extraneous tissue under a stereomicroscope and

their diameters recorded. A small slit was made in the follicle wall to allow the antral fluid to escape into a Petri dish whence it was aspirated through a finely drawn-out Pasteur pipette, taking care not to remove clumps or sheets of granulosa cells. The fluid was frozen for subsequent hormone assay. The granulosa cells were recovered and their number quantitated as previously described (McNatty, Moore-Smith, Makris, Osathanondh & Ryan, 1979). During recovery of follicular fluid or granulosa cells the oocyte was also isolated and its status assessed subjectively as healthy or degenerate as previously described (McNatty *et al.*, 1983a). To help classify the follicles as healthy or atretic, the presence or absence of thecal blood capillaries (at  $\times 10$  magnification) and debris in follicular fluid was noted. In addition, after removal of the granulosa cells, the colour of the theca interna (i.e. red, pink or white) was noted. For the purpose of this study a healthy follicle was defined as one with: (a) thecal blood capillaries visible at  $\times 10$  magnification, (b) follicular fluid devoid of debris, (c) an apparently healthy oocyte, (d) a pink-to-red coloured theca interna, and (e)  $\geq 50\%$  of the maximum number of granulosa cells for a given follicle size (McNatty *et al.*, 1982b). Conversely, an atretic follicle was one for which any of these criteria did not apply.

#### *The granulosa cell aromatase assay*

From some of the follicles recovered during the cloprostenol-induced follicular phase, Day 9–10 of the cycle or anoestrus, the granulosa cells were collected into Medium 199 containing sodium bicarbonate (0.85 g/l), Earle's salts, L-glutamine (0.10 g/l), Hepes buffer (20 mM) and 1% BSA (w/v), Medium A. These were washed and resuspended in Medium A so that the final cell concentration was 60–600  $\times 10^3$  granulosa cells per ml; 0.5 ml aliquants of these cell suspensions were placed in 10  $\times$  75 mm plastic tubes containing 0.5 ml of a solution of 2000 ng testosterone/ml Medium A, gassed with 5% CO<sub>2</sub> in air, stoppered and then incubated for 3 h in a shaking water bath at 37°C. At the end of the incubation, the tubes, containing medium plus cells, were frozen at  $-20^\circ\text{C}$ . Subsequently the tubes were thawed, centrifuged and the supernatant assayed for oestradiol. Preliminary studies revealed that the rate of oestradiol formation was linear for the first 3 h for granulosa cells from all atretic follicles and from most healthy follicles. In some of the healthy follicles, however, the rate of metabolism was not linear after 2 h so that the results in some instances underestimated extant oestrogen synthetase (aromatase) activity by up to 20%.

#### *Hormone assays*

**LH.** The radioimmunoassay for LH was identical to that reported by McNatty, Gibb, Dobson, Thurley & Findlay (1981a). The LH antibody raised in a rabbit against NIH-LH-S11 was used at an initial dilution of 1:40 000. The antiserum exhibited low cross-reactions with other ovine pituitary hormones: prolactin (NIH-P-S12), 0.09%; TSH (NIH-TSH-S8), 2.4%; GH (NIH-GH-S11), 0.4%; and FSH (NIH-FSH-S10), 0.4%. The within- and between-assay coefficients of variation were 6.8 and 10.3% respectively. The limit of detection was 0.2 ng LH/ml plasma.

**FSH.** The radioimmunoassay for FSH was based on that described by Salamonsen *et al.* (1973). The standard preparation was NIH-FSH-10 and a Papkoff preparation, G4-150C, was used as the iodinated tracer. Rabbit anti-human FSH (M94) was supplied by Dr W. Butt, and was used at an initial dilution of 1:8000. Cross-reactions with other ovine pituitary hormones were: LH (NIH-LH-S21),  $< 0.5\%$ ; prolactin (NIH-P-S18),  $< 0.5\%$ ; GH (NIH-GH-S11),  $< 0.5\%$ ; and TSH (NIH-TSH-S8)  $< 0.5\%$ . The within- and between-assay coefficients of variation were 8.5 and 15.0% respectively. The limit of detection was 20 ng FSH/ml plasma.

Only a limited amount of FSH antiserum was available for this study. Therefore, of the 85 sheep blood sampled for seasonal changes in LH secretion, 43 (2–6 each month) were selected for FSH analysis.

**Prolactin.** Prolactin in plasma was measured by the method of Munro *et al.* (1980). Ovine prolactin NIH-P-S11 was used as the reference standard and ovine prolactin LER-860-2 as the

iodinated tracer. Rabbit antiserum to ovine prolactin (NIH-P-S6) was supplied by Dr J. R. McNeilly. Details of cross-reactions and characterization of the antiserum have been described by Lamming, Moseley & McNeilly (1974). The within- and between-assay coefficients of variation were 6.5 and 15.0% respectively. The minimal detectable concentration of prolactin was 2.0 ng/ml plasma.

*Steroids.* All steroids were measured using previously published radioimmunoassay procedures (McNatty *et al.*, 1981a, 1982b). Progesterone, androstenedione and oestradiol were measured directly in diluted (10–100-fold with 0.1 M-phosphate-buffered saline (PBS), pH 7.2) aliquants of follicular fluid. Testosterone was measured in follicular fluid after it had been diluted (10–100-fold) with PBS and extracted twice with 5 volumes of freshly redistilled diethyl ether.

Oestradiol was measured directly in Medium A (the aromatase assay solution) without extraction.

For the progesterone antiserum (WA-26), the major cross-reacting steroids were 11 $\alpha$ -hydroxyprogesterone (120%), 11 $\beta$ -hydroxyprogesterone (25%), 20 $\alpha$ -dihydroprogesterone (3.5%) and androstenedione (0.4%). The minimal detectable quantity of progesterone was 1 ng/ml antral fluid.

The androstenedione antiserum (WA-965) was used at an initial dilution of 1:3000. Major cross-reacting steroids were 4-androsten-3,11,17-trione (40%), 11 $\beta$ -hydroxyandrostenedione (31%) and testosterone (0.4%). The detection limit of this assay was 2 ng/ml antral fluid.

The testosterone antiserum (WA-36) was used at an initial dilution of 1:800. Major cross-reacting steroids were 5 $\alpha$ -dihydrotestosterone (75%), 5 $\beta$ -dihydrotestosterone (75%), 5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (37.5%), 5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (16.5%) and androstenedione (0.1%). The detection limit of the assay was 1 ng/ml antral fluid. Since the first mentioned 5 $\alpha$ - or 5 $\beta$ -reduced androgens cross-reacted in significant amounts, the testosterone concentrations may have been overestimated in samples containing appreciable quantities of 5 $\alpha$ - or 5 $\beta$ -dihydrotestosterone (DHT). Forty-two individual samples of follicular fluid were fractionated by celite chromatography to separate DHT from testosterone (McNatty *et al.*, 1982b) and to determine the extent of DHT interference in the testosterone assay. The fluid samples were from large ( $\geq 5$  mm diam.), intermediate (3–4 mm diam.) and small ( $\leq 2$  mm diam.) healthy and atretic follicles. The mean testosterone concentration was  $39 \pm 6$  (s.e.m.) ng/ml and the mean DHT concentration  $4 \pm 1$  (s.e.m.) ng/ml. Overall the fraction of DHT as a percentage of the total amount of testosterone plus DHT was  $12.2 \pm 2.5$  (s.e.m.)%. In 3 samples the DHT concentrations were high ( $> 20$  ng/ml) and exceeded those of testosterone; these fluids were from small atretic follicles.

The oestradiol antiserum (WA-27) was used at an initial dilution of 1:16 000 and major cross-reacting steroids with this antiserum were oestrone (7.3%), oestriol (1.4%) and oestradiol-17 $\alpha$  (1.4%). The detection limit for antral fluid was 1 ng/ml and for the aromatase assay 50 pg/ml.

The intra- and inter-assay coefficients of variation for all the above steroid assays were  $< 12\%$ .

#### *Seasonal daylength changes at Wallaceville*

The Wallaceville Animal Research Centre is situated at 41°08'S and 173°03'E. The mean monthly daylength changes for 1980 were calculated from sunrise and sunset tables supplied by the New Zealand Meteorological Service (Government Printing Office, Wellington, New Zealand). The mean numbers of daylight hours for each month from January to December were 14.5, 13.6, 12.2, 10.9, 9.9, 9.1, 9.3, 10.4, 12.0, 13.1, 14.5 and 15.1 h. These data were used when correlating possible seasonal changes in basal gonadotrophin concentrations or follicle number with daylength.

#### *Definitions*

*LH peak.* The criteria used to define an LH peak were modifications of those described by Van Look (1976) and an LH peak was identified if (a) the concentrations of LH in at least 2 consecutive

samples ('the peak samples') were higher than the mean of the previous 2 samples ('baseline' samples), and if (b) the increase above baseline in at least one of the 'peak samples' was greater than 4 times the within-assay variation.

*LH amplitude.* The amplitude of the LH peak was defined as the difference between the highest LH value of the peak and the mean of the two baseline samples. A high-amplitude peak was defined as one with an LH value of  $\geq 1$  ng/ml, whereas a low-amplitude peak was one with an LH value of  $< 1$  ng/ml (McNatty *et al.*, 1981b).

### Statistics

When appropriate the normalized data were subjected to analysis of variance followed by Student's *t* test.

## Results

### *Changes in ovarian weight, follicle size, distribution and incidence of healthy follicles throughout the year*

These data are summarized in Text-fig. 1. The mean ( $\pm$  s.e.m.) weight of ovaries during anoestrus was  $0.95 \pm 0.03$  g ( $n = 46$ ) and, during Day 9–10 of the breeding season, the mean ( $\pm$  s.e.m.) weights of ovaries with and without CL, were, respectively,  $1.63 \pm 0.04$  g ( $n = 22$ ) and  $0.93 \pm 0.04$  g ( $n = 22$ ). During the transitional period of oestrus–anoestrus (August–September), the mean weight of an ovary not containing a CL was uniformly low, i.e.  $0.77 \pm 0.04$  g ( $n = 14$ ).

Throughout the year, most follicles  $\geq 1$  mm diameter (i.e. 60–80%) were 1–2 mm in diameter. Proportionately, preovulatory-sized follicles represented the smallest population (i.e. 2–10%) but they were present every month of the year.

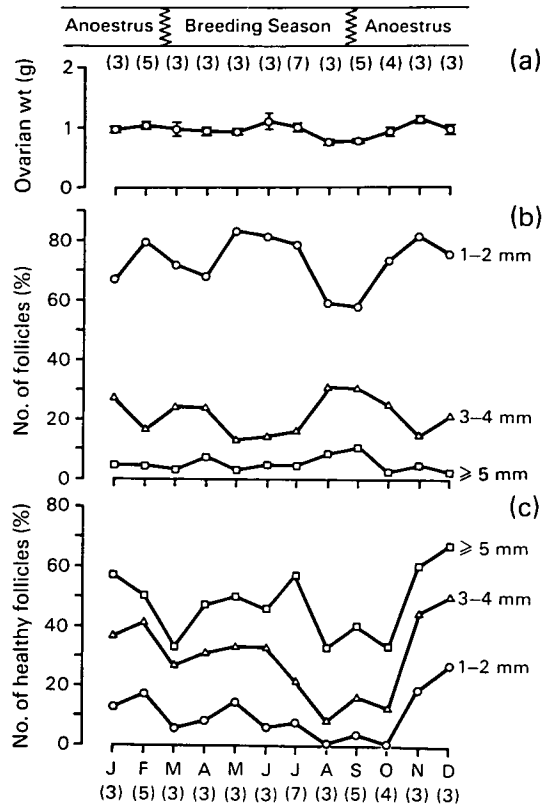
The mean number of healthy follicles of each size range (i.e. 1–2, 3–4 or  $\geq 5$  mm diam.) varied throughout the year with the proportions of each size range changing in a similar pattern (Text-fig. 1c). The majority of 1–2 mm diameter follicles ( $\geq 70\%$ ) were atretic. For the 1–2 mm and 3–4 mm diameter follicles the lowest mean proportions of healthy follicles were recorded during August, September and October. For most of anoestrus (i.e. November, December, January and February) the mean proportions of healthy follicles for all diameters were high compared to the rest of the year (Text-fig. 1c).

### *Changes in the number of antral follicles ( $\geq 1$ mm diameter) throughout the year*

These data are summarized in Text-fig. 2. Overall, there were significantly more antral follicles ( $\geq 1$  mm diam.) during anoestrus compared to the breeding season (anoestrus,  $35 \pm 3$  (s.e.m.), 23 sheep; Day 9–10 of the oestrous cycle,  $24 \pm 1$  (s.e.m.), 22 sheep;  $P < 0.01$ ). The mean number of preovulatory-sized follicles ( $\geq 5$  mm diam.) per ewe did not differ significantly between anoestrus and the breeding season (anoestrus  $1.3 \pm 0.2$  (s.e.m.), 23 sheep; Day 9–10 of the oestrous cycle,  $1.0 \pm 0.1$  (s.e.m.), 22 sheep). Linear regression analysis of the mean monthly follicle number (Text-fig. 2) with the mean monthly hours of daylength (see 'Materials and Methods') showed that there was an association between the two parameters with a correlation coefficient of 0.82 ( $P < 0.001$ ).

### *Steroid concentrations in follicular fluid of healthy and atretic follicles*

These data are summarized in Table 1. The pattern of steroidogenesis during anoestrus (as represented by steroid concentrations in follicular fluid) was similar to that during the luteal phase of the oestrous cycle. Of the steroids measured, the major ones in healthy follicles  $\geq 3$  mm diameter throughout the year were consistently oestradiol and testosterone. Regardless of the time of the



**Text-fig. 1.** Mean monthly changes in: (a) ovarian weight; (b) number of follicles of different diameter per ewe expressed as percentage of the total population of follicles  $\geq 1$  mm in diameter; and (c) number of healthy follicles per ewe expressed as a percentage of follicles in each size range. During the breeding season the data refer to ovaries recovered on Day 9–10 of the oestrous cycle. In (a) the vertical bars =  $\pm$  s.e.m., and the mean monthly weights refer to ovaries devoid of corpora lutea. In (b) and (c) the follicular data refer to all ovaries and do not discriminate between those with or without corpora lutea. The number of ewes examined in each month is indicated.

oestrous cycle or year, small (1–2 mm diam.) antral follicles were steroidogenically active and the healthy ones, at least, were capable of synthesizing some oestradiol.

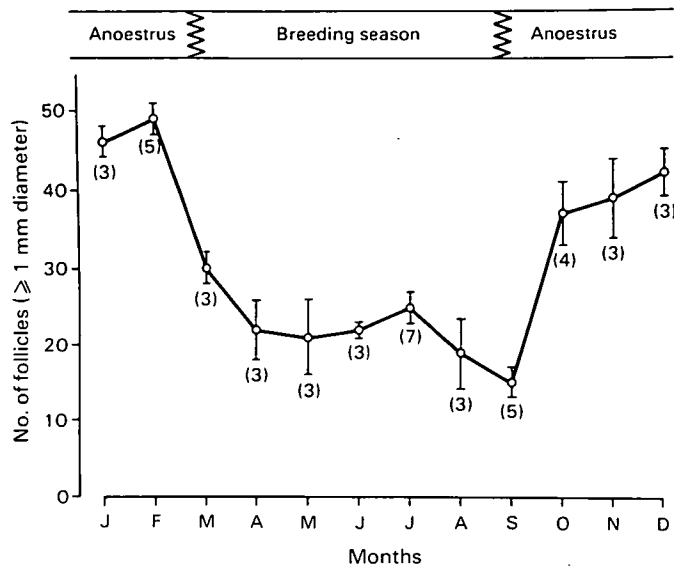
Only large healthy follicles ( $\geq 5$  mm diam.) during the follicular phase contained intrafollicular levels of oestradiol most frequently above 90 ng/ml.

#### *Extant aromatase activity in granulosa cells*

These data with respect to follicle size and health are summarized in Table 2. Since the mean level of aromatase activity in granulosa cells from large healthy follicles ( $\geq 5$  mm diam.) on Day 9–10 of the oestrous cycle ( $6.6 \pm 1.0$  ng/ $10^6$  cells/3 h,  $n = 5$ ) was not different from that in cells from healthy follicles of similar size during the follicular phase ( $5.7 \pm 0.9$ ,  $n = 9$ ), all the data from the breeding season were pooled as indicated above.

There was no effect of season on the level of extant aromatase activity in cells from small (1–2 mm diam.), medium (3–4 mm diam.) or large follicles ( $\geq 5$  mm diam.). In the healthy follicles, extant aromatase activity increased with increasing follicular size. In general, aromatase activity,





**Text-fig. 2.** Mean  $\pm$  s.e.m. monthly changes in the antral follicle population ( $\geq 1$  mm diam.) per ewe (number in parentheses). During the breeding season the follicle numbers refer only to ovaries recovered on Day 9–10 of the oestrous cycle.

when assessed using 1000 ng testosterone substrate, was higher in healthy than atretic follicles; this difference was most obvious in the preovulatory-sized follicles.

#### LH concentration

The data summarizing the concentration of LH, LH peak frequency and LH peak amplitude in Romney ewes on Day 9–10 of the oestrous cycle and during anoestrus are shown in Table 3. There was no significant difference in the mean LH concentration between the two intervals although differences in the pattern of LH secretion were noted. On Day 9–10 there were significantly more ( $P < 0.001$ ) high-amplitude peaks and significantly fewer ( $P < 0.001$ ) low-amplitude peaks compared to those in anoestrus ewes. The respective mean amplitudes of the high- and low-amplitude peaks during each season were similar.

When the data for LH concentrations (Text-fig. 3; Table 3), the overall LH peak frequency and the low amplitude peak frequency (Table 3) were analysed month by month no obvious seasonally-related trends or differences were noted. However, the monthly data for the high-amplitude peak frequency showed consistently high peak frequencies from March until July; the respective means  $\pm$  s.e.m. of the high-amplitude peak frequency per 9 h for these months were  $6.2 \pm 0.6$  (9 ewes),  $4.7 \pm 1.0$  (6),  $5.0 \pm 0.5$  (5),  $5.6 \pm 1.6$  (4),  $6.4 \pm 0.8$  (4). Thereafter from August, the high-amplitude LH peak frequency declined steadily until November; the respective means  $\pm$  s.e.m. per 9 h for each of these months were:  $4.0 \pm 0.4$  (4 ewes),  $3.4 \pm 0.5$  (4),  $2.5 \pm 0.2$  (18) and  $1.8 \pm 0.4$  (12). During December, January and February, the high-amplitude LH peak frequencies remained  $< 3$  peaks/9 h (i.e.  $2.4 \pm 0.4$ ,  $2.3 \pm 0.4$  and  $2.5 \pm 0.5$  peaks/9 h respectively) until the onset of the breeding season.

#### FSH concentrations

The mean plasma concentrations of FSH each month of the year are shown in Text-fig. 3. Overall, the mean ( $\pm$  s.e.m.) concentration during the breeding season ( $117 \pm 8$  ng/ml, 20 sheep)

**Table 1.** Steroids in ovine follicular fluid (values are geometric means and 95% confidence limits)

Reproductive status	Follicle status	Follicle diameter (mm)	n	Steroid concentration (ng/ml)			
				P	$\Delta_4$	T	E <sub>2</sub>
Luteal*	Healthy	≥5	10	8 (5, 14)	7 (4, 12)	35 (16, 76)	44 (14, 137)
	Atretic		5	15 (5, 47)	8 (2, 37)	32 (15, 65)	9 (6, 14)
Follicular†	Healthy		12	15 (11, 22)	16 (10, 27)	37 (21, 66)	184 (90, 378)
	Atretic		15	20 (10, 38)	10 (5, 19)	9 (5, 15)	10 (6, 17)
Anoestrous‡	Healthy		21	8 (6, 12)	9 (6, 12)	16 (10, 26)	31 (21, 47)
	Atretic		22	9 (7, 11)	11 (8, 16)	25 (16, 37)	8 (3, 18)
Luteal*	Healthy	3-4	15	13 (8, 13)	11 (7, 16)	37 (22, 64)	37 (20, 67)
	Atretic		13	13 (8, 21)	10 (7, 16)	17 (11, 25)	9 (6, 14)
Follicular†	Healthy		16	9 (5, 16)	14 (7, 27)	25 (15, 40)	29 (22, 51)
	Atretic		19	22 (14, 33)	28 (18, 44)	33 (21, 53)	20 (14, 29)
Anoestrous‡	Healthy		18	12 (8, 16)	7 (5, 10)	30 (21, 44)	25 (16, 39)
	Atretic		45	12 (10, 15)	12 (10, 14)	37 (25, 40)	7 (2, 9)
Luteal*	Healthy	1-2	15	13 (7, 20)	10 (6, 19)	43 (29, 64)	28 (9, 28)
	Atretic		6	20 (15, 29)	10 (8, 12)	33 (22, 49)	12 (7, 22)
Follicular†	Healthy		19	9 (6, 12)	19 (11, 31)	50 (37, 67)	32 (22, 49)
	Atretic		3	15 (8, 28)	64 (28, 148)	37 (18, 77)	8 (2, 32)
Anoestrous‡	Healthy		12	11 (7, 17)	15 (11, 22)	54 (37, 81)	12 (7, 20)
	Atretic		74	12 (10, 16)	14 (12, 17)	54 (22, 135)	6 (5, 7)

\* Ovaries recovered on Days 9 and 10 of the oestrous cycle (Day 0 = day of oestrus).

† Ovaries recovered between 10 and 48 h after induction of luteolysis with cloprostenol (125 µg s.c.) (see McNatty *et al.*, 1982b).

‡ Ovaries recovered from anovulatory ewes between September and February.

n = number of follicles tested; P = progesterone;  $\Delta_4$  = androstenedione; T = testosterone; E<sub>2</sub> = oestradiol-17 $\beta$ .

was significantly lower ( $P < 0.05$ ) than that during the non-breeding season ( $158 \pm 16$  ng/ml,  $n = 23$ ). In general the range of values for each animal over a 9 h interval varied by  $< 20\%$ . But although the within-animal variation was small, the monthly variation between animals was at times as much as 300% (Text-fig. 3). Despite this between-animal variation, the mean concentrations of FSH were persistently elevated, 170–220 ng/ml, during October, November and December. These high mean values were the result of 8/12 (66.7%) sheep (3/4 during both October and November and 2/4 during December) having FSH values in excess of 170 ng/ml. In contrast, mean FSH values  $> 170$  ng/ml were recorded on only 3/31 (9.7%) other occasions throughout the year.

**Table 2.** Extant aromatase activity (ng oestradiol/10<sup>6</sup> granulosa cells/3 h) in follicles of sheep in the breeding season and during anoestrus

Follicle diameter (mm)	Healthy follicles		Atretic follicles	
	Breeding season	Anoestrus	Breeding season	Anoestrus
≥5	6.00 ± 0.79 <sup>a,c</sup> (14)	6.59 ± 0.71 <sup>a,c</sup> (15)	0.30 ± 0.11 <sup>b,c</sup> (6)	0.26 ± 0.08 <sup>b,c</sup> (12)
3-4	3.77 ± 0.55 <sup>a,c</sup> (14)	3.59 ± 0.56 <sup>a,d</sup> (17)	0.21 ± 0.06 <sup>b,c</sup> (35)	0.29 ± 0.06 <sup>b,c</sup> (34)
≤2.5	0.34 ± 0.09 <sup>a,d</sup> (8)	0.39 ± 0.10 <sup>a,f</sup> (24)	0.16 ± 0.02 <sup>a,c</sup> (42)	0.18 ± 0.03 <sup>a,c</sup> (46)

Values are mean ± s.e.m. with the no. of follicles tested in parentheses.

Mean values with different superscripts (a & b) in the same row are different, superscripts (c, d, e & f) in the same column are significantly different (a vs b, c vs d, e vs f, d vs f,  $P < 0.01$ ; e vs d,  $P < 0.05$ ) (analysis of variance (ANOVA) followed by Student's *t* test between pairs using the variance from the ANOVA).

**Table 3.** The concentration of LH, LH peak frequency and LH peak amplitude (high = ≥1 ng/ml; low = <1 ng/ml) in Romney ewes on Day 9-10 of the oestrous cycle and during anoestrus (results are means ± s.e.m.)

Reproductive status	No. of sheep	Overall LH conc. (ng/ml)	Overall no. of LH peaks per 9 h	Amplitude of all recorded peaks (ng/ml)	High-amplitude peaks		Low-amplitude peaks	
					No./9 h	Amplitude (ng/ml)( <i>n</i> <sub>1</sub> )	No./9 h	Amplitude (ng/ml)( <i>n</i> <sub>2</sub> )
Day 10	32	1.8 ± 0.1	8.4 ± 0.4	2.0 ± 0.2	5.3 ± 0.4	2.8 ± 0.2 (32)	3.1 ± 0.4	0.5 ± 0.04 (28)
Anoestrus	53	1.7 ± 0.1	7.4 ± 0.3*	1.5 ± 0.1†	2.4 ± 0.2‡	3.2 ± 0.3 (49)	5.0 ± 0.3‡	0.6 ± 0.02 (51)

Not all sheep gave records of low- or high-amplitude peaks; *n*<sub>1</sub> and *n*<sub>2</sub> indicate the number from which the respective mean amplitudes were calculated.

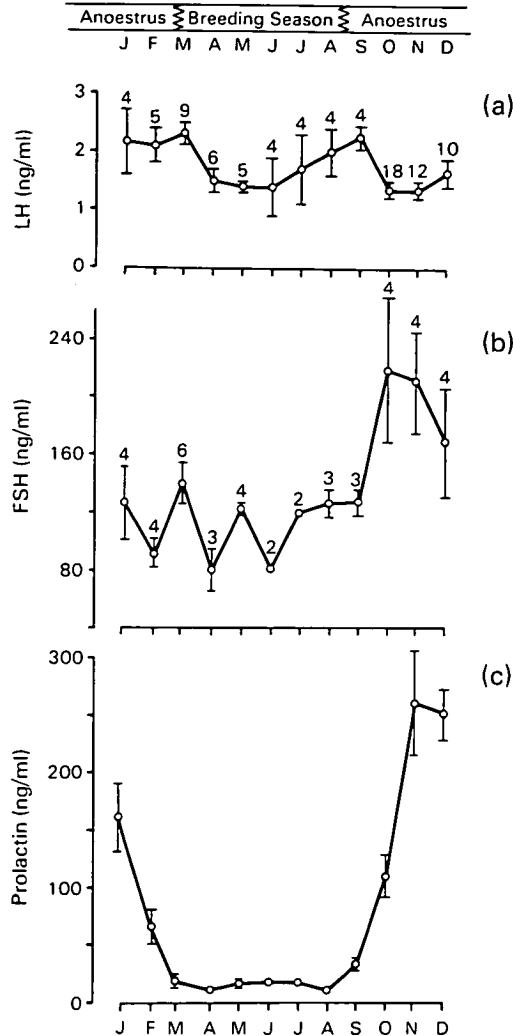
Significantly different from Day 9-10 value: \* $P < 0.05$ ; † $P < 0.005$ ; ‡ $P < 0.001$  (analysis of variance between each variable).

#### *Prolactin concentrations during the breeding season and anoestrus*

The mean plasma concentrations of prolactin each month for the year are shown in Text-fig. 3. Overall, the mean ± s.e.m. concentration during the breeding season (15 ± 1 ng/ml, *n* = 48 mean monthly values) was significantly lower ( $P < 0.001$ ) than that during the non-breeding season (148 ± 16 ng/ml, *n* = 48). Linear regression analysis of the log-transformed mean monthly data for prolactin and the mean hours of daylight each month showed there was an association between the two parameters with a correlation coefficient of 0.64 ( $P < 0.01$ ). Moreover, linear regression analysis of the mean monthly data for prolactin and follicle number gave a correlation coefficient of 0.84 ( $P < 0.01$ ).

#### Discussion

These data demonstrate for the Romney ewe that antral follicular development is not constant throughout the year. During the breeding season and the first month of anoestrus, the number of antral follicles (≥1 mm diam.) per ewe was uniformly below 35. In contrast, during most of anoestrus the antral follicle population was uniformly above 35. The correlation between antral



**Text-fig. 3.** Mean  $\pm$  s.e.m. monthly changes in plasma hormone concentrations of sheep (no. indicated). For LH and FSH, each ewe was sampled every 10 min for 9 h on Day 9 or 10 of the oestrous cycle or during anoestrus. Thus the mean value for each ewe was determined from 55 samples. For the monthly prolactin values, the same 8 ewes were bled by venepuncture every 3rd day throughout the year so that the mean monthly value was determined from 9–10 samples. For further details see 'Materials and Methods'.

follicle number and hours of daylength suggest that perhaps the level of follicular activity is regulated by photoperiod. Although unexpected, the rapid decline in the follicle population after February coincided with the onset of the breeding season. However, at the end of the breeding season, the increase (or rebound) in the antral follicle number to that observed in January or February did not occur until October, i.e. not until 4–6 weeks after the last ovulation of the breeding season. The results of this study suggest that the end of the breeding season coincided with an overall reduction in ovarian activity. For example, the ovarian weights were uniformly lower in August and September than any other month of the year, the mean proportions of healthy, small- and medium-sized antral follicles were lowest in August, September and October, and, although some individual animal variation existed, the mean follicle population ( $\geq 1$  mm diam.) was lowest

in August and September. Whether these reductions in ovarian activity are associated with a lowered level of ovarian steroid secretion remains to be determined.

In a previous study, Cahill (1979) reported a significant correlation ( $P < 0.05$ ) between the mean prolactin concentration during the luteal phase and the number of non-atretic preantral follicles. In the present study, the circannual pattern of antral follicular activity correlated significantly with the circannual patterns of prolactin secretion and daylength. Although these data could be used to reinforce the generally accepted theories that the environment, and primarily the photoperiodic component, influence hypothalamic-pituitary function and thereby gonadal activity (Lincoln & Short, 1980; Arendt, Symons & Laud, 1981; Karsch, Bittman & Legan, 1981), the possibility exists that the high number of antral follicles during anoestrus are, in part, a consequence of a direct stimulatory effect of prolactin on the preantral follicle population.

The present studies confirm a previous report for Romney ewes that the mean plasma concentration of LH during the luteal phase is not significantly different from that during anoestrus (McNatty *et al.*, 1981b). But, during the breeding season (on Day 9–10 of the oestrous cycle), the frequency of LH peaks (i.e. all peaks) per 9 h was 8.4 whereas during anoestrus the total peak frequency per 9 h was 7.4 ( $P < 0.05$ ). By contrast, on Day 9–10 of the cycle, the number of high and low amplitude LH peaks was 2.2 times and 0.6 times respectively of those values recorded during anoestrus. Any interpretation of the above LH data is critically dependent on the criteria used to define an LH peak (Van Look, 1976; see also Merriam & Wachter, 1982) as well as the physiological significance of the LH peaks at the level of the ovary. In-vitro studies on the ovaries of cows and ewes have shown that the steroidogenic response of theca interna cells to LH is significantly enhanced by LH concentrations  $\geq 1$  ng/ml but not by those  $< 1$  ng/ml (unpublished data). It is therefore possible that a low-amplitude LH peak reflects a low-amplitude secretion of pituitary LH and that some or all of these small pulses are without effect at the level of the ovary. If the low-amplitude peaks truly represent secretory episodes, then changes in LH secretory activity during the year could be due to changes in the amplitude as well as frequency of hypothalamic GnRH release. This line of reasoning suggests that seasonal changes in the frequency of GnRH release are small relative to the seasonal changes in amplitude, assuming that each pulse of GnRH releases a pulse of LH (McIntosh & McIntosh, 1982). However, the possibility cannot be discounted that some of the so-called low-amplitude peaks represent interference ('noise') in the radioimmunoassay due to changes in gonadotrophin subunit activity or other immunoreactive materials. From October until February, the high amplitude LH peak frequency was consistently below 1 peak per 3 h and prolactin concentrations in plasma consistently above 50 ng/ml. During this time, the number of antral follicles ( $\geq 1$  mm diam.) was above 35 per ewe and the proportion of healthy follicles uniformly higher than in most other months of the year. Moreover, the healthy follicles during anoestrus contained similar levels of granulosa-cell aromatase activity as did follicles from the breeding season when the appropriate quantities of testosterone were made available. Two questions arise from these findings of a high level of follicular activity during anoestrus: (1) why do oestrogen-enriched follicles (i.e. with follicular fluid oestradiol  $\geq 90$  ng/ml) not develop and (2) why does ovulation not occur? One possibility is that the frequency of high-amplitude LH discharges is too low to sustain an adequate supply of thecal androgen substrate for the synthesis of oestradiol (see McNatty, 1982, for review). This hypothesis is also supported by the finding that the administration of exogenous LH to anoestrous ewes at a frequency of one injection per 2 h is able to induce ovulation and cyclic progestational activity (McNatty *et al.*, 1983b).

An important time of the year with respect to seasonal changes in FSH secretion appeared to be the months of October, November and December, when there is a greater antral follicular activity relative to that during the previous 6 months. Although this increased output of FSH varied markedly between animals, it coincided with the reduction in high-amplitude LH peak frequency and the entry of animals into a state of hyperprolactinaemia. Whilst the timing of this FSH rise is of interest, the role of FSH on antrum formation, and follicular maturation in sheep, remains to be elucidated (Carson, Findlay, Burger & Trounson, 1979; McNatty, 1982).

In conclusion, the present studies show that the level of antral follicular activity changes throughout the year in synchrony with the circannual patterns of prolactin secretion and daylength. Moreover, these data support the notion that anovulation during seasonal anoestrus is due to a reduced frequency of high amplitude LH peaks in plasma.

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## Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary

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**Summary.** The patterns of ovarian follicular development and the steroidogenic properties of individual follicles ( $\geq 2$  mm diam.) were assessed in Angus cows from Day  $-5$  until Day  $+1$  of the oestrous cycle (oestrus = Day 0). Individual follicles were judged to be healthy or atretic using a new classification system incorporating assessments of thecal vascularity and colour, the number of granulosa cells, the presence or absence of debris in follicular fluid and the status of the oocyte.

The results suggest that the theca interna of small antral follicles ( $< 5$  mm diam.) responds to LH and synthesizes androstenedione before the granulosa cells develop an appreciable ability to metabolize androgen to oestrogen. Regardless of follicle size, the output of thecal androstenedione per unit mass of tissue remained unchanged in healthy but not in atretic follicles. On a per cell basis, aromatase activity increased in granulosa cells from healthy but not from atretic follicles with increasing follicle size. Peak levels of aromatizing activity were consistently observed in dominant oestrogen-enriched follicles on Day 0 although similar activity was also observed in some healthy follicles ( $\geq 8$  mm diam.) on other days of the cycle. Early atresia in bovine follicles was characterized by an absence or lowering of aromatase activity in granulosa cells which always preceded any reduction in the thecal steroidogenic response to LH.

It was estimated that between 20 and 60 antral follicles ( $\geq 2$  mm diam.) per cow may respond to LH by synthesizing androgen whereas only 1–3 follicles ( $> 5$  mm diam.) have granulosa cells capable of metabolizing androstenedione or testosterone to oestradiol.

### Introduction

It is generally accepted that bovine antral follicles grow and regress continuously throughout the oestrous cycle (Rajakoski, 1960; Donaldson & Hansel, 1965; Erickson, 1966; Priedkalns, Weber & Zemjanis, 1968). It has been estimated that a bovine follicle takes about 22 days to grow from 0.4 mm (i.e. early antral) to 10 mm (i.e. preovulatory size) in diameter, and more than 4 days to enlarge from 5 mm to preovulatory size (Scaramuzzi, Turnbull & Nancarrow, 1980; Staigmiller & England, 1982). From Day 4 of the oestrous cycle until ovulation there is usually one large follicle ( $\geq 8$  mm diam.) present every day (Rajakoski, 1960; Choudary, Gier & Marion, 1968; Ireland, Coulson & Murphree, 1979; Matton, Adalakoun, Couture & Dufour, 1981; Ireland & Roche, 1983). From histological criteria it is thought that the number of non-atretic follicles  $> 5.0$  mm in diameter is low ( $\leq 3$  per cow) due to a high incidence of atresia in follicles enlarging from 2 to 5 mm in diameter (Rajakoski, 1960; Marion, Gier & Choudary, 1968; Scaramuzzi *et al.*, 1980).

Androstenedione, testosterone and oestradiol are major secretory products of bovine follicles and their concentrations in peripheral plasma fluctuate markedly, and often asynchronously, both within days and between each day of the oestrous cycle (Dobson & Dean, 1974; Lemon & Saumande, 1974; Peterson, Fairclough, Payne & Smith, 1975; Peterson, Fairclough & Smith, 1978; Wise, Caton, Thatcher, Lehrer & Fields, 1982). However, it has been difficult to extrapolate from hormone concentrations in peripheral plasma, the degree of follicular activity within the ovary. This is in part due to the fact that the relationship between the health of a follicle and its potential to synthesize steroids is poorly understood (Merz, Hauser & England, 1981; Kruip & Dieleman, 1982). Nevertheless, it is well established that large healthy ( $\geq 8$  mm diam.) and/or preovulatory bovine follicles may synthesize large quantities of oestrogen (Short, 1962; Lunaas, 1964; England, Karavolas, Hauser & Casida, 1973; Merz *et al.*, 1981; Staigmiller, England, Webb, Short & Bellows, 1982; Kruip & Dieleman, 1982). For optimal oestradiol biosynthesis it is thought that a functional interaction between theca interna and granulosa cells is required with the two key variables being the amount of LH-induced androstenedione or testosterone synthesized by the theca interna cells and the level of aromatase activity in granulosa cells (Hillier, 1981; McNatty *et al.*, 1984a).

The objectives of this study were to determine (1) the degree of antral follicle development ( $\geq 2$  mm diam.) from Day -5 until Day +1 of the oestrous cycle (oestrus = Day 0) and (2) the LH responsiveness of theca interna cells and amount of extant (or residual) aromatase activity in granulosa cells from healthy and atretic follicles.

### Materials and Methods

*Animals, blood sampling and recovery of ovaries.* Thirty-three Angus cows aged 2-8 years (mean 4.7 years) were studied. Six animals were parous and 27 nulliparous. These animals were grazed on open pasture with a vasectomized bull and examined twice daily for oestrous activity. The mean oestrous cycle length ( $20.6 \pm 0.2$  days) in all 33 cows was determined from  $7 \pm 0.5$  (s.e.m.,  $n = 33$ ) consecutive oestrous cycles. For each cow the mean  $\pm$  s.e.m. coefficient of variability with respect to cycle length was  $8.4 \pm 0.9\%$  ( $n = 33$ ). After monitoring oestrous cycle activity, 10 of the cows were slaughtered on a known day of the oestrous cycle at a local slaughterhouse, and the ovaries recovered within 30 min of slaughter. The remaining 23 animals underwent ovariectomy on a known day of the oestrous cycle at the Wallaceville Animal Research Centre, after blood had first been obtained from the jugular and ovarian veins ( $\sim 20$  ml/per vein/cow) of most animals. Ovariectomy and blood sampling was performed while the animals were under thiopentone sodium anaesthesia (Intraval; May and Baker, New Zealand). The ovaries were obtained within 1 h of induction of anaesthesia. All blood samples were collected into heparinized centrifuge tubes and centrifuged at 4000 g for 30 min at 6°C within 1 h of collection. Aliquots of the plasma were frozen to -20°C for subsequent hormone determinations.

From all cows, the ovaries were collected into chilled Medium A (Medium 199 containing Earle's salts, L-glutamine (0.1 g/l), Hepes buffer (20 mmol/l) (Gibco, Santa Clara, CA, U.S.A.) and 0.1% BSA (w/v, fraction V, Sigma Chemical Co., St Louis, MO, U.S.A.).

*Ovarian studies.* Excised ovaries were weighed, and their gross morphology recorded. All individual antral follicles ( $\geq 2$  mm diam.) and corpora lutea were dissected free of extraneous tissue under a stereomicroscope and their diameters recorded to the nearest 0.5 mm. Dissection procedures, collection of follicular fluid, granulosa cell recovery and quantitation, isolation of theca interna as well as recovery and subsequent classification of oocytes (at  $\times 40$  magnification) have all been described in detail elsewhere (McNatty *et al.*, 1983, 1984a). Some oocytes were also fixed in a sodium cacodylate buffer containing 3% glutaraldehyde (0.1 M, pH 7.35) for a more detailed histological analysis. After 1 h in fixative, the oocytes were rinsed twice in 0.1 M-sodium cacodylate buffer containing 1% (w/v) osmium tetroxide. After further rinsing in cacodylate buffer, the

oocytes were then stained with 2.0% (w/v) aqueous uranyl acetate for 40 min, dehydrated through a graded series of alcohols (30, 50, 70, 95 and 100%) and embedded in Spurr's Epoxy Resin. Light microscope sections were stained with 0.5% toluidine blue in 0.5% borax.

*Classification of atresia in antral follicles.* Current methodologies for classifying follicles into various stages of atresia before undertaking biochemical studies (Moor, Hay, Dott & Cran, 1978; Kruip & Dieleman, 1982; McNatty, 1982) were, in our hands, too unreliable for identifying those with a functionally active aromatase system in the granulosa cells and/or an LH-responsive theca interna (in terms of androstenedione synthesis). Accordingly, we developed a revised classification system which incorporated some of the criteria identified in the earlier studies. To assess the state of atresia of the various follicles, the following factors were considered: the presence or absence of thecal blood capillaries when the intact (but cleanly dissected) follicles were observed at  $\times 10$  magnification; the presence or absence of debris in follicular fluid; the presence and status of the oocyte (healthy, degenerate or absent); the total number of granulosa cells expressed as a percentage of the maximum number of cells observed in a follicle of that size (McNatty *et al.*, 1984a); and the colour (or appearance) of the theca interna (red, pink or white). When these variables were collectively subjected to hierarchical cluster analysis (Genstat, 1981), they separated into four groups termed Grades 1, 2a, 2b and 3, with a similarity coefficient within clusters of 0.85. Grade 1 contained follicles with: a vascularized theca interna which was red, pink, or white, no debris in follicular fluid,  $> 75\%$  of the maximum number of granulosa cells for a given follicle size and a healthy-looking oocyte. Grade 2a contained follicles with: a vascularized red to pink theca interna, debris in the follicular fluid,  $< 75\%$  of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. Grade 2b contained follicles with: an avascular white theca interna, no debris in the follicular fluid,  $< 75\%$  of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate oocyte or no oocyte. Grade 3 contained follicles with a white avascular theca interna, debris in the follicular fluid,  $< 50\%$  of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. Grade 1, which was considered to contain the healthiest follicles, was arbitrarily divided into two subgroups (1a and 1b). All other characteristics being similar Grade 1a follicles were those with a vascularized theca interna which was red to pink and Grade 1b follicles had a vascularized theca interna which was white. Based on these 5 subgroups it was presumed that follicles would degenerate along two possible pathways namely: 1a  $\rightarrow$  1b  $\rightarrow$  2b  $\rightarrow$  3 or 1a  $\rightarrow$  2a  $\rightarrow$  3.

*Classification of follicles based on size and dominance.* For each cow, follicles were classified as dominant, large ( $\geq 8$  mm diam.), intermediate (5–7.5 mm diam.) or small (2–4.5 mm diam.). With the exception of Day + 1, the dominant follicle was, for each cow, the Grade 1a follicle with the highest concentration of oestradiol in the follicular fluid and having granulosa cells with the highest aromatase activity. In 30/33 cows, one follicle in each animal was clearly identifiable as the dominant follicle. In the 3 remaining cows the above criteria fitted 2 follicles in each animal; in each of these animals both follicles were classified as dominant.

*Dating the oestrous cycle.* For each cow, the day of ovariectomy relative to the next presumed day of oestrus was calculated from a knowledge of the previous mean oestrous cycle length and the last date of oestrus. Subsequently, this was confirmed or modified by the histology of the corpus luteum (CL), the weight of CL, the binding characteristics of  $^{125}\text{I}$ -labelled human chorionic gonadotrophin (hCG) to homogenized luteal tissue, the progesterone secretory characteristics of the dispersed luteal cells over 3 h *in vitro* and, in some instances, the LH and progesterone concentrations in peripheral plasma.

The histological assessment of CL age was especially useful in dating the oestrous cycle on Days – 3 to – 1. The histological criteria were as follows: on Days – 4 or – 5 the luteal cells had granular cytoplasm and were of approximately equal size with large nuclei and well distributed chromatin.

The arterioles had large lumina. The general picture was of little connective tissue and a uniform appearance. On Day -3 occasional shrunken, darkly staining cells with pycnotic nuclei were scattered unevenly throughout the tissue. A few of the luteal cells were vacuolated. The lumina of the arterioles were smaller than on Day -4 or -5. In some places, the small arterioles were sclerotic. On Day -2 increasing numbers of shrunken cells with or without pycnotic nuclei were present and there was more variation in the size of the luteal cells. The granules in the luteal cell cytoplasm were less extensive and vacuolation was more pronounced. The arterioles were similar to those on Day -3 but a greater proportion had small lumina. More very small arterioles were evident than before, presumably because of an increase in the thickness of their walls. There was a general increase in the quantity of connective tissue which gave a marbled appearance throughout the CL. There were a few inflammatory cells and cell fragments. On Day -1 the number of shrunken cells was less than on Day -2. However, there was more variation in the size of the luteal cells which, overall, were smaller relative to those on Day -2. There was also a reduction in luteal cell number and more connective tissue was evident between the remaining cells. The arterioles had very small lumina and were numerous. There were small but scattered groups of inflammatory cells and debris. On Day 0, the extent of degeneration was much more obvious than on Day -1 and by Day +1 no luteal cells were apparent and most arterioles were sclerotic.

The mean  $\pm$  s.e.m. weight of the CL differed little between Days -5 and -1 ( $4.77 \pm 0.14$  g,  $n = 22$ ). On Days 0 and +1 the mean  $\pm$  s.e.m. CL weight was  $3.49 \pm 0.25$  g ( $n = 7$ ) and  $1.62 \pm 0.2$  g ( $n = 4$ ), respectively.

From the criteria outlined by Henderson, Kieboom, McNatty, Lun & Heath (1984), the calculated equilibrium dissociation constants ( $K_d$ ) and maximum binding capacities ( $B_{max}$ ) for specific binding of  $^{125}I$ -labelled hCG to homogenized luteal tissue did not differ for CL recovered from Day -5 to -1; over this period the respective mean  $\pm$  s.e.m. for  $B_{max}$  and  $K_d$  was  $38.3 \pm 3$  fM/mg protein and  $0.324 \pm 0.027$  nM ( $n = 11$ ). On Day 0 these values were  $16.9 \pm 5.7$  fM/mg protein and  $0.239 \pm 0.059$  nM ( $n = 4$ ) and on Day 1, 0 and 0 ( $n = 2$ ).

Using the method of Henderson & Moon (1979) to isolate bovine luteal cells, it was found that the luteal progesterone output over 3 h in response to stimulation by LH (NIH-LH-B10; 100 ng/ml) varied with the day of the cycle. From Days -5 to -2 the mean output was relatively constant, i.e.  $159 \pm 24$  ng/ $10^6$  cells/3 h ( $n = 6$ ), but from cells recovered on Days -1 and 0 the respective outputs were  $30 \pm 10$  ( $n = 4$ ) and  $19 \pm 9$  ( $n = 3$ ) ng/ $10^6$  cells/3 h.

The mean  $\pm$  s.e.m. concentrations (ng/ml) of LH in plasma were  $1.6 \pm 0.06$  ( $n = 19$ ) on Day -5,  $1.5 \pm 0.07$  (23) on Day -4,  $1.6 \pm 0.08$  (20) on Day -3,  $1.4 \pm 0.1$  (15) on Day -2,  $1.7 \pm 0.2$  (10) on Day -1,  $17 \pm 6$  (7) on Day -0 and  $2.6 \pm 0.4$  (3) on Day +1. Since only one blood sample was recovered for LH analysis it was not possible to determine whether the ovaries on Day 0 were recovered during the ascending or descending limb of the preovulatory LH surge.

The mean  $\pm$  s.e.m. progesterone concentrations (ng/ml) in plasma were  $5.1 \pm 0.3$  ( $n = 19$ ) on Day -5,  $4.6 \pm 0.3$  (23) on Day -4,  $3.8 \pm 0.3$  (20) on Day -3,  $2.6 \pm 0.4$  (15) on Day -2,  $1.3 \pm 0.3$  (10) on Day -1,  $0.9 \pm 0.2$  (7) on Day 0 and  $0.3 \pm 0.1$  (3) on Day +1.

By assessment of all of the above criteria 30 of the 33 cows were judged to be at the day of oestrous cycle that would have been expected from the previous history, one at Day 0 instead of Day +1, one at Day 0 instead of -1 and one at Day -2 instead of Day 0. Overall, 2 cows were judged to be at Day -5 of the cycle, 3 at Day -4, 5 at Day -3, 5 at Day -2, 7 at Day -1, 7 at Day 0, and 4 at Day +1.

*The granulosa cell aromatase assays.* Granulosa cells from all individual follicles were collected into fresh Medium A. In most instances aromatase assays were performed on individual healthy (Grade 1a) follicles. On some occasions with small healthy follicles (< 5 mm diam.) and on many occasions with atretic (Grades 1b, 2b and 3) follicles too few cells were available for individual assays and so several follicles were pooled. Care was taken to ensure that pools of cells originated from follicles of comparable diameter and stage of atresia. The cells were washed and resuspended

in Medium A containing sodium bicarbonate (0.85 g/l) so that the final cell concentration was  $100\text{--}600 \times 10^3$  granulosa cells/ml; 0.5 ml aliquants of these cell suspensions were placed in  $10 \times 75$  mm test tubes containing 0.5 ml of a solution of testosterone or androstenedione (for amounts added see below) in Medium A with sodium bicarbonate (0.85 g/l), gassed with 5%  $\text{CO}_2$  in air, stoppered, and then incubated for 0, 0.5, 1, 2, 3, 4 or 6 h in a shaking water bath at  $37^\circ\text{C}$ . All assays were performed in duplicate. At the end of the incubation, the assay tubes were snap frozen to  $-70^\circ\text{C}$ . Subsequently, the tubes were thawed, centrifuged and the supernatants assayed for oestradiol.

Three types of aromatase assays were performed. In one experiment the cells were incubated with androstenedione or testosterone at doses of 0, 3, 30, 300, 3000, 30 000 or 300 000 ng/ml to compare their respective potencies as substrates. In a second experiment cells from 40 individual healthy follicles (2.5–14.5 mm diam.) were subdivided into appropriate aliquants and incubated with 1000 ng testosterone for variable periods of time (see above) or for a fixed time (3 h) with variable amounts of testosterone. Preliminary studies revealed that the rate of oestradiol accumulation by granulosa cells exposed to different amounts of testosterone or androstenedione could be fitted to the Lineweaver–Burk or Eadie–Hofstee forms of the Michaelis–Menten equation with a correlation coefficient of  $>0.9$  ( $P < 0.01$ ). Consequently, the characteristics of aromatase enzyme activity in entire bovine cells were analysed using the enzyme kinetic programme of Crabbe (1982). In the third and most common experiment, the cells (from follicle pools or individual follicles) were incubated in testosterone at a final concentration of 1000 ng/ml for 3 h. Preliminary studies with 1000 ng testosterone substrate revealed that the rate of oestradiol formation was linear for the first 3 h for granulosa cells from atretic follicles and for most healthy follicles. However, in some healthy follicles, the rate of metabolism was not always linear after 2 h and for these follicles the results underestimated the true activity by up to 25%.

*Theca interna perfusions.* To determine the ability of theca interna to secrete androstenedione in the presence or absence of LH, samples of theca interna were perfused *in vitro* as described previously (McNatty *et al.*, 1984a). Briefly 25–50 mg theca interna tissue were recovered from the internal face of the follicle wall by microdissection, split into 4 fractions with 2 of these being placed into glass columns ( $4 \times 0.7$  cm, Econo-column: Bio-Rad Laboratories, CA, U.S.A.) containing 50 mg Sephadex G-25, pre-washed for 1 h with a perfusion medium consisting of sterile Krebs–Ringer–bicarbonate buffer containing 1% BSA (w/v) which was gassed continuously with 50%  $\text{O}_2$ , 45%  $\text{N}_2$  and 5%  $\text{CO}_2$ . The 3rd thecal fraction was homogenized in 1 ml ethanol to determine the endogenous steroid content and the 4th was fixed for histological examination to test the purity of the tissue before perfusion. The thecal tissue on the columns was perfused at  $37^\circ\text{C}$  for 3 h at a flow-rate of 1.4 ml/min with 12 min fractions being collected. For 1 of the 2 theca fractions on the columns LH (200 ng/ml; NIAMDD-bLH-4) was added to the perfusion medium for 20 min after the theca had been perfused for 1 h. The other perfused theca was not exposed to LH and served as an unstimulated control. At the end of the 3-h period, the tissues were fixed for histological examination and the medium from each tube frozen until assayed for androstenedione by a specific radioimmunoassay (RIA) (McNatty, Gibb, Dobson, Thurley & Findlay, 1981). The androstenedione output from theca was expressed as the cumulative output (ng) per 10 mg theca interna over the 3-h period during and after LH stimulation or after control media perfusion. Analysis of all thecae fixed for histological examination revealed that 70–100% of the cell population was theca interna: the major contaminants were theca externa, stroma and/or red blood cells and only a few granulosa cells (i.e.  $<5$  per  $7 \mu\text{m}$  section) were ever present.

*Hormone assays.* All steroids were measured using previously published RIA procedures (McNatty *et al.*, 1981, 1982). Androstenedione, testosterone and oestradiol were extracted from peripheral blood (10 ml) or ovarian venous blood (1 ml) with diethyl ether ( $2 \times 5$  vol) and then separated by the Lipidex 5000, or Sephadex LH-20 column chromatographic methods outlined elsewhere (McNatty *et al.*, 1981). Progesterone in peripheral plasma (1 ml) was measured using an RIA procedure (Thorneycroft & Stone, 1972). In follicular fluid progesterone, androstenedione and

oestradiol were measured directly in diluted (10–100-fold with 0.1 M-phosphate-buffered saline, PBS, pH 7.2) aliquants of follicular fluid. Testosterone was measured in follicular fluid after it had been diluted (10–100-fold) with PBS and extracted twice with 5 volumes of diethyl ether.

Androstenedione in Krebs–Ringer solution (from the thecal columns) and oestradiol in the aromatase assay solutions were measured directly without extraction.

Details regarding the working dilutions and specifications of the progesterone (WA-26), androstenedione (WA-965), testosterone (WA-36) and oestradiol (WA-27) antisera are provided elsewhere (McNatty *et al.*, 1981, 1984b).

The detection limit of steroids in follicular fluid was 2 ng/ml. The detection limits of progesterone, androstenedione, testosterone and oestradiol in plasma were 200, 2, 1 and 1 pg/ml respectively. The intra- and inter-assay coefficients of variation for all the above steroid assays were <12%.

*Statistical procedures.* The criteria used to investigate the various stages of follicular atresia were subjected to cluster analysis using the single-linkage procedure outlined in Genstat (1981). With this method, the similarity coefficient between two clusters is the greatest between any two units, one in each cluster. All units start in separate clusters and the algorithm clusters those with similarity levels greater than some preset level (between 0 and 1).

When analysing the data on hormones in follicular fluid or plasma, they were first normalized by log transformation and then subjected to analysis of variance (ANOVA) followed by pair-wise comparisons of means using the error mean square of the ANOVA. The results from these studies were expressed as geometric means and 95% confidence limits.

## Results

### *Hormones in peripheral and ovarian venous plasma*

The concentrations (geometric means and 95% confidence limits) of androstenedione, testosterone and oestradiol in peripheral plasma and in the veins draining the ovary containing a dominant follicle or the contralateral ovary on different days of the oestrous cycle are summarized in Table 1. In general, the mean concentrations of steroids in ovarian venous blood exceeded those in peripheral plasma, but the range of values in the former was large and significant differences for all steroids were only observed on Day 0. With the exception of Day –3, the oestradiol concentrations in veins draining ovaries with a dominant follicle were always significantly greater than those in peripheral plasma ( $P < 0.05$ ). With the exception of Day –1, the androstenedione concentrations in the veins draining contralateral ovaries were always significantly greater than in peripheral plasma ( $P < 0.05$ ). Oestradiol concentrations from the dominant ovary were significantly greater than those from the contralateral ovary ( $P < 0.05$ ) only on Day 0.

### *Ovarian weights and follicle numbers*

The weights of ovaries with and without CL (means  $\pm$  s.e.m.) were  $10.07 \pm 0.44$  g ( $n = 31$ ) and  $5.71 \pm 0.25$  g (33) respectively. The mean  $\pm$  s.e.m. number of follicles  $\geq 2$  mm diameter per cow was  $52.4 \pm 4.1$  ( $n = 33$  cows). The mean  $\pm$  s.e.m. number of follicles in ovaries with and without CL were  $26.5 \pm 2.3$  (31) and  $25.5 \pm 2.2$  (33) respectively.

### *Follicular health and atresia*

The distribution of healthy ovarian follicles (Grade 1a) with respect to diameter and day of the oestrous cycle is shown in Text-fig. 1. On each day, there was at least 1 large ( $\geq 8$  mm diam.) healthy follicle in each cow. On Days –5, –4, –3, –2, –1, 0 and +1, the chance that the largest follicle in the ovary was the dominant follicle was 50 (1/2 cows), 66.7 (2/3), 60.0 (3/5), 80 (4/5), 85.7 (6/7), 71.4

**Table 1.** Steroid concentrations in peripheral plasma, and in ovarian veins draining ovaries with a dominant follicle and from the contralateral ovaries

Plasma source	Day from oestrus	No. of cows	Plasma steroid concentrations (pg/ml)		
			Androstenedione	Testosterone	Oestradiol
Peripheral			7 <sup>a,p</sup> (2-29)	14 <sup>p</sup> (5-40)	15 <sup>a</sup> (5-43)
Ovary with dominant follicle*	-3	5	37 <sup>a</sup> (3-459)	27 <sup>p</sup> (18-42)	98 <sup>a</sup> (4-1939)
Contralateral ovary			464 <sup>b,q</sup> (45-4722)	142 <sup>a</sup> (37-556)	30 <sup>a</sup> (11-78)
Peripheral			20 <sup>a,p</sup> (5-74)	23 <sup>a</sup> (4-132)	19 <sup>a</sup> (6-62)
Ovary with dominant follicle*	-2	5	493 <sup>b,q</sup> (90-2724)	104 <sup>a</sup> (36-299)	144 <sup>b</sup> (20-706)
Contralateral ovary			178 <sup>b</sup> (45-2018)	110 <sup>a</sup> (27-441)	38 <sup>a</sup> (15-95)
Peripheral			11 <sup>a</sup> (2-144)	14 <sup>a</sup> (2-171)	18 <sup>a</sup> (16-21)
Ovary with dominant follicle*	-1	3	185 <sup>a</sup> (5-7044)	86 <sup>a</sup> (23-314)	164 <sup>b</sup> (19-1380)
Contralateral ovary			118 <sup>a</sup> (4-3790)	61 <sup>a</sup> (6-614)	106 <sup>b</sup> (8-1352)
Peripheral			10 <sup>a,p</sup> (4-27)	7 <sup>a</sup> (2-23)	14 <sup>a,p</sup> (7-31)
Ovary with dominant follicle*	0	5	191 <sup>b,q</sup> (31-1164)	94 <sup>b</sup> (34-284)	1326 <sup>b,q</sup> (270-6568)
Contralateral ovary			86 <sup>c,q</sup> (38-240)	60 <sup>b</sup> (17-209)	162 <sup>b,q</sup> (46-1635)

Values are geometric means with 95% confidence limits in parentheses.

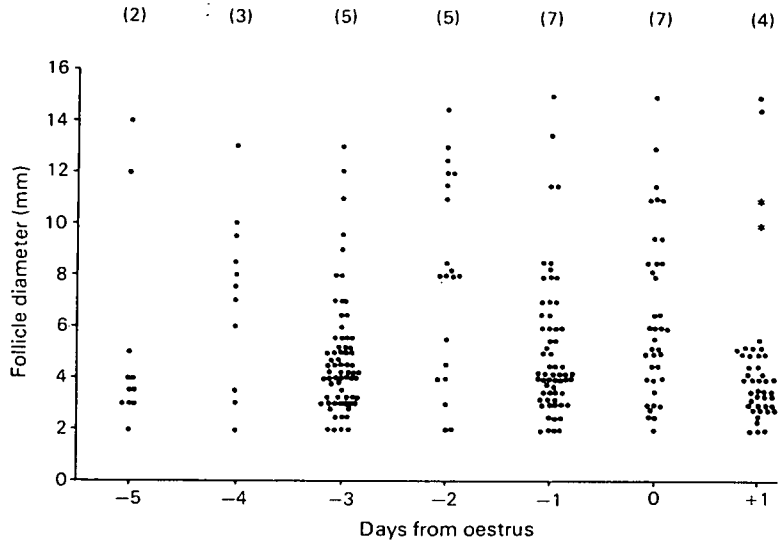
Numbers in columns for each day from oestrus with a different alphabetical superscript are significantly different from one another: <sup>a,b,c</sup> $P < 0.05$ ; <sup>p,q</sup> $P < 0.01$  (analysis of variance).

\* Dominant refers to the Grade 1a (healthy) follicles in each cow with the highest concentration of oestradiol in follicular fluid and with granulosa cells having the highest level of extant aromatase activity.

(5/7) and 100% (4/4). The mean  $\pm$  s.e.m. number of healthy follicles ( $\geq 2$  mm diam.) per cow was  $7.6 \pm 1.0$ ; this represented  $14.9 \pm 1.6\%$  of the follicle population.

Most (i.e.  $85.1 \pm 1.5\%$ ) antral follicles ( $\geq 2$  mm diam.) were atretic (i.e. Grades 1b, 2a, 2b and 3). The proportions of large ( $\geq 8$  mm diam.), intermediate (5-7.5 mm diam.) or small follicles (2-4.5 mm diam.) in atresia did not vary with the day of the oestrous cycle. There was, however, an influence of follicular diameter on the distribution of follicles at each stage of atresia. The mean number of large follicles per cow was  $2.4 \pm 0.2$ ; of this number  $30 \pm 6\%$  per cow were atretic. Nearly all, 16/17 (94.1%) of these atretic follicles were in Grade 2a atresia; only one (5.9%) was in Grade 1b atresia. The mean number of intermediate-sized follicles per cow was  $5.3 \pm 0.6$ ; of this number  $67.3 \pm 7.4\%$  were atretic. Overall the distribution of intermediate-sized follicles at Grade 1b, 2a, 2b and 3 atresia was 37.0, 48.9, 5.4 and 8.7% respectively. The mean number of small follicles (2-4.5 mm diam.) per cow was  $45.9 \pm 4.5$ ; of this number  $92.9 \pm 1.4\%$  were atretic. Overall, the distribution of small follicles at Grades 1b, 2a, 2b and 3 atresia was 25.6, 22.7, 15.0 and 36.8%.

Of all Grade 2a, 2b or 3 follicles, 53% contained oocytes which appeared healthy, 13% contained oocytes which were clearly degenerate and from 34% of the follicles no oocyte was recovered. Healthy oocytes were not uncommon even in Grade 3 follicles which were severely deficient in granulosa cells.



**Text-fig. 1.** Total number of healthy follicles of different size with respect to day from oestrus (Day 0). Numbers in parentheses refer to number of cows studied on each day of the cycle.  
\* Haemorrhagic presumptive preovulatory follicles.

#### *Follicular diameter and cell number*

There was no effect of day of oestrous cycle on the diameter of the largest healthy (Grade 1a) follicle (Text-fig. 1), the diameter of the dominant follicle or their respective numbers of granulosa cells.

#### *Steroids in follicular fluid*

There was no difference for any steroid when the results from anaesthetized animals were compared with those from the slaughterhouse. Consequently, the results from these two groups of animals were pooled when appropriate. The concentrations (geometric means and 95% confidence limits) of progesterone, androstenedione, testosterone and oestradiol in the fluid of dominant, large, intermediate or small healthy (Grade 1a) follicles regardless of the day of oestrous cycle are shown in Table 2. As healthy follicles increased in size there were significant increases in the concentrations of progesterone and oestradiol ( $P < 0.05$ ) and significant decreases in the concentrations of androstenedione and testosterone ( $P < 0.01$ ). The dominant follicles differed from the large follicles in that they contained significantly higher concentrations of oestradiol ( $P < 0.01$ ). When the data for each size of healthy follicle were analysed with respect to the day of the oestrous cycle, no significant changes in the concentration of progesterone, androstenedione or testosterone were observed. The only exception to this was noted for progesterone concentrations in dominant follicles on Day +1. On this day the progesterone concentrations in two follicles were 485 and 775 ng/ml; these concentrations were at least 16-fold greater than in all other dominant follicles on all other days of the cycle. There were no significant changes in oestradiol concentrations for small or intermediate-sized follicles with respect to day of cycle. For the large healthy follicles or dominant follicles, however (see Table 3), the mean concentrations of oestradiol on Day 0 were 2–10-fold higher than those on other days of the cycle although high oestradiol concentrations were also present in some follicles on Days -5, -4, -3, -2 and -1, but not on Day +1.

The concentrations (geometric means and 95% confidence limits) of progesterone, androstenedione, testosterone and oestradiol in the fluid of all atretic follicles (Grades 1b to 3) with respect to follicle diameter are summarized in Table 2. The data refer to follicles at Grades 1b, 2a, 2b or 3 of



Table 2. Steroid concentrations (geometric means and 95% confidence limits) in healthy and atretic bovine follicles

Follicle type or diameter (mm)	No. of follicles	Steroid (ng/ml)			
		Progesterone	Androstenedione	Testosterone	Oestradiol
Healthy					
Dominant*	32	30 <sup>a,p</sup> (24-39)	11 <sup>a,p</sup> (9-15)	17 <sup>a,p</sup> (11-27)	60 <sup>a,p</sup> (35-105)
≥ 8	56	27 <sup>a,p</sup> (22-33)	10 <sup>a,p</sup> (8-12)	14 <sup>a,p</sup> (10-19)	27 <sup>b,q</sup> (17-42)
5-7.5	45	20 <sup>b,q</sup> (16-23)	20 <sup>b,p</sup> (15-25)	31 <sup>b,q</sup> (23-42)	7 <sup>c,r</sup> (5-9)
2-4.5	77	15 <sup>c,q</sup> (13-18)	34 <sup>c,q</sup> (27-43)	59 <sup>c,r</sup> (47-74)	4 <sup>d,r</sup> (3-4)
Atretic					
≥ 8	17	176 <sup>a,p</sup> (100-305)	9 <sup>a,p</sup> (5-15)	15 <sup>a,p</sup> (6-38)	2 <sup>a</sup> (2-2)
5-7.5	73	33 <sup>b,q</sup> (25-45)	17 <sup>b,p</sup> (14-21)	23 <sup>b,p</sup> (18-30)	3 <sup>a</sup> (2-3)
2-4.5	707	30 <sup>b,q</sup> (28-33)	35 <sup>c,q</sup> (32-37)	42 <sup>b,q</sup> (38-46)	3 <sup>a</sup> (2-3)

Numbers (in columns) for either healthy or atretic follicles with a different alphabetical superscript are significantly different from one another: <sup>a,b,c,d</sup> $P < 0.05$ ; <sup>p,q,r</sup> $P < 0.01$  (analysis of variance).

\* A dominant follicle was the Grade 1a follicle in each cow with the highest concentration of oestradiol in follicular fluid, and with the granulosa cells having the highest level of extant aromatase activity.

Table 3. Oestradiol concentrations (ng/ml) in dominant\* or large healthy (≥ 8 mm diam.) follicles during the bovine oestrous cycle

Follicle type or diameter	Days from oestrus (Day 0 = oestrus)					
	-5 and -4	-3	-2	-1	0	+1
Dominant						
Oestradiol conc.	44 <sup>a</sup> (13-141)	30 <sup>a</sup> (3-324)	21 <sup>a</sup> (4-129)	83 <sup>a,b</sup> (37-185)	256 <sup>b</sup> (87-750)	20
No. of follicles	5	5	5	8	7	2
≥ 8 mm						
Oestradiol conc.	10 <sup>a</sup> (3-32)	16 <sup>a</sup> (5-60)	15 <sup>a</sup> (7-32)	57 <sup>a,b</sup> (27-123)	104 <sup>b</sup> (24-455)	20
No. of follicles	8	9	16	11	10	2

Values are geometric means with 95% confidence limits in parentheses.

Numbers (in rows) with a different alphabetical superscript are significantly different from one another,  $P < 0.05$  (analysis of variance).

\* A dominant follicle was the Grade 1a follicle in each cow with the highest concentration of oestradiol in follicular fluid and with granulosa cells having the highest level of extant aromatase activity.

atresia since no differences in steroid concentrations between these different grades of atresia were observed. Compared to small or intermediate sized atretic follicles, large atretic follicles contained the highest concentrations of progesterone, the lowest concentrations of androstenedione and testosterone and uniformly low or undetectable concentrations of oestradiol.

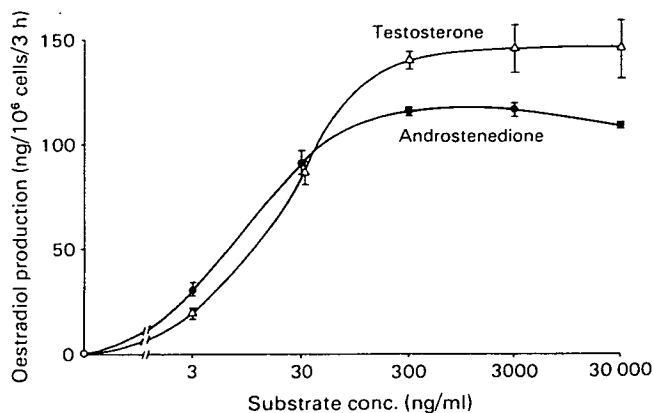
When the data for each size of atretic follicles were analysed with respect to day of the oestrous cycle, no significant changes were noted. Of the 17 large atretic follicles, 16 were assessed at Grade 2a atresia. When the steroid concentrations in these predominantly Grade 2a atretic follicles were compared to those in healthy or dominant follicles of comparable size, the atretic follicles contained the highest progesterone ( $P < 0.01$ ) and the lowest oestradiol concentrations ( $P < 0.01$ ) whereas the concentrations of androstenedione and testosterone were similar (see Table 2). Moreover, in small and intermediate atretic follicles the progesterone concentrations were significantly greater (both  $P < 0.01$ ) than those in the respective healthy follicles (Table 2).

#### Aromatase activity in granulosa cells

The respective abilities of testosterone and androstenedione to be utilized by bovine granulosa cells for the synthesis of oestradiol are shown in Text-fig. 2. These androgens were equipotent as substrates for oestrogen biosynthesis.

The levels of aromatase activity in granulosa cells from healthy (Grade 1a), small (2–4.5 mm diam.), intermediate (5–7.5 mm diam.), large ( $\geq 8$  mm diam.) and dominant follicles were  $2.8 \pm 0.4$  (23 follicles),  $12.5 \pm 1.3$  (33),  $65.0 \pm 11.2$  (36) and  $92.5 \pm 15.6$  (23) ng/ $10^6$  cells/3 h respectively (intermediate *versus* small,  $P < 0.01$ ; large *versus* intermediate,  $P < 0.01$ ; dominant *versus* large,  $P > 0.05$ ). Granulosa cells from all atretic follicles, regardless of grade of atresia or follicle size, contained little or no discernible aromatase activity ( $< 2$  ng/ $10^6$  cells/3 h, 195 follicles tested).

Aromatase activity in the granulosa cells was influenced by substrate concentration and incubation time. The characteristics of aromatase enzyme activity in cells from dominant follicles during the oestrous cycle are summarized in Table 4. Dominant follicles on Day  $-4$  to  $-1$  sometimes contained granulosa cells with similar aromatase enzyme characteristics to those at oestrus. Overall, the apparent  $K_m$  values in healthy but not dominant follicles ( $\geq 5$  mm diam.;  $n = 14$ ) were highly variable (i.e. undetectable or ranged from 40 to 448 nM) whereas the  $V_{max}$  values which ranged from 0 to 50 ng oestradiol/ $10^6$  cells/3 h were consistently lower than those in cells from dominant follicles. The  $V_{max}$  and apparent  $K_m$  values in cells from a 10-mm presumptive preovulatory follicle on Day  $+1$  were 0 nM and undetectable respectively. In cells from small healthy follicles (2–4.5 mm diam.) the apparent  $K_m$  values on Days  $-5$  to 0 were undetectable or ranged from 621 to 1022 nM (9 follicles tested), whereas on Day  $+1$  (2 follicles) they ranged from 27 to 37 nM. The  $V_{max}$  values in small healthy follicles on Days  $-5$  to 0 (9 follicles) ranged from 0–4 ng/ $10^6$  cells/3 h, whereas on Day  $+1$  (2 follicles) they ranged from 12 to 26 ng/ $10^6$  cells/3 h.



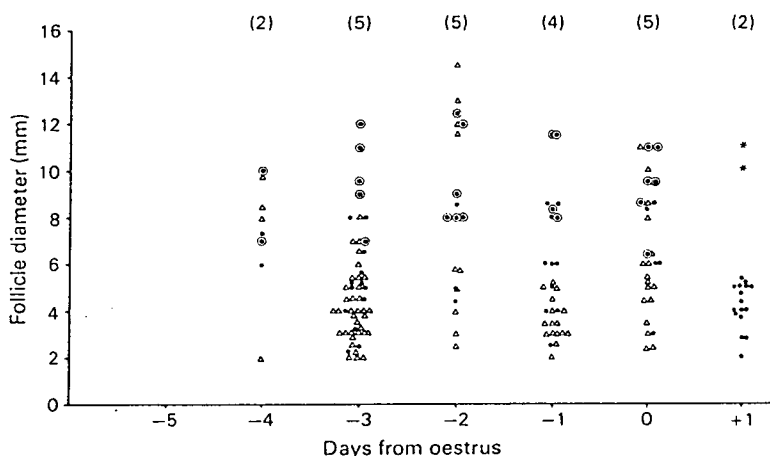
**Text-fig. 2.** Oestradiol output by bovine granulosa cells (250 000 cells/tube) from a pool of healthy large ( $\geq 8$  mm diam.) follicles incubated with different concentrations of testosterone or androstenedione.

**Table 4.** Characteristics of aromatase enzyme activity in dispersed bovine granulosa cells from dominant\* follicles during the oestrous cycle

	Days from oestrus (Day 0)		
	-4 and -3	-2 and -1	0
$K_m$ (nM)	136 (118-156)	89 (18-132)	144 (74-209)
$V_{max}$ (ng oestradiol/ $10^6$ cells/3 h)	56 (21-296)	86 (38-120)	116 (109-244)
No. of follicles	3	4	5

Values are medians with ranges in parentheses.

\* Dominant refers to the Grade 1a (healthy) follicles in each cow with the highest concentration of oestradiol in follicular fluid and with the highest level of extant aromatase activity.



**Text-fig. 3.** Scatter graph showing the relationship between diameter of healthy follicles and day of oestrous cycle relative to the day of oestrus (Day 0). ○, Dominant follicles with aromatase activity; ● and Δ, healthy but not dominant follicles containing granulosa cells with or without aromatase activity respectively; \* haemorrhagic presumptive ovulatory follicles which were devoid of aromatase activity. Aromatase activity was assessed after incubation of granulosa cells with 1000 ng testosterone. The points refer to the total number of healthy ovarian follicles ( $\geq 2$  mm diam.) from the number of cows indicated in parentheses.

Aromatase activity was examined in cells from all healthy follicles (using 1000 ng testosterone and a 3-h incubation time) from 23/33 cows with respect to day of the oestrous cycle (Text-fig. 3). Follicles with cells producing  $> 2$  ng oestradiol/ $10^6$  cells/3 h were recorded as having activity. On Days -5 to 0, the number of intermediate and large healthy follicles with aromatase activity was variable (0-100%). On Day +1, the recently ruptured or presumptive preovulatory follicles had no discernible aromatase activity (i.e.  $< 2$  ng oestradiol/ $10^6$  cells/3h) although all the small and intermediate-sized follicles had some activity ( $\geq 2$  ng oestradiol/ $10^6$  cells/3 h).

#### *LH induced steroidogenesis in theca interna*

Theca interna tissue from small, intermediate or large healthy follicles (Grade 1a,  $n = 67$ ) responded to LH and produced 2.8-15-fold more ( $P < 0.01$ ) androstenedione than did the

unstimulated tissues. The only exception to this was for LH-treated theca interna recovered from presumptive preovulatory follicles ( $n = 2$ ) on Day +1; these tissues did not respond to LH and produced only trivial amounts of androstenedione (i.e. 3 and 9 ng/10 mg/3 h). For the other follicles that were 2–4.5 mm, 5–7.5 mm,  $\geq 8$  mm (on Days –5 to +1) or dominant (on Days –5 to 0), the respective androstenedione outputs by LH-stimulated thecae were  $427 \pm 101$  (mean  $\pm$  s.e.m.;  $n = 7$ ),  $393 \pm 47$  (14),  $425 \pm 35$  (23) and  $453 \pm 36$  (23) ng/10 mg theca/3 h while the respective outputs from the unstimulated controls were 58 (2),  $86 \pm 13$  (7),  $96 \pm 5$  (19) and  $87 \pm 12$  (15) ng/10 mg theca/3 h. These data show that there was no relationship between follicular diameter and thecal androgen output when the results were expressed per 10 mg tissue. It was also evident that the thecal androstenedione output from dominant follicles was similar to that from small, intermediate or large healthy follicles. When the data were analysed with respect to day from oestrus, no significant differences were observed. Even for the dominant follicle on Day 0, the level of LH-induced androstenedione biosynthesis was similar to that on other days of the oestrous cycle.

The LH-stimulated thecae from large atretic (Grade 2a) follicles ( $n = 9$ ) produced significantly less androstenedione than did the controls (LH-treated theca,  $27 \pm 6$  androstenedione/10 mg theca/3 h; control theca,  $61 \pm 8$  ng/10 mg/3 h;  $P < 0.01$ ). In contrast LH-stimulated theca interna from small and intermediate atretic (Grades 1b and 2a) follicles ( $n = 10$ ) produced 1.2–8-fold more androstenedione than did their respective controls (LH-treated theca,  $310 \pm 32$  ng/10 mg/3 h; controls,  $68 \pm 7$  ng/10 mg/3 h;  $P < 0.01$ ). Thecae internae from Grades 2b and 3 follicles were not studied because of the difficulties in identifying this tissue at these stages of atresia.

#### *Relationship between LH-induced steroidogenesis in theca interna and extant aromatase activity in granulosa cells*

The relationship between the LH-responsiveness of theca interna with respect to androstenedione production and the level of aromatase activity in granulosa cells from 57 individual healthy follicles ( $\geq 5$  mm diam.) was examined. An LH-responsive theca interna was considered to be that producing more than twice the control outputs whereas granulosa cells having aromatase activity were those producing  $\geq 2$  ng oestradiol/ $10^6$  cells/3 h. Most (i.e. 39/57 or 68%) of the healthy follicles contained measurable activity in both cell types although 18/57 (32%) contained an LH-responsive theca but no granulosa cell activity. No follicles were found with aromatase activity in granulosa cells in the absence of an LH-responsive theca or an absence of steroidogenic activity in both cell types.

### Discussion

The results of the present study confirm and extend the notion that there is at least one large healthy follicle present in the ovaries of the cow on each day from Day –5 until ovulation, and that on each of these days there are 1–3 ovarian follicles  $\geq 5$  mm in each cow with an LH-responsive theca interna and extant aromatase activity in granulosa cells. The demonstration of highly variable aromatase activity in dominant follicles, highly variable concentrations of oestradiol in ovarian venous blood and follicular fluid and variable numbers of atretic follicles each day from Day –5 to Day 0 is consistent with the possibility that there is a continual turnover of follicles with a potential to ovulate (Matton *et al.*, 1981). Alternatively, and as a consequence of episodic gonadotrophin secretion (Hansel & Convey, 1983), it is possible that there are variable periodicities of activity and quiescence with respect to oestrogen biosynthesis in healthy follicles ( $\geq 5$  mm diam.) which have a relatively slow turnover time (Scaramuzzi *et al.*, 1980).

In small healthy follicles (i.e.  $< 5$  mm diam.) the presence of high androstenedione and testosterone, and low or undetectable concentrations of oestradiol in follicular fluid (Table 2), low or undetectable extant aromatase activity in the granulosa cells (Text-fig. 4; Table 4) and an LH-

responsive theca provide strong evidence that early developing antral follicles develop a competence to synthesize androgens before appreciable aromatase activity is expressed in the granulosa cells. Presumably the increasing concentration of oestradiol at the expense of androgen as healthy follicles enlarge beyond 4.5 mm diameter (Table 2) is a direct consequence of an induction or activation of aromatase activity in granulosa cells.

In large healthy or dominant follicles, the LH responsiveness of theca interna (per unit mass of tissue) was similar to that in intermediate or small healthy follicles and did not differ with the day of the oestrous cycle. In dominant follicles, the level of androgen substrate required for aromatization by granulosa cells at 50%  $V_{max}$  can be derived from the apparent  $K_m$  value (i.e. 18–296 nM, Table 4). These amounts of androgen can readily be synthesized by LH-stimulated thecal tissues under in-vitro conditions (see 'Results' and McNatty *et al.*, 1984a). Therefore, the level of oestradiol biosynthesis by a dominant follicle *in vivo* is probably dependent upon the plasma concentration of LH and/or the LH pulse frequency as well as on the mass of theca interna which is known to increase with increasing follicular diameter (McNatty *et al.*, 1984a).

The follicle classification system described herein has provided a framework in which to examine some aspects of atresia. Of the intermediate and large follicles, between 1 and 5 per cow were assessed to be healthy (Grade 1a): 2–4 of these contained low or undetectable extant aromatase activity in their granulosa cells even though the theca from such follicles responded to LH by secreting androstenedione. Early atresia in intermediate or large follicles may be due to a failure to stimulate aromatase activity or to a loss of enzyme activity preceding any degenerative changes in the theca interna. In either case any treatment to enhance twin ovulations in cattle may need to sustain or activate aromatase activity in more than one of the large and/or intermediate-sized healthy follicles while these follicles await the preovulatory rise in LH secretion.

Of the small follicle population, ~93% were assessed to be atretic leaving on average only 3 healthy follicles per cow per day of cycle. Although the mean level of aromatase activity in small healthy follicles was indistinguishable from that in atretic follicles, it was clear that some individual follicles had enzyme activity whereas others did not. The substrate requirement in small follicles for half-maximum oestradiol biosynthesis (i.e. the apparent  $K_m$  value) was very high on Days -5 to 0 (521–1022 nM) whereas on Day +1 it was relatively low (27–37 nM). Furthermore, <25% of all small healthy follicles on Days -4 and 0 had measureable aromatase activity whereas all the small and intermediate-sized follicles on Day +1 had some activity (Text-fig. 3). A major difference between follicles on Day +1 compared to Days -4 to 0 is that all follicles on Day +1 would have experienced the preovulatory surge of LH and FSH (see Hansel & Convey, 1983, for review). Whilst the gonadotrophin surge may down-regulate aromatase activity in large follicles and initiate preovulatory luteinization (Dieleman, Kruij, Fontijne, de Jong & van der Weyden, 1983), it may concomitantly have increased the oestrogen-secreting potential of the small healthy follicles by altering the kinetic properties of the aromatizing enzyme system. The thecae from the small follicles on Day +1 still retained their ability to respond steroidogenically to LH *in vitro* (data not shown), but it is not known whether the increased incidence of aromatase activity in follicles on Day +1 was due to increased exposure to FSH, LH or androgen or any combination thereof.

Henderson *et al.* (1984) have shown that the theca interna from healthy and atretic follicles has specific LH/hCG receptors and that the binding characteristics of these receptors do not change with increasing atresia. However, as large follicles degenerate from Grade 1a to 1b or 2a, the thecae internae were no longer capable of secreting androgen or progesterone in response to LH (see 'Results'; Henderson *et al.*, 1984; McNatty *et al.*, 1984a). In conjunction with this loss of steroidogenesis in thecal tissue there were significantly higher progesterone concentrations in follicular fluid together with significantly lower (30-fold) oestradiol concentrations relative to those in dominant follicles (Table 2). Since small- and intermediate-sized as well as the large atretic follicles contained higher ( $P < 0.01$ ) progesterone concentrations than healthy follicles of equivalent size, it seems reasonable to conclude that increased progesterone synthesis by granulosa cells is a common event during atresia in bovine follicles.

In contrast to the inability of LH to stimulate androgen synthesis in large atretic follicles, small- or intermediate-sized atretic follicles (Grades 1b and 2a) still retained a potential to synthesize androgen when stimulated by LH. Therefore, if all the healthy and many of the atretic follicles are capable of synthesizing androgen in response to LH, one could expect between 20 and 60 follicles (>2 mm diam.) per cow to secrete androgen in response to LH whereas only 1–3 would have the ability to metabolize the newly synthesized androgen to oestrogen. This finding may partly explain the extremely large variation in the concentrations of androstenedione in ovarian venous blood, the relatively high concentrations of androgen in small healthy and atretic follicles as well as the low concentrations of androgen in large healthy follicles (see also Wise *et al.*, 1982).

In conclusion our data suggest that most, if not all, healthy follicles ( $\geq 2$  mm diam.) and many atretic follicles are biochemically competent to secrete androstenedione in response to LH whereas only 1–3 follicles ( $\geq 5$  mm diam.) per cow have granulosa cells capable of metabolizing androstenedione to oestrogen. The aromatizing enzyme complex seems to be highly variable in activity and perhaps even unstable. The absence of aromatase activity in large or intermediate healthy follicles may indicate one of the earliest signs of atresia. Therefore, an understanding of the factors regulating granulosa cell aromatizing activity may help to provide new insights into methods of enhancing the ovulation rate in cattle.

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## Seasonal differences in ovarian activity in cows

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### ABSTRACT

The plasma concentrations of LH and prolactin and various parameters of ovarian function were examined in cows on known days of the oestrous cycle during May and June (autumn and winter) and during October (spring).

Luteinizing hormone peak frequency and plasma prolactin concentrations were significantly higher in October than during the May–June period (LH,  $P < 0.05$ ; prolactin,  $P < 0.01$ ). The mean diameters of large healthy follicles ( $\geq 8$  mm diameter) and the dominant oestrogen-secreting follicles were significantly larger ( $P < 0.01$  for both follicle types) and each follicle contained more granulosa cells (both  $P < 0.01$ )

in May–June than in October. The LH responsiveness of theca interna with respect to androstenedione production and the levels of aromatase activity in granulosa cells did not differ with time of year. The corpora lutea were heavier ( $P < 0.05$ ) and secreted more progesterone ( $P < 0.01$ ) in May–June than in October.

It is concluded that seasonal differences in ovarian activity exist in cows and that these differences are probably the consequence of seasonal differences in gonadotrophin secretion.

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### INTRODUCTION

There is evidence to suggest that seasonal reproductive mechanisms are present in cattle even though this species ovulates and breeds throughout the year. Cows tend to have a shorter postpartum anoestrus in a long photoperiod than they do in a shorter photoperiod (Thibault, Courot, Martinet *et al.* 1966; Bulman & Lamming, 1978; Montgomery, Davis & Hurrell, 1980; Peters & Riley, 1982). In non-pregnant cows, there is a seasonal pattern of prolactin secretion with the highest plasma concentrations in the summer and the lowest in the winter (Tucker, 1982). In ovariectomized cows, there is a seasonal pattern of luteinizing hormone (LH) secretion with the highest concentrations in the winter and the lowest in the summer (Critser, Miller, Gunsett & Ginther, 1983).

The aim of this study was to investigate whether seasonal differences in ovarian activity were discernible in oestrous cyclic cows and whether these differences were correlated with those of LH and prolactin secretion. The seasonal parameters of ovarian

activity measured were: the patterns of follicular atresia; the diameters of healthy antral follicles; the number of granulosa cells in healthy follicles of a given size; extant (i.e. residual) aromatase activity in granulosa cells; LH-induced androstenedione synthesis in theca interna tissue; the weight of corpora lutea (CL); the concentration of progesterone in peripheral plasma.

### MATERIALS AND METHODS

#### Animals and recovery of ovaries

Thirty-three Angus cows aged  $4.7 \pm 0.3$  (S.E.M.) years were used for the study of ovarian function and/or LH and progesterone concentrations in plasma; four other animals aged  $4.3 \pm 0.5$  years were bled weekly for the seasonal study of prolactin production. Six of the animals were parous and 31 nulliparous. All animals were grazed on ryegrass/white clover pasture throughout the study. During July, August and September, pasture growth rates were low and insufficient pasture

was available to maintain cow liveweights. Over these months the animals were supplemented with meadow hay (ranging from 5–10 kg hay/cow per day). All animals were grazed with a vasectomized bull and examined at least twice daily for oestrous activity. The mean oestrous cycle lengths in individual cows were determined from  $7 \pm 0.5$  ( $n = 33$ ) consecutive oestrous cycles in animals in which ovarian function was to be investigated. Ten of these cows were slaughtered at a local abattoir in May or June on a known day of the oestrous cycle and both ovaries from each animal recovered within 30 min of slaughter. The remaining 23 animals underwent ovariectomy also on a known day of the oestrous cycle at the Wallaceville Animal Research Centre; 11 during May or June and 12 in October. The ovaries were removed about 1 h after the animals had been anaesthetized with thiopentone sodium (Intraval; May and Baker, Wellington, New Zealand). In all instances the ovaries were collected immediately into chilled Medium 199 containing Earles' salts, L-glutamine (0.68 mmol/l), Hepes buffer (20 mmol/l) (Gibco, Santa Clara, California, U.S.A.) and 0.1% (w/v) bovine serum albumin (BSA; fraction V; Sigma Chemical Co., St Louis, Missouri, U.S.A.) (medium A).

#### Blood sampling

Once daily from 7 days before oestrus (day - 7) in the 23 animals undergoing ovariectomy, a peripheral blood sample (10 ml) was collected into a heparinized tube from a jugular vein, for the measurement of progesterone. One day before ovariectomy, 10 ml blood samples were also collected every 10 min for 6 h via a jugular venous cannula inserted the previous day. From the animals studied for prolactin, blood was collected into a heparinized vacutainer (Becton-Dickinson, Rutherford, New Jersey, U.S.A.) after insertion of a 20 gauge needle into a tail vein. The regular weekly blood samples were obtained at 15.30 h after the animals were brought into the yards with a herd of milking cows. The animals were well used to the procedures described since they had been herded and blood sampled regularly for at least 6 months prior to the present study. All blood samples were centrifuged (4000 g at 6 °C for 20 min) within 1 h of collection, the plasma samples subsequently being stored at -20 °C until analysed.

#### Ovarian studies

Excised ovaries were weighed and their gross morphology was recorded. All individual follicles ( $\geq 2$  mm diameter) and CL were dissected free of extraneous tissue under a stereomicroscope and their diameters recorded. Dissection procedures, collection of follicular fluid, granulosa cell recovery and quantitation

and isolation of purified theca interna have all been described in detail elsewhere (McNatty, Hillier, van den Boogaard *et al.* 1983; McNatty, Heath, Lun *et al.* 1984).

#### Classification of atresia in bovine follicles

The bovine follicles were assessed to be in grade 1a, 1b, 2a, 2b or 3 of atresia. Grade 1a follicles (the healthiest follicles) had a pink to red coloured vascularized theca interna, no debris in follicular fluid, >25% of the maximum number of granulosa cells for a given follicle size, and a healthy looking oocyte. Grade 1b follicles had a vascularized theca which was white in appearance, follicular fluid free of debris, >25% of the maximum number of granulosa cells for a given follicle size and a healthy looking oocyte. Grade 2a had a pink to red coloured vascularized theca interna, debris in follicular fluid, <75% of the maximum number of granulosa cells for a given follicle size and a healthy or degenerate-looking oocyte. Grade 2b had an avascular theca interna which was white in appearance, no debris in follicular fluid, <75% of the maximum number of granulosa cells for a given follicle size and a healthy or degenerate-looking oocyte or no oocyte. Group 3 had a white avascular theca interna, debris in follicular fluid, <50% of the maximum number of granulosa cells for a given follicle size and an oocyte which was healthy or degenerate or no oocyte. Follicles in grades 1, 2a, 2b and 3 were distinguishable by hierarchical cluster analysis, the similarity coefficient between clusters being 0.85 (Genstat, 1981). Grade 1a follicles were not separable from grade 1b follicles by cluster analysis; however, 1a follicles were characterized by their higher concentrations of oestradiol in follicular fluid compared with 1b follicles ( $P < 0.01$ ) and high levels of aromatase activity in their granulosa cells ( $P < 0.01$ ) (K. P. McNatty, D. A. Heath, K. M. Henderson, S. Lun, P. Hurst, L. Ellis, G. W. Montgomery, L. Morrison & D. C. Thurley, unpublished data).

#### Classification of follicles on size and dominance

For each cow follicles were classified as dominant or large ( $\geq 8$  mm diameter), intermediate (5–7.5 mm) or small (2–4.5 mm). With the exception of day +1 (day 0 = presumed day of oestrus) the dominant follicle for each cow was the grade 1a follicle with the highest concentrations of oestradiol in follicular fluid and having granulosa cells with the highest extant aromatase activity. In 30/33 cows one follicle in each animal was clearly identifiable as the dominant follicle. In the three remaining cows the above criteria fitted two follicles in each animal and both were classified as dominant.

### Granulosa cell aromatase assays

Granulosa cells from all individual follicles were collected into fresh medium A. In most instances aromatase assays were performed on individual healthy (grade 1a) follicles. On some occasions, with atretic follicles (grades 1b, 2a, 2b and 3), too few cells were available for individual assays so that several follicles were pooled. Care was taken to ensure that pools of cells originated from follicles of comparable diameter and stage of atresia. The cells were washed and resuspended in medium A containing sodium bicarbonate (0.01 mol/l) so that the final cell concentration was  $100 \times 10^3$  to  $600 \times 10^3$  granulosa cells/ml; 0.5 ml aliquots of these cell suspensions were placed in  $10 \times 75$  mm tubes containing 0.5 ml of a solution of testosterone (6.67  $\mu$ mol/l) in medium A with sodium bicarbonate (0.01 mol/l), gassed with 5% CO<sub>2</sub> in air, stoppered and then incubated for 3 h in a shaking water bath at 37 °C. All assays were carried out in duplicate. At the end of the incubation the assay tubes were snap frozen to -70 °C. The tubes were subsequently thawed, centrifuged and the supernatant fractions assayed for oestradiol.

### Theca interna perfusions

The theca interna from individual bovine follicles was isolated and perfused as described previously (McNatty *et al.* 1984). Briefly, 10–50 mg theca interna was placed into glass columns (4 × 0.7 cm Econocolumn, Bio-Rad Laboratories, California, U.S.A.) containing 50 mg Sephadex G-25 (Sigma), prewashed for 1 h with a perfusion medium consisting of sterile Krebs–Ringer bicarbonate buffer containing 1% (w/v) BSA which was gassed continuously with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. An aliquot of the theca was homogenized in 1 ml ethanol at the start of the experiment to determine the endogenous steroid content. Also, a small strip of theca was fixed for histological examination to test the purity of the tissue before perfusion. The theca on the column was perfused at 37 °C for 3 h at a flow rate of 1.4 ml/min and 12-min fractions were collected. Luteinizing hormone (NIADDK-bLH-4; 200  $\mu$ g/l) was introduced into the perfusion medium for 20 min after the theca had been perfused for 1 h. At the end of the 3-h period, the tissue was fixed for histological examination and the medium from each tube frozen until assayed for androstenedione. The androstenedione output from the theca interna was expressed as the cumulative output (nmol) per 10 mg theca interna over the 3-h period during and after LH stimulation.

### Dating the oestrous cycle

The day of ovariectomy was chosen, for each cow, according to the next presumed day of oestrus (day 0)

and was determined from a knowledge of the previous mean oestrous cycle length and the last date of oestrus. Subsequently this date was confirmed or modified after assessing the histology of the CL, the weight of the CL (see Results), the binding characteristics of human chorionic gonadotrophin (hCG) to homogenized CL tissue, the progesterone-secreting characteristics of dispersed luteal cells over 3 h *in vitro* and the progesterone concentrations in peripheral plasma (see Results). The histological evaluation of luteal cell size, appearance, and nuclear to cytoplasmic ratio as well as the size of the lumina in the arterioles and the extent of the connective tissue (Donaldson & Hansel, 1965) permitted the age of the CL on days -5 or -4, -3, -2, -1, 0 and +1 to be assessed with some precision. For example, in a blind study of the same tissue sections at three consecutive monthly intervals, an expert observer (Dr D. C. Thurley, Wallaceville Animal Research Centre) achieved a 100% success rate when dating the age of the CL. The CL weights on the days presumed to be 0 and +1 were distinguishable from those on days -5 to -1 (see Results). The calculated equilibrium dissociation constants ( $K_d$ ) and maximum binding capacities ( $B_{max}$ ) for specific <sup>125</sup>I-labelled hCG binding to homogenized luteal tissue (Henderson, Kieboom, McNatty *et al.* 1984) did not differ for CL recovered from days -5 to -1 (i.e.  $B_{max} = 38 \pm 3$  fmol/mg protein;  $K_d = 0.324 \pm 0.027$  nmol,  $n = 11$ ). However, on days 0 ( $n = 4$ ) and +1 ( $n = 2$ ), these parameters were  $17 \pm 6$  fmol/mg protein ( $B_{max}$ ),  $0.239 \pm 0.059$  nmol ( $K_d$ ) and 0 fmol/mg protein ( $B_{max}$ ), 0 nmol ( $K_d$ ) respectively. Using the method of Henderson & Moon (1979) to isolate bovine luteal cells, it was found that the luteal progesterone output over 3 h in response to stimulation by LH (NIH-LH-B10; 100 ng/ml) varied with the day of the cycle. From days -5 to -2, the mean output was  $159 \pm 24$  ng/10<sup>6</sup> cells per 3 h ( $n = 6$ ). From cells recovered on days -1 and 0, however, the respective outputs were  $30 \pm 10$  ( $n = 4$ ) and  $19 \pm 9$  ( $n = 3$ ) ng/10<sup>6</sup> cells per 3 h.

From the above criteria, ovaries were recovered during October from two cows on day -4, one on day -3, two on day -2, two on day -1, four on day 0 and one on day +1. For the May–June interval, ovaries were recovered from two cows on day -5, one on day -4, four on day -3, three on day -2, four on day -1, two on day 0 and three on day +1.

### Hormone assays

All steroids were measured using previously published radioimmunoassay procedures (McNatty, Gibb, Dobson *et al.* 1981; McNatty, Gibb, Dobson *et al.* 1982). Progesterone was extracted from peripheral blood (1 ml) with diethyl ether (2 × 5 vol.) before radio-

immunoassay as described by Thorneycroft & Stone (1972). Oestradiol in an aliquot of follicular fluid was measured directly after dilution (10 to 100-fold) with 0.1 M-phosphate-buffered saline, pH 7.2. Androstenedione in Krebs-Ringer solution (from the thecal columns) and oestradiol in the aromatase assay solutions were measured directly without extraction after first validating these procedures (Neal, Baker, McNatty & Scaramuzzi, 1975).

Details regarding the working dilutions and specificities of the progesterone (WA-26), androstenedione (WA-965) and oestradiol (WA-27) antisera are provided elsewhere (McNatty *et al.* 1981, 1982); all antisera were prepared at the Wallaceville Animal Research Centre.

The detection limits of oestradiol in follicular fluid and progesterone in peripheral plasma were 7.3 and 0.6 nmol/l respectively. The detection limits of androstenedione in perfusion media and oestradiol in aromatase assay media were 300 and 180 pmol/l respectively. The intra- and interassay coefficients of variation for all the above steroid assays were <12%.

Luteinizing hormone in plasma was assayed by a specific radioimmunoassay described elsewhere (McNatty *et al.* 1981). Differences to the published assay procedure were that the bovine LH preparation LER 1716-2 was used as the iodination standard and NIADDK-bLH-4 for generating the standard curves. The within- and between-assay coefficients of variation were 6.0 and 7.0% respectively. The detection limit was 0.3 µg/l.

Prolactin in plasma was assayed by a specific heterologous radioimmunoassay procedure similar to that outlined by Munro, McNatty & Renshaw (1980). The antiserum was raised in a rabbit against ovine prolactin (NIH-P-S6) and used in the assay at an initial dilution of 1:4000. Ovine prolactin NIH-P-S11 was used as the iodination standard and bovine prolactin NIH-P-B4 to generate the standard curves. The antiserum exhibited low cross-reactivity against the bovine standards tested: bovine gamma globulin, <0.1%; bovine LH (NIH-LH-B9), <0.01%; bovine follicle-stimulating hormone (NIH-FSH-B1), <0.1%; BSA (fraction V), <0.01%; bovine thyroid-stimulating hormone (NIH-TSH-B7), <0.01%; bovine growth hormone (NIH-GH-B17), 1%. The within- and between-assay coefficients of variation were 5 and 9.6% respectively. The detection limit was 2 µg/l.

#### Analysis of LH data

The LH data were analysed by the Pulsar program of Merriam & Wachter (1982). Luteinizing hormone peaks were identified from the Pulsar program using the following values of 3.8, 2.6, 1.9, 1.5 and 1.2 for G(1), G(2), G(3), G(4) and G(5) respectively. These

values permit a peak to be defined by one, two, three, four or five consecutive points; however, the smaller the number of points incorporated into the peak, the higher the peak needs to be before it becomes distinguishable from the noise. The G values were chosen to restrict the probability of false peaks as assessed by eye to ≤5%. The mean concentration of LH was calculated from the baseline values (i.e. after all the peak samples were omitted). For the sampling period the baseline was established using a weighted moving average procedure and a smoothing time of 12 h. In essence the baseline can be described as a smooth curve near the middle of the noise and the base of the peaks. The amplitude of the LH peak was the difference between the peak value and the smoothed baseline.

#### Seasonal daylength and temperature changes at Wallaceville

The Wallaceville Animal Research Centre is situated at latitude 41°08'S and longitude 175°03'E. The mean monthly daylength changes were calculated from data supplied by the New Zealand Meteorological Service (1982). The mean monthly temperatures were calculated from routine daily recordings made at the Wallaceville Animal Research Centre. The mean number of daylight hours for each month from January to December were 14.9, 13.8, 12.1, 11.0, 10.0, 9.2, 9.5, 10.5, 12.0, 13.0, 14.5 and 15.1 h. The mean monthly temperatures from January to December were 16.8, 17.8, 14.8, 11.0, 10.5, 7.3, 6.5, 8.5, 9.5, 10.2, 13.8 and 13.4 °C. These data were used when relating seasonal changes in prolactin concentrations with daylength or temperature.

#### Statistics

Unless stated otherwise, the raw or log-transformed data were subjected to analysis of variance.

#### RESULTS

The mean ± s.e.m. duration of the oestrous cycles in cows from which ovaries were recovered in the spring (i.e. October,  $n = 12$ ) was  $20.7 \pm 0.2$  days whereas the duration of those in the autumn-winter (May-June,  $n = 21$ ) was  $20.5 \pm 0.2$  days. The within-animal coefficient of variation with respect to oestrous cycle length was  $6.3 \pm 0.8\%$  in the spring and  $8.3 \pm 1.4\%$  in the autumn-winter period.

The mean ± s.e.m. weight of the cows ovariectomized in May and June was  $467 \pm 13$  kg. This reflected a mean increase of 14 kg (i.e. from  $453 \pm 10$  kg) over the period in which their oestrous cycles were monitored. The mean weight of the cows ovariectomized in October was  $484 \pm 10$  kg; this was an increase of 27 kg (i.e. from  $457 \pm 13$ ) over the period

during which their oestrous cycles were monitored. The weights of the animals slaughtered at the abattoir were not recorded. The weights of ovaries, follicle numbers and patterns of atresia (see below) in the cows which were slaughtered in May-June were not different from those found in the cows ovariectomized at that time.

**Ovarian weights, follicle numbers and patterns of atresia**

The weights of ovaries (means ± S.E.M.) with and without CL were 9.93 ± 0.57 and 5.69 ± 0.33 g respectively for May-June (n = 21) and 10.31 ± 0.67 and 5.76 ± 0.39 g respectively for October (n = 12). In the May-June interval the numbers of follicles ≥ 2 mm diameter on ovaries with and without CL were 24.9 ± 2.6 and 25.7 ± 2.8 respectively while in October the respective numbers were 29.0 ± 4.2 and 25.3 ± 3.4.

Irrespective of follicle diameter for those ≥ 2 mm diameter, the patterns of atresia in May-June and October were similar. The proportion of follicles in grades 1a, 1b, 2a, 2b and 3 of atresia were 17.4 ± 2.3, 21.6 ± 3.2, 20.8 ± 2.7, 11.1 ± 2.1 and 29.1 ± 3.5% respectively for May-June and 13.0 ± 1.1, 21.9 ± 2.4, 22.9 ± 4.8, 12.8 ± 3.6 and 29.4 ± 3.2% respectively for October. When the patterns of atresia were examined on specific days of the oestrous cycle (i.e. days -4 to +1) no seasonal differences were apparent.

**Follicular diameter and cell number**

The day of the oestrous cycle had no effect on the mean diameter of the large healthy follicles (grade 1a), the diameter of the dominant follicle or their respective numbers of granulosa cells (Table 1). When the data for all large follicles (≥ 8 mm diameter) or dominant follicles from days -5 to 0 were pooled however, there were significant time of year (seasonal) effects on both

follicular diameter and granulosa cell number. From the large (grade 1a) follicles studied in October (n = 19), the respective diameter was 9.3 ± 0.4 mm and the granulosa cell number was 6.4 ± 0.06 × 10<sup>6</sup> cells whereas for May-June (n = 23) the respective means were 11.2 ± 0.05 mm (P < 0.01 compared with October; Student's *t*-test) and 11.5 ± 0.8 × 10<sup>6</sup> cells (P < 0.01 compared with October; Student's *t*-test).

For dominant follicles the respective diameter and granulosa cell numbers were 8.9 ± 0.05 mm and 7.6 ± 0.8 × 10<sup>6</sup> for October (n = 14) whereas for May-June (n = 22) the respective values were 11.2 ± 0.5 mm (P < 0.01 compared with October) and 11.6 ± 0.9 × 10<sup>6</sup> cells (P < 0.01 compared with October).

For the intermediate-sized (5-7.5 mm) and small (2-4.5 mm) healthy (grade 1a) follicles there were no significant time-of-year effects on the number of granulosa cells. The respective diameters and numbers of granulosa cells for intermediate follicles were 5.7 ± 0.2 mm and 3.5 ± 0.4 × 10<sup>6</sup> cells for October (n = 23 follicles) and 5.6 ± 0.1 mm and 3.7 ± 0.3 × 10<sup>6</sup> cells for May-June (n = 35 follicles). The respective diameters and numbers of granulosa cells for small follicles were 3.6 ± 0.2 mm and 1.3 ± 0.2 × 10<sup>6</sup> cells for October (n = 16 follicles) and 3.4 ± 0.06 mm and 1.5 ± 0.06 × 10<sup>6</sup> cells for May-June (n = 51 follicles).

**Oestradiol concentrations in dominant follicles**

The oestradiol concentrations in dominant follicles on days -5, -4, -3, -2 and -1 were similar and therefore the results over these days of the cycle were pooled with respect to time of year. The respective concentrations (geometric mean and 95% confidence limits) in May-June (n = 13 dominant follicles on days -5 to -1) and October (n = 11) were 84 (33,212) and

TABLE 1. Effect of day of oestrous cycle on diameters of large healthy (≥ 8 mm diameter) and dominant bovine follicles and their respective numbers of granulosa cells relative to oestrus (= day 0). Values are means ± S.E.M.

	Day of oestrous cycle						
	-5	-4	-3	-2	-1	0	+1
Diameter (mm) of large healthy follicles	13.0	10.3 ± 0.9	9.3 ± 0.6	10.0 ± 0.7	9.9 ± 0.8	10.3 ± 0.7	13.5
No. of granulosa cells (× 10 <sup>6</sup> ) in large healthy follicles	16.1	9.9 ± 0.9	9.4 ± 0.6	8.2 ± 1.5	8.5 ± 1.8	9.1 ± 1.0	14.5
No. of follicles	2	3	7	7	9	14	2
Diameter (mm) of dominant follicle	13.0	10.0 ± 1.4	9.7 ± 0.8	9.4 ± 0.7	10.1 ± 0.9	10.8 ± 1.0	13.5
No. (× 10 <sup>6</sup> ) of granulosa cells in dominant follicle	16.1	8.8 ± 1.5	9.9 ± 0.9	8.3 ± 1.5	10.4 ± 1.6	10.6 ± 1.3	14.5
No. of follicles	2	3	5	7	8	7	2

117 (76,180) nmol/l. On day 0 the respective concentrations in May–June ( $n = 4$ ) and October ( $n = 3$ ) were 889 (301,2649) and 672 (264,1714) nmol/l.

#### Extant aromatase activity

The data summarizing extant aromatase activity in granulosa cells from small, intermediate, large and dominant healthy bovine follicles with respect to time of year are shown in Table 2.

Regardless of follicle size or time of year, aromatase activity in atretic (grades 1b, 2a, 2b and 3) follicles was low ( $< 7$  nmol/10<sup>6</sup> granulosa cells per 3 h).

#### LH-induced androstenedione synthesis by theca interna

As assessed by histological examination, the theca interna ranged from 65 to 100% (mean 75%) in purity; the major contaminant was theca externa or stroma and the level of granulosa cell contamination was minimal, i.e.  $< 20$  cells/5  $\mu$ m cross-section of tissue. The steroid outputs were not corrected for tissue purity. Regardless of time of year, androstenedione synthesis by LH-stimulated theca interna produced 2.8- to 15-fold more ( $P < 0.01$ ) androstenedione than did the unstimulated tissues. For dominant, large, intermediate and small follicles, the respective mean outputs of androstenedione for LH-stimulated and control tissues were  $1.51 \pm 0.12$  (LH-stimulated,  $n = 23$ ) and  $0.29 \pm 0.003$  (control,  $n = 15$ );  $1.42 \pm 0.12$

$n = 32$ ) and  $0.32 \pm 0.02$  ( $n = 19$ );  $1.31 \pm 0.16$  ( $n = 14$ ) and  $0.29 \pm 0.04$  ( $n = 7$ );  $1.42 \pm 0.34$  ( $n = 7$ ) and  $0.23$  ( $n = 2$ ) nmol/10 mg theca interna per 3 h. When the LH-induced androstenedione outputs from different sized follicles were examined with respect to time of year (Table 3) no significant size or time-of-year effects were observed.

#### Corpus luteum weight and function

Overall, progesterone concentrations in plasma ( $n = 23$  animals monitored) declined progressively from days  $-5$  to  $+1$  but a decline in luteal weight was only obvious on days 0 and  $+1$  (Fig. 1a). The weight of corpora luteal tissue in bovine ovaries was significantly ( $P < 0.05$ ) lower in October than in May–June (Fig. 1a). Also the mean progesterone concentrations in plasma were significantly ( $P < 0.01$ ) lower in October than in May–June (Fig. 1b).

#### Prolactin and LH concentrations

The mean plasma concentrations of prolactin of cows throughout the year are shown in Fig. 2. During May–June, the mean  $\pm$  s.e.m. concentration of prolactin was  $24 \pm 3$   $\mu$ g/l ( $n = 9$  sampling days) and this was significantly lower than that during October when the mean  $\pm$  s.e.m. concentration was  $124 \pm 18$   $\mu$ g/l ( $n = 4$  sampling days;  $P < 0.01$ , Student's *t*-test). Linear regression analysis of the log-transformed mean

TABLE 2. Extant aromatase activity in granulosa cells from different sized healthy bovine follicles. Values are means  $\pm$  s.e.m.

	Oestradiol formation (pmol/10 <sup>6</sup> granulosa cells per 3 h)			
	Small	Intermediate	Large	Dominant
Time of year				
May–June	$14.5 \pm 3.6^a$	$32.7 \pm 0.7^b$	$222 \pm 40^c$	$265 \pm 40^c$
No. of follicles	17	21	16	12
October	$7.3 \pm 3.3^{a,d}$	$72.7 \pm 29.0^b$	$251 \pm 73^b$	$415 \pm 95^c$
No. of follicles	6	12	20	12

For each row, the values not sharing a common alphabetical superscript are significantly different from one another: a vs b,  $P < 0.05$ ; b vs c,  $P < 0.01$ ; a vs c,  $P < 0.01$ ; d vs c,  $P < 0.01$ . There were no significant differences between values in each column (analysis of variance).

TABLE 3. Luteinizing hormone-induced androstenedione secretion by bovine theca interna *in vitro*. Values are means  $\pm$  s.e.m.

	Androstenedione output (nmol/10 mg tissue per 3 h)			
	Small	Intermediate	Large	Dominant
Time of year				
May–June	1.53	$1.30 \pm 0.29$	$1.60 \pm 0.14$	$1.49 \pm 0.11$
No. of follicles	1	5	13	10
October	$1.89 \pm 0.52$	$1.34 \pm 0.20$	$1.32 \pm 0.15$	$1.45 \pm 0.19$
No. of follicles	4	9	19	13

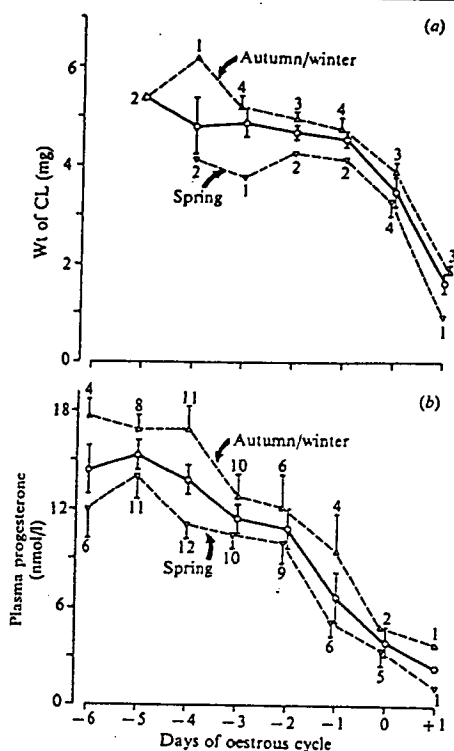


FIGURE 1. Changes in the (a) weight of corpora lutea (CL) and (b) plasma progesterone concentration in all cows (O), those during May-June (i.e. autumn-winter) or October (i.e. spring) on different days of the oestrous cycle relative to the day of oestrus (day 0). Values are means  $\pm$  S.E.M. Numbers of (a) CL or (b) animals are indicated.

monthly data for prolactin and the mean hours of daylight each month showed there was an association between the two parameters with a correlation coefficient of 0.92 ( $P < 0.01$ ;  $n = 12$ ). Linear regression

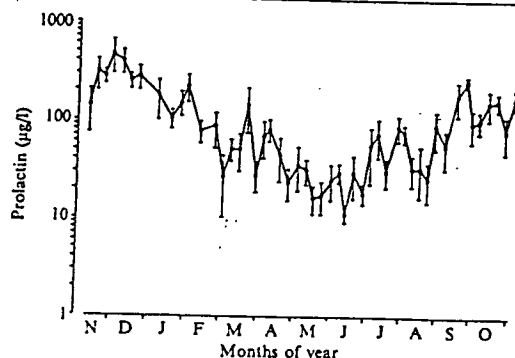


FIGURE 2. Plasma prolactin concentration (mean  $\pm$  S.E.M.) in four oestrous cows throughout the year.

analysis of the log-transformed data and the mean monthly temperatures indicated that the association was not significant at the 5% level ( $r = 0.56$ ,  $P > 0.05$  but  $< 0.10$ ,  $n = 12$ ).

For all animals ( $n = 20$ ), the mean concentration of LH for 20 cows sampled on days -5 to -1 ranged between 1.3 and 1.9  $\mu\text{g/l}$ , the peak frequency ranged between 0 and 7 peaks/6 h (median = 1 peak/6 h), the LH peak amplitudes ranged between 0.5 and 3.6  $\mu\text{g/l}$  (median = 0.9  $\mu\text{g/l}$ ) and the peak lengths ranged between 10 and 40 min (median = 20 min). No differences were observed between the above days either in mean concentrations or peak frequency. On day 0, two animals were sampled and the smoothed mean LH concentrations were  $3.6 \pm 0.3$  and  $4.1 \pm 0.4$   $\mu\text{g/l}$  respectively; the LH peak frequency for both animals was 4 peaks/6 h with the duration of each peak varying from 48.0 to 52.5 min. It is not known whether the blood samples on day 0 were taken before or immediately after the preovulatory LH surge in plasma.

The data summarizing the concentration of LH, LH peak frequency, peak amplitude and length on days -5 to -1 with respect to time of year are shown in Table 4. There were no significant differences in the

TABLE 4. Plasma concentrations of LH and LH peak frequency, amplitude and duration between days -5 and -1 of the oestrous cycle (day 0 = oestrus) with respect to time of year. Results are means  $\pm$  S.E.M.

Time of year	Mean LH ( $\mu\text{g/l}$ )	LH peaks (no./6 h)	LH peak† amplitude ( $\mu\text{g/l}$ )	LH peak length (min)	No. of animals
October	$1.6 \pm 0.07$	$2.8 \pm 0.7$	$1.0 \pm 0.1$	$19.2 \pm 2.0$	11
May-June	$1.6 \pm 0.08$	$0.6 \pm 0.2^*$	$0.7 \pm 0.2$	$20.0 \pm 4.7$	9

\*  $P < 0.01$  compared with October (Student's  $t$ -test on log-transformed data).  
† Amplitude of all recorded peaks: October = 31 peaks, May-June = 5 peaks.

mean LH concentrations or in peak length between October and May–June. However, there were significantly ( $P < 0.01$ ) more LH peaks observed in October than May–June although the mean amplitudes of the peaks at these times of the year were not different from one another.

## DISCUSSION

These results show that seasonal differences in ovarian activity exist in cattle even though the animals were cycling regularly throughout the study. The seasonal differences that were observed related specifically to the development of the preovulatory follicle and the CL. During late autumn and winter (i.e. May–June in New Zealand), the mean diameter of the dominant oestrogen-secreting follicles and of large healthy follicles ( $\geq 8$  mm diameter) were both larger (both  $P < 0.01$ ) with each containing more granulosa cells (both  $P < 0.01$ ) than in the spring (i.e. October). Also, during May–June the CL were heavier ( $P < 0.05$ ) and produced more progesterone ( $P < 0.01$ ) than in October. The above seasonal differences in preovulatory follicular development appeared not to be due to seasonal differences in the capacity of theca cells to synthesize androgen in response to LH, or of granulosa cells to metabolize androgen to oestrogen, but perhaps in part to seasonal differences in LH secretion. In the bovine ovary, specific high affinity LH receptors are present in theca interna tissue (Henderson *et al.* 1984). In response to an LH pulse of  $\geq 1$   $\mu\text{g/l}$  amplitude, theca interna may secrete androstenedione for up to 3 h *in vitro*. These LH-mediated events are demonstrable in most healthy (i.e. grade 1a) as well as some atretic follicles ( $\geq 2$  mm diameter) (Table 3, McNatty *et al.* 1984; K. P. McNatty, D. A. Heath, K. M. Henderson, S. Lun, P. Hurst, L. Ellis, G. W. Montgomery, L. Morrison & D. C. Thurley, unpublished data). Although the theca output of androstenedione per unit mass of tissue remains constant as a bovine follicle enlarges from 2 mm to  $\geq 8$  mm in diameter (Table 3), the greatest production rate is probably achieved by large healthy follicles because of their greater thecal mass compared to that in intermediate and small follicles (McNatty *et al.* 1984). Oestrogen synthesis is limited to granulosa cells from healthy (i.e. grade 1a) follicles (K. P. McNatty, D. A. Heath, K. M. Henderson, S. Lun, P. Hurst, L. Ellis, G. W. Montgomery, L. Morrison & D. C. Thurley, unpublished data) with the greatest production rate originating from large and/or dominant follicles (Table 3). When these observations are considered together with the finding of a higher LH peak frequency in October than in May–June ( $P < 0.05$ ) it would seem reasonable to suggest that large healthy follicles have a greater potential for

androgen and oestrogen biosynthesis in October than in May or June. Another possible consequence of seasonal differences in LH pulse frequency might be that a high level of oestrogen biosynthesis is initiated in healthy follicles of a smaller diameter in October than in May or June. This notion is supported by the finding of a similar range of oestradiol concentrations in dominant follicles in October and May and June.

The CL is formed from the preovulatory follicle. After the preovulatory surge mitosis in granulosa cells is arrested (Peters & McNatty, 1981) and is absent in the CL after day 4 (Donaldson & Hansel, 1965). Thus the composition of the CL is predetermined to some extent by the composition of the preovulatory follicle. The finding of heavier CL during May–June compared to October is consistent with the observation that dominant follicles during the former months contain more granulosa cells than those during October.

The nutritional qualities of the pastures available to the animals at the different times of the year were not studied. Although the difference was not significant, animals in October were 17 kg heavier than those in May and June. But whether this was a factor contributing to the differences at the pituitary or ovarian levels is not known.

Little is known about seasonal control of gonadotrophin secretion in cattle. In one study in ovariectomized cattle (Critser *et al.* 1983), LH concentrations were highest during the winter months and lowest during summer in contrast to the present results. Moreover, ovariectomized cows given oestradiol implants had higher LH concentrations than did the controls even though the seasonal changes in LH concentration were still apparent. It was suggested by these authors that the underlying seasonality of LH concentrations in ovariectomized cattle was similar to that in ewes (Karsch, Goodman & Legan, 1980). In our study, the low LH pulse frequency in the autumn and winter was associated with higher plasma concentrations of progesterone relative to those in the spring (Tables 4 and Fig. 1). Perhaps the inverse relationship between progesterone and LH pulse frequency on days  $-5$  to  $-1$  indicate a causal relationship between the two hormones as progesterone is known to inhibit LH pulse frequency (Goodman, 1978; Goodman, Bittman, Foster & Karsch, 1981).

Seasonal differences in ovarian activity occurred at two markedly different levels of prolactin secretion. During May–June the plasma concentrations of prolactin were around the lowest values recorded for the year (i.e. 10–20  $\mu\text{g/l}$ ) whereas during October the concentrations were approaching the highest values recorded for the year (i.e. 100–300  $\mu\text{g/l}$ ). The seasonal differences in prolactin secretion are similar to those observed in ewes (Walton, McNeilly, McNeilly & Cunningham, 1977; Munro *et al.* 1980). It is possible that



these differences in prolactin were the result of the marked seasonal changes in air temperature and/or daylength. Prolactin has been shown to suppress LH-induced cyclic AMP release from bovine thecal tissue *in vitro* (Weiss, Nancarrow, Armstrong & Donnelly, 1981) as well as FSH-induced aromatase activity in rat granulosa cells (Dorrington & Gore-Langton, 1981). In ewes, hyperprolactinaemia is associated with a reduced level of ovarian oestradiol secretion (McNeilly & Baird, 1983). In cattle, there is insufficient knowledge on the interrelationships between prolactin, LH pulse frequency and ovarian follicular development to infer that the seasonal alterations in LH pulse frequency and/or ovarian activity were a direct consequence of seasonal changes in prolactin secretion.

One of the factors which contributes to the duration of post-partum anoestrus in cattle might be the time required to restore a pulsatile pattern of LH secretion (Lamming, Wathes & Peters, 1981). The finding that the frequency of LH release is influenced by season in oestrous cows suggests that this may also be a factor contributing to the duration of anoestrus in post-partum cows. However, further endocrine studies on postpartum cattle are obviously needed to confirm or refute this view.

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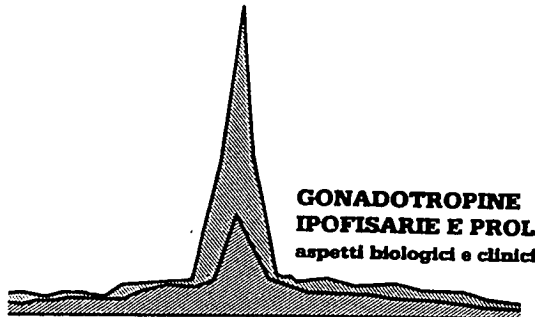
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**GONADOTROPINE  
IPOFISARIE E PROLATTINA:  
aspetti biologici e clinici**

FIRENZE, Palazzo dei Congressi, 19-20 Ottobre 1984

**GONADOTROPIC REGULATION  
OF FOLLICULAR MATURATION AND ATRESIA**

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## SUMMARY

Follicle stimulating hormone (FSH) acts specifically on ovarian granulosa cells to influence a number of biochemical processes which involve the viability of the developing follicle and the regulation of oestrogen biosynthesis. Granulosa cells acquire specific receptors for FSH during preantral development. FSH-stimulable cAMP production increases in granulosa cells with increasing follicle size. FSH (via cAMP) stimulates granulosa cell aromatase (oestrogen-synthetase) enzyme activity and modulates its kinetic properties (i.e.,  $V_{(max)}$  and  $K_m$ ) throughout follicular maturation. Temporal changes in the blood concentrations of FSH significantly influence the proportion of healthy and atretic antral follicles without altering the size of the antral follicle pool. The interrelationships between atresia, FSH-stimulable cAMP and aromatase activity are poorly understood but low levels of aromatase activity are evident in atretic follicles and also when there are low concentrations of FSH in plasma.

Luteinizing hormone (LH) acts on ovarian theca interna cells to influence the synthesis and secretion of follicular androgens which, in part, may serve as substrates for oestrogen biosynthesis. Theca interna cells probably acquire specific receptors for LH during preantral development. The LH-adenylate cyclase-steroidogenic system is fully functional during early antral development. The theca interna is the major follicular source of androstenedione. The output of thecal androstenedione is influenced *in vitro* by the rate of media perfusion, LH concentration and duration of LH exposure. It is speculated that during follicular atresia, the thecal steroidogenic system first uncouples from the adenylate cyclase system which on further degeneration uncouples from the LH receptor whose characteristics remain unchanged during this process. However, from studies on intermediate to large sized bovine follicles (>5 mm diameter), a reduced level of granulosa cell aromatase activity was always apparent before any biochemical lesions were discernible in the theca interna.

It is concluded that the viability and steroidogenic activity of follicular granulosa cells are highly sensitive to temporal changes in FSH secretion and that FSH is a principal determinant of the number of follicles which may synthesize oestradiol. In addition, it is suggested that theca interna cells are acutely sensitive to low amplitude fluctuations in LH secretion and that the frequency (and amplitude) of these fluctuations may influence the level of

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follicular androgen biosynthesis by developing follicles.

## INTRODUCTION

Follicular growth is initiated when the oocyte of a small resting follicle (i.e., a primordial follicle) begins to enlarge and its granulosa cells start to proliferate. The initiation of follicular growth is a continuous process; it occurs throughout infancy and adult life and is not interrupted by pregnancy or other periods of anovulation (1-2). Hypophysectomy or neutralization of gonadotrophic activity does not prevent the initiation of follicular growth (3-5). In the absence of gonadotrophins oocytes continue to grow and granulosa cells proliferate but the organization of the membrana granulosa and theca layer is abnormal. Moreover, antral follicles do not develop (4,6). In addition to an absolute requirement for gonadotrophins, antral follicles appear to have temporal threshold requirements to sustain their development to ovulation (7-11). For example, partial suppression of follicle-stimulating hormone (FSH) secretion during the early to mid-follicular phase of the menstrual cycle in Rhesus monkey or humans may lead to a reduced level of oestradiol secretion, a delay in ovulation and/or inadequate luteal function (7,9,12). Similarly, derangements in luteinizing hormone (LH) secretion during the follicular phase may also result in anovulation or inappropriate luteal function. For example, a reduced frequency and/or amplitude of pituitary LH release is causally related to seasonal infertility in some breeds of sheep (11,13-15).

Throughout most of follicular development specific LH receptors are present on theca interna cells whereas specific FSH receptors are present on granulosa cells (16). As follicles acquire the capacity to synthesize oestradiol, specific LH receptors are also induced in granulosa cells (16-18).

The purpose of this paper is to review some aspects of gonadotrophin interaction with theca and granulosa cells during follicular growth and atresia.

### Follicle-stimulating hormone

Follicle stimulating hormone binding sites are thought to develop in granulosa cells of large preantral follicles (19). Information on the binding characteristics of FSH to granulosa cells has been somewhat difficult to obtain. This has been mainly due to technical difficulties arising from high non-specific

binding and the inhibitory properties of certain buffers and polyamines (20-22). In bovine granulosa cells from large healthy follicles (>8 mm diameter), the FSH binding dissociation constant (Kd) and binding capacity [ $B_{(max)}$ ] have been estimated in our laboratory to be 1.0 nM and 3.4 fmol/10<sup>6</sup> cells respectively. These findings are similar to those of Darga & Reichert Jr. (20) who report a Kd value of 5 nM for bovine granulosa cells across all follicle sizes and health. The influence of follicular atresia on the FSH binding characteristic is unknown.

It has been reported that circulating FSH levels may influence the proportion of healthy and atretic follicles in the ovary (23,24). This has recently been confirmed in sheep treated with steroid-free bovine follicular fluid (bFF) and/or exogenous ovine FSH (NIH-FSH-S12) (Table 1). Bovine follicular fluid is a rich source of inhibin which is a potent suppressor of FSH secretion (25). Subcutaneous injection of bFF (i.e., 5 ml on two occasions, 12 h apart) resulted in a reduced output of FSH within 6 h of the first injection to values 60% lower than those in control animals; LH levels following this treatment were unaffected. Moreover, the low plasma concentrations of FSH persisted throughout the 24 h treatment period. After bFF treatment, a significant reduction was noted in the proportion of healthy follicles over all sizes ( $p < 0.01$ ) without any significant alteration in the total follicle population (>1 mm diameter; Table 1). Addition of FSH to control ewes (10 µg NIH-FSH-S12 i.v. per h for 24 h) did not influence the total number of follicles or the proportion of healthy follicles. However, this dose regime was sufficient to neutralise the inhibitory influence of bFF. In contrast, when the FSH dose in bFF-treated animals was increased 5-fold (i.e., to 50 µg i.v. per h for 24 h), there was a significant increase in the proportion of healthy follicles compared to all other treatments ( $p < 0.01$ ) without any concomitant increase in the total follicle population.

Follicle-stimulating hormone may interact with granulosa cells to stimulate <sup>3</sup>H-glucosamine incorporation into mucopolysaccharides (26) and increased inhibin (27), cAMP (28,29), aromatase enzyme (28,30,31) and progesterone synthesis (28, 30). Follicle-stimulating hormone induced cAMP synthesis is significantly associated with follicle size with the maximum effects of FSH being observed in large follicles (Fig. 1). The sensitivity of granulosa cells to FSH also changes with follicle size (Fig. 1, ref. 29). For example, in ovine granulosa

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cells from large follicles (i.e., >4 mm diameter), the cAMP response to 10 ng/ml FSH (NIH-FSH-S12) is similar to that observed in cells from small follicles (<2 mm diameter) to 100 ng/ml FSH.

The relationship between the FSH-adenylate cyclase system and the formation of steroidogenic enzymes in granulosa cells is poorly understood. It seems that  $17\beta$ -hydroxysteroid dehydrogenase enzyme activity is present in cells during preantral development, and that cholesterol side-chain cleavage enzymes as well as  $3\beta$ -hydroxysteroid dehydrogenase and aromatising enzymes are formed in the cells during antral follicle development. A generally-held view is that granulosa cells do not develop appreciable levels of  $17\alpha$ -hydroxylase or C17-C20 desmolase activity (30), but this has been disputed (32). When considering the factors influencing oestrogen biosynthesis in developing follicles one of the most important of the above enzyme systems is the aromatase enzyme complex (30). Induction and/or activation of aromatase activity involves FSH (28,31). In rats and sheep, the aromatase enzyme complex is functionally coupled to the FSH-adenylate cyclase system (28,30,31). Peak aromatase activity is only present in granulosa cells which are capable of a maximum cAMP response to FSH (Table 2).

Follicle-stimulating hormone, possibly acting via cAMP, influences follicular oestradiol biosynthesis by modulating the kinetic properties of the aromatase enzyme complex. Supporting evidence for this notion has recently been gained from studies on dispersed ovine granulosa cells incubated with testosterone as substrate. The rate of oestradiol formation varied with both time and substrate concentration. The data from these studies could be fitted to the Michaelis-Menten equation with a correlation coefficient of >0.85. When large healthy follicles (>5 mm diameter) were recovered from ewes treated with saline or FSH (10  $\mu$ g i.v. once per h for 24 h), the  $V_{(max)}$  and apparent  $K_m$  values for the aromatase enzyme complex in granulosa cells were as follows: saline-treated ewes,  $V_{(max)} = 7$  ng oestradiol/ $10^6$  cells/3 h,  $K_m = 83$  nM; FSH-treatment,  $V_{(max)} = 32$  ng oestradiol/ $10^6$  cells/3 h,  $K_m = 245$  nM. The enhancement of the  $V_{(max)}$  by FSH also occurred in follicles over all size ranges. Treatment of ewes with ovine FSH (NIH-FSH-S12; 50  $\mu$ g i.v. once per h for 24 h) increased aromatase activity 2-10 fold in small (1-2.5 mm), and medium (2-4.5 mm) follicles as well as in large healthy follicles compared to those from control ovaries (Fig. 2). During antral follicle development, aromatase



activity increased in granulosa cells (on a per cell basis) with increasing follicle size (34,35). Perhaps this increase in aromatase activity is a direct consequence of the increased sensitivity of granulosa cells to FSH (29).

The interrelationships between FSH induction of aromatase enzyme and/or cAMP synthesis during atresia is poorly understood. In sheep, cattle and man, there is a significant direct relationship between atresia and aromatase activity in intermediate to large sized follicles (sheep  $>2.5$  mm; cattle  $>5$  mm; man  $>5$  mm diam; refs. 10,35; Table 3).

Collectively, the evidence suggests that granulosa cell aromatase activity varies with follicle size, health and the plasma concentrations of FSH. Moreover, the evidence also suggests that the FSH concentrations in plasma determine the number of healthy follicles as well as the number with a potential for oestradiol biosynthesis.

#### Luteinizing hormone

The development of LH receptors in theca and/or interstitial cells has recently been reviewed by Erickson & Magoffin (36). From studies on hypophysectomised rats, they proposed that theca-interstitial cells in preantral follicles develop 'spontaneously' a single class of specific and high affinity LH receptors ( $K_d = 1.0 \pm 0.08 \times 10^{-11}$  M) and that these LH receptors are functionally coupled to the adenylate cyclase but not to the steroidogenic enzyme systems. Of the various steroidogenic enzymes present in functionally active theca interna cells (e.g.,  $3\beta$ -hydroxysteroid dehydrogenase,  $17\alpha$ -hydroxylase and C17-C20 desmolase), the first mentioned is already present in preantral follicles whereas the other two, together with the cholesterol side-chain cleavage enzymes are only detectable after LH-induced gene expression (36-38). When the enzymes are coupled to the LH receptor-adenylate cyclase system, theca interna cells emerge as a major intraovarian site of androstenedione biosynthesis. To a lesser extent, they also become a source of progesterone, dehydroepiandrosterone, testosterone and oestradiol (1,39-41). In cattle, sheep and man, small antral follicles (i.e.,  $<5$  mm in cattle) contain high concentrations of androstenedione and/or testosterone but only low concentrations of oestradiol (Table 4, see also ref. 10,34,35). The findings in Table 4 suggest that the thecal steroidogenic system is coupled to the LH receptor either

before or during early antral development and hence that thecal steroidogenesis is initiated before appreciable aromatase activity is evident in granulosa cells.

Because of the small diameter of antral follicles in rats, rabbits, etc., theca interna is not readily distinguishable (e.g., by microdissection) from interstitial/stromal tissue. In contrast, bovine ovaries provide an abundant source of theca interna tissue which is readily distinguishable from theca externa, stroma and granulosa cells (39,42). From studies on bovine ovaries, a single class of specific high affinity LH receptors was found in the theca interna but not the theca externa or stroma of either healthy or atretic antral follicles (2-16 mm diameter) (42). The binding characteristics of the LH receptor was the same for healthy and atretic follicles irrespective of follicle size but only the theca interna from healthy follicles (>8 mm diameter) responded to LH to produce androstenedione when perfused *in vitro* (Table 5).

Androstenedione output by perfused theca interna was regulated by the flow rate of medium (Fig. 3), the LH concentration and also the duration of exposure to LH (Fig. 4). The influence of medium flow across the tissue suggests that the regulation of thecal blood flow *in vivo* may be a factor of some significance with respect to follicular androgen production. The finding that as little as 30 seconds exposure to LH or an LH concentration of 1 ng/ml were each sufficient to cause a significant increase in androstenedione over a 3 h period suggests that the normally vascularized tissue is acutely sensitive to abrupt changes in LH concentration or to low amplitude pulses of the hormone (39,43).

The output of androstenedione from LH-primed theca (i.e., per unit mass of tissue) was found not to vary with follicular diameter (in antral follicles) nor with stage of cycle in either the ewe or the cow (34,35,44). It therefore seems likely that the supply of steroid substrate for follicular oestrogen biosynthesis is regulated by the frequency (and amplitude) of LH secretory episodes (10,39,43)

The interrelationships between the LH receptor, adenylate cyclase and androstenedione synthesis during atresia are poorly understood. Recently three types of theca interna from atretic bovine follicles were identified: (i) a theca with an LH receptor coupled to adenylate cyclase and capable of

synthesizing androstenedione in response to LH; (ii) a theca with an LH receptor coupled to adenylate cyclase but incapable of synthesizing androstenedione, progesterone and pregnenolone; and (iii) a theca with an LH receptor uncoupled to adenylate cyclase and incapable of steroid synthesis (Fig. 5). Perhaps during atresia, the LH receptor-adenylate cyclase-steroidogenic complex in theca interna uncouples in the reverse sequence to that described for their coupling during follicular maturation (36).

The interrelationships between follicular health and an LH-responsive, steroidogenically active theca interna and granulosa cell aromatase activity in intermediate to large diameter antral follicles are obscure. This was recently investigated in 57 bovine follicles (5-14 mm diameter) which had been classified by hierarchical cluster analysis as healthy follicles (35). Most (i.e., 39/57 or 68%) of the follicles contained an LH-responsive theca and granulosa cell aromatase activity although some (i.e., 18/57 or 32%) contained an LH-responsive theca but no granulosa cell aromatase activity. No follicles were found with aromatase activity in granulosa cells and an LH unresponsive theca or an absence of steroidogenic activity in both cell types.

#### CONCLUSION

One purpose of this paper was to highlight the importance of FSH in regulating follicular development and oestradiol biosynthesis. For example, in studies with sheep it was shown that a 2.5-fold reduction in the plasma concentration of FSH over 24 h resulted in most antral follicles  $\geq 1$  mm diameter undergoing atresia. In human ovaries, the proportion of healthy follicles  $\geq 1$  mm diameter declines from  $\sim 20\%$  during the late-follicular, early luteal phase to  $< 1\%$  during the mid luteal phase (10). This massive increase in atresia is accompanied by a 30-50% reduction in FSH secretion suggesting that the two events are causally related. This paper also describes the importance of FSH in stimulating aromatase enzyme activity in granulosa cells in developing antral follicles. Under normal physiological conditions it appears that the level of enzyme activity in individual follicles may be submaximal and probably limiting for oestradiol biosynthesis. By raising the blood levels of FSH in sheep it has been possible to stimulate a 5-fold increase in aromatase enzyme activity in preovulatory follicles as well as in other antral follicles over all size ranges

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These data show that FSH may directly influence the number of follicles which can metabolise (i.e., aromatise) androgen to oestradiol.

The second purpose of this paper was to highlight the significance of LH concentration, LH exposure time and thecal blood flow on androgen biosynthesis in thecal tissue. It seems very likely that the frequency and amplitude of LH secretory episodes may determine the level of substrate for oestrogen synthesis by granulosa cells. In this context it is attractive to speculate that a primary effect of reduced LH secretion is to limit androgen output and thereby impair preovulatory oestrogen biosynthesis by the dominant follicle(s).

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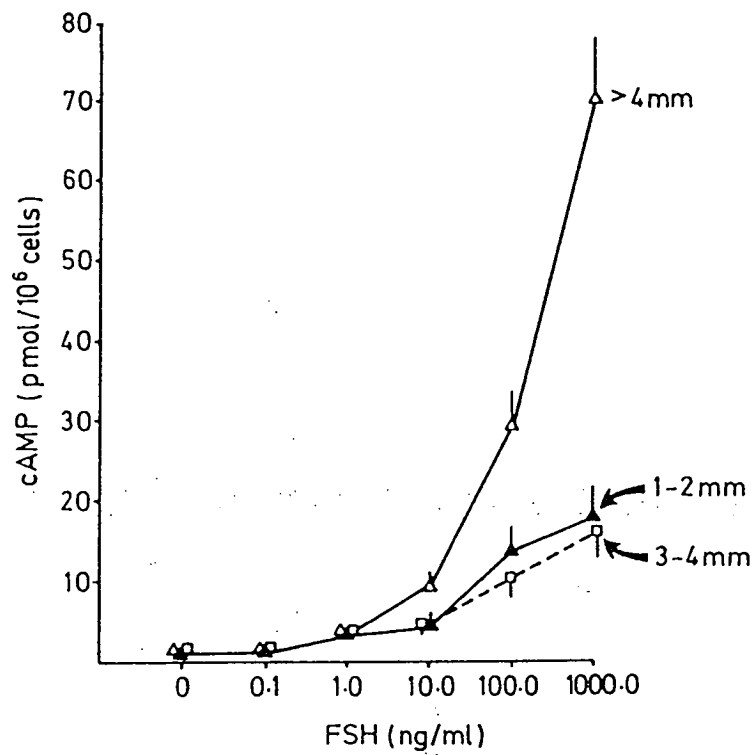


Fig. 1. Cyclic AMP response to different doses of ovine FSH (NIH-FSH-S12) by ovine granulosa cells ( $50 \times 10^3$ - $600 \times 10^3$ ) from small (1-2 mm diam.  $\Delta$  —  $\Delta$ ), intermediate (3-4 mm,  $\square$  —  $\square$ ), and large (>4 mm,  $\nabla$  —  $\nabla$ ) follicles, after incubation at 37 °C for 1 h in 1 ml Dulbecco's buffer + 0.1% BSA (w/v) (33).

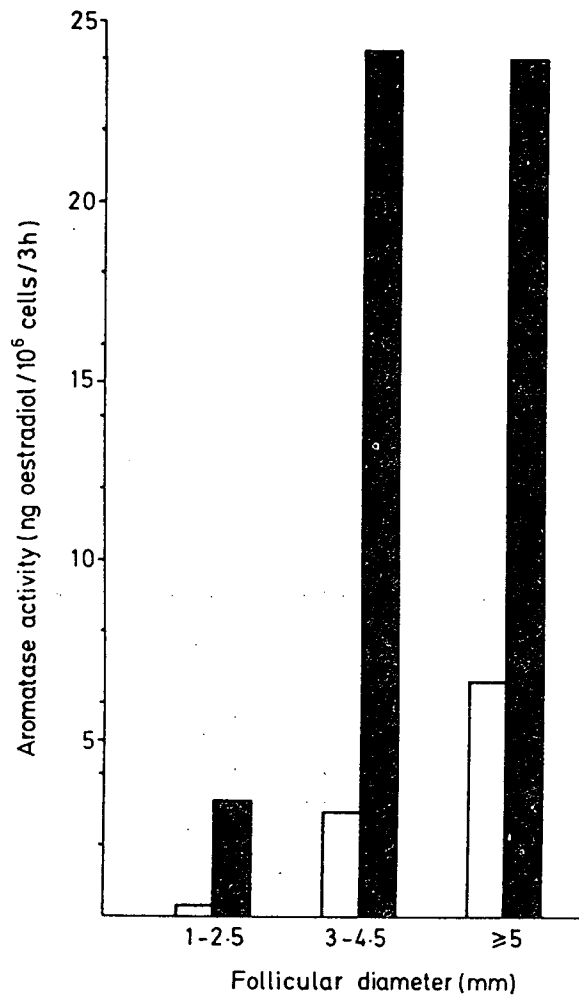


Fig. 2. Aromatase activity in granulosa cells pooled from healthy follicles (1-2.5 mm, 3-4.5 mm or  $\geq 5$  mm diameter) recovered from 4 ewes 'pulsed' with saline (white histograms) or 50  $\mu\text{g}$  ovine FSH (i.v.) once per h for 24 h (black histograms). Cells ( $\sim 1 \times 10^6$ /tube) were incubated with 1  $\mu\text{g}/\text{ml}$  testosterone for 3 h in Medium 199 + Heparin's buffer (20 mM) + Earle's salts and 5%  $\text{CO}_2$  in air (34).

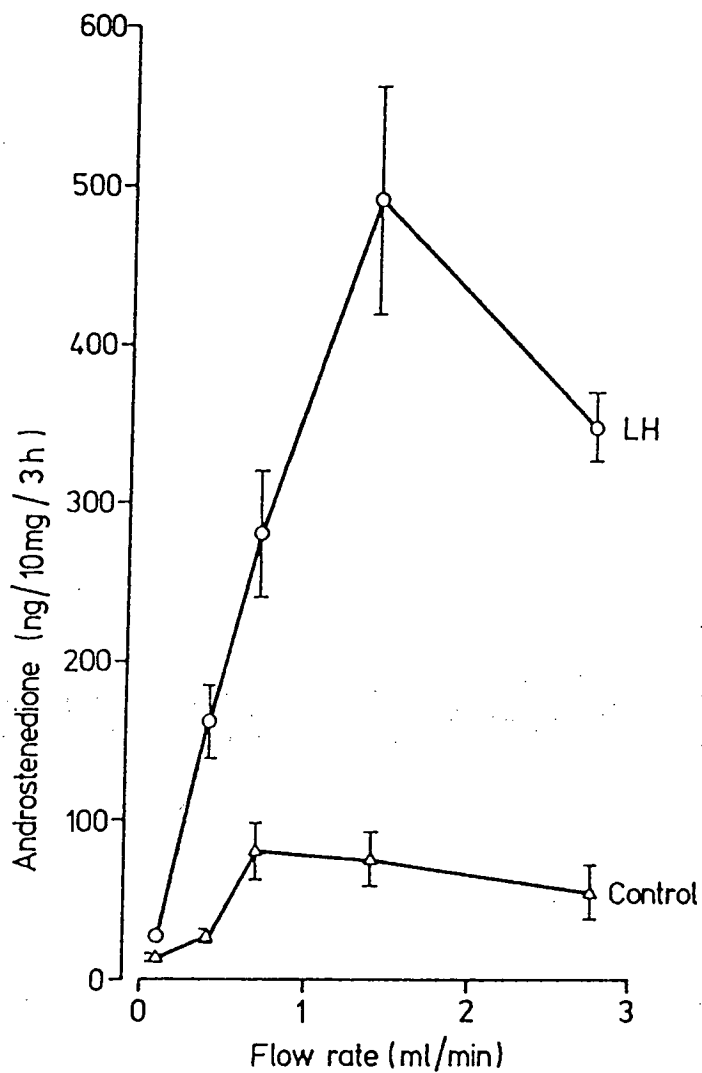


Fig. 3. The relationship between medium (Krebs-Ringer bicarbonate + 0.1% BSA) flow rate across bovine theca interna and the cumulative 3 h production-rate of androstenedione with and without exposure to LH (NIAMDD-bLH-4; 200 ng/ml). In these experiments LH was added for 20 min and the cumulative (3 h) output of steroid represents the total amount produced during and after LH stimulation. Each point was determined from 5-16 experiments. The thecae were recovered from preovulatory bovine follicles ( $\geq 10$  mm diameter; ref. 39).

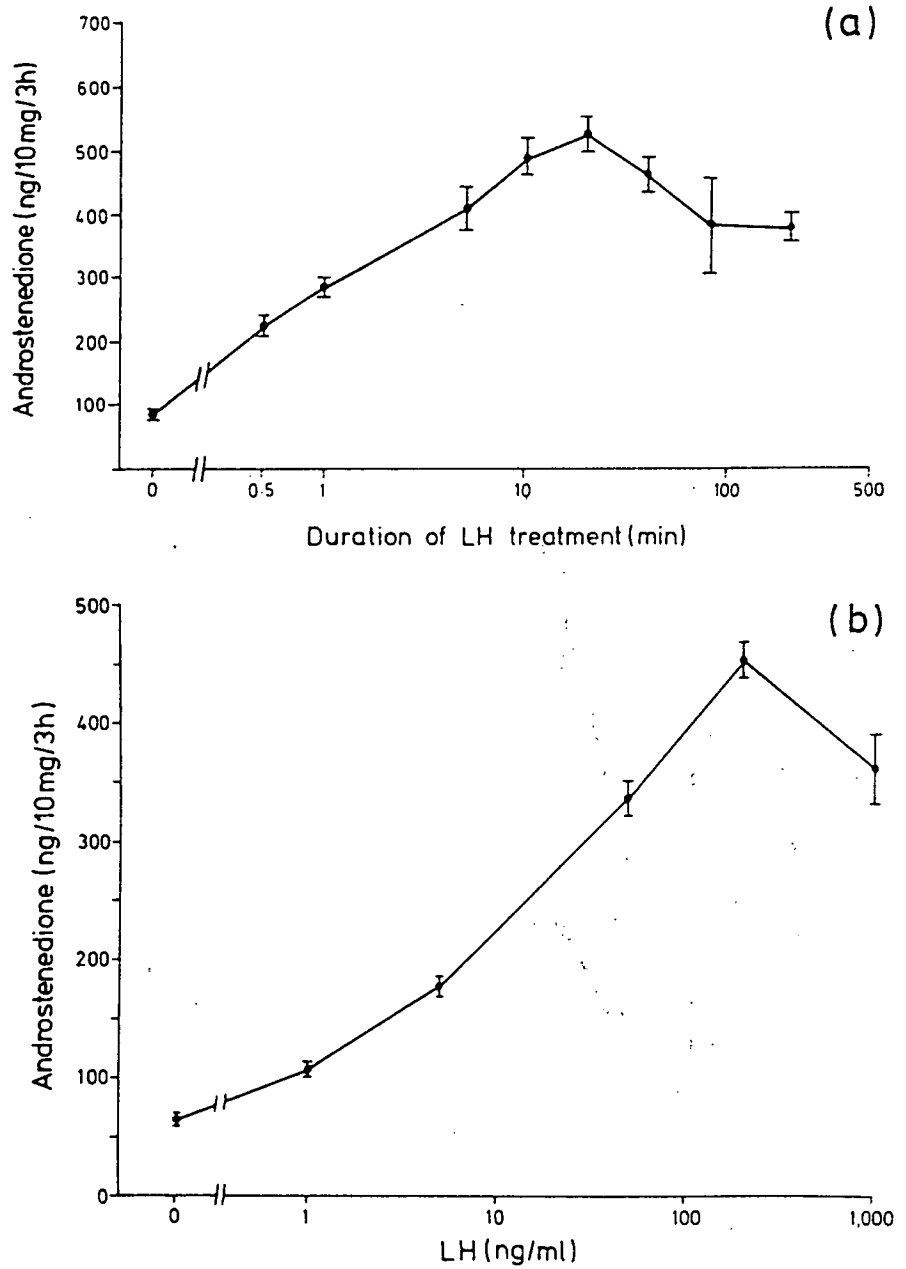


Fig. 4. The relationship between duration of LH treatment of theca interna (a) or concentration of LH (b) on the cumulative 3 h production of androstenedione (see Fig. 3). The bovine thecae were perfused with Krebs-Ringer bicarbonate 0.1% BSA at 1.4 ml at 37 °C. The results in (a) and (b) are the means  $\pm$  s.e.m from 6 and 7 experiments respectively (ref. 39).

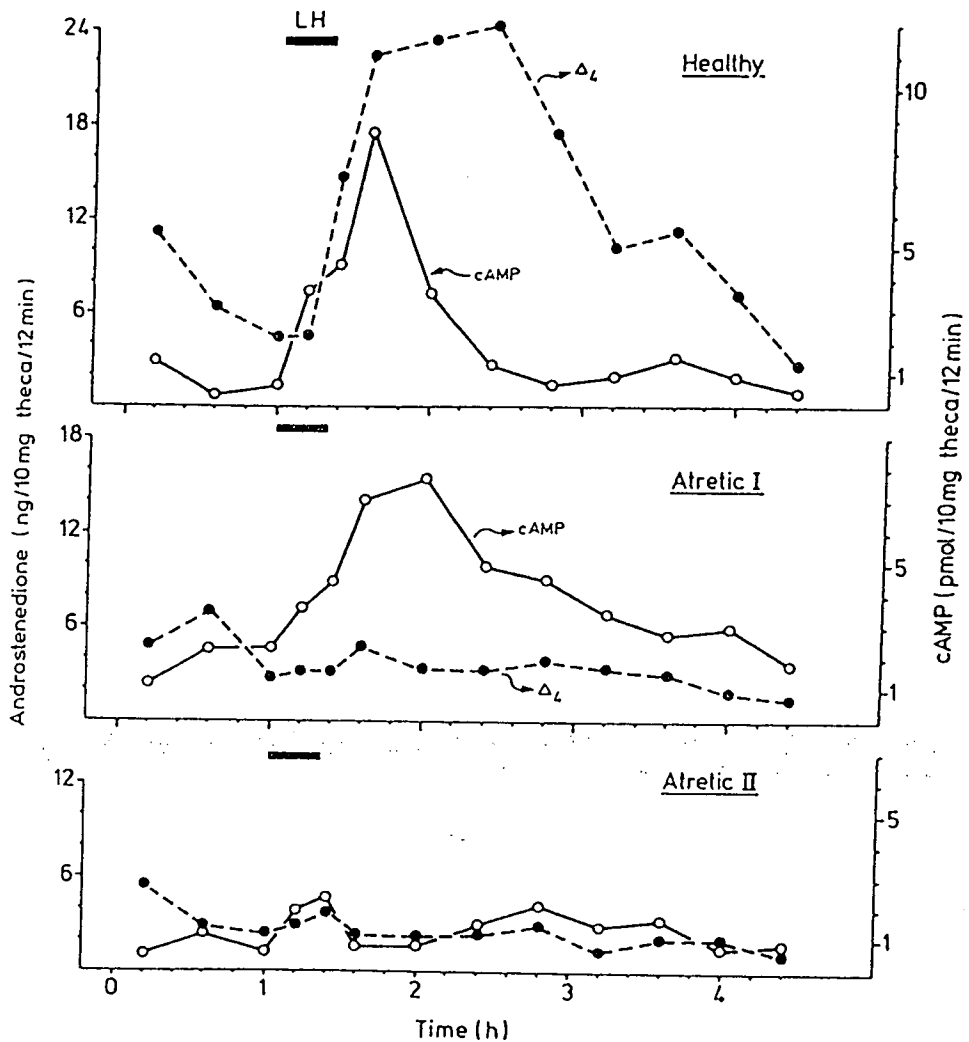


Fig 5. Theca interna output of cAMP and androstenedione before, during and after 20 min exposure to LH (NIAMDD-bLH-4; 200 ng/ml). The thecae interna were from bovine ovaries and perfused at 1-4 ml/min as described in Fig. 4. The healthy follicle was 11 mm in diameter with  $11.8 \times 10^6$  granulosa cells, no debris in follicular fluid, a healthy-looking oocyte and a follicular fluid oestradiol concentration of 280 ng/ml. The Atretic I follicle was 12.5 mm in diameter with  $5.8 \times 10^6$  granulosa cells, debris in follicular fluid, a healthy-looking oocyte, and an oestradiol concentration of 16 ng/ml. The Atretic II follicle was 11 mm in diameter with  $1.8 \times 10^6$  granulosa cells, debris in follicular fluid, a healthy looking oocyte and an oestradiol concentration of 2 ng/ml.

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Table 1. Effect of steroid-free bovine follicular fluid (FF) and/or FSH inoculation in sheep on the number of healthy follicles and their distribution with respect to follicular diameter. Values are means  $\pm$  s.e.m.

Treatment (n)	Total number of follicles ( $\geq 1$ mm diam.) per ewe	Percent of healthy follicles ( $\geq 1$ mm diam.) per ewe	No. of healthy follicles with respect to follicular diameter (mm)	
			$\geq 3$	1-2.5
Control (8)	34 <sup>a</sup> $\pm 4$	23 <sup>a</sup> $\pm 3$	3 <sup>a</sup> $\pm 0.5$	4 <sup>a</sup> $\pm 0.6$
FF (6)	31 <sup>a</sup> $\pm 4$	4 <sup>b</sup> $\pm 1$	0.2 <sup>b</sup> $\pm 0.2$	1 <sup>b</sup> $\pm 0.5$
10 $\mu$ g FSH (6)	29 <sup>a</sup> $\pm 3$	31 <sup>a</sup> $\pm 3$	2 <sup>a</sup> $\pm 0.3$	7 <sup>a</sup> $\pm 0.8$
10 $\mu$ g FSH + FF (4)	35 <sup>a</sup> $\pm 7$	21 <sup>a</sup> $\pm 7$	3 <sup>a</sup> $\pm 1$	4 <sup>a</sup> $\pm 1$
50 $\mu$ g FSH + FF (4)	39 <sup>a</sup> $\pm 5$	58 <sup>c</sup> $\pm 1$	18 <sup>c</sup> $\pm 4$	6 <sup>a</sup> $\pm 1$

FF (s.c., 5 ml) was injected at 24 h and 12 h before ovariectomy.

Ovine FSH (NIH-FSH-S12): FSH at dose indicated in parenthesis was injected i.v. once per hour for 24 h prior to ovariectomy.

Healthy follicles were defined as those with: a vascularized pink to red theca interna (at 10x magnification); no debris in follicular fluid, <25% the maximum number of granulosa cells for a given follicular diameter (see ref. 34) and a healthy-looking oocyte. Atretic follicles were those in which one or more of these criteria did not apply. For each column values with different superscripts are significantly different from one another, a,b or a,c or b,c =  $p < 0.01$  (ANOVA on log-transformed data).

Table 2. Contingency table showing distribution of granulosa cell aromatase activity and FSH-stimulated cAMP production in sheep

Aromatase activity (ng oestradiol/10 <sup>6</sup> cells/3 h)	cAMP (pmol/10 <sup>6</sup> cells/h) response		
	<10	10-19	≥20
<1	66	13	2
1-19	15	16	5
≥20	0	0	13

There was a significant relationship between aromatase activity and FSH-stimulated cAMP production ( $p < 0.001$ ; contingency table analysis).

Each value refers to the cAMP/aromatase response to 100 ng ovine FSH (NIH-FSH-S12) in granulosa cells from ovine follicles ( $\geq 1$  mm diameter).

Aromatase activity was determined by incubating cells ( $50 \times 10^3$ - $600 \times 10^3$ ) with 1  $\mu$ g/ml testosterone. Data from ref. 32.

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Table 3. Contingency table showing influence of follicular health on aromatase activity in ovine granulosa cells

Follicular health	Aromatase activity (ng oestradiol/10 <sup>6</sup> cells/3 h)	
	<1	1-5
Healthy	38	19
Atretic	48	10

Each value refers to the activity in cells from individual follicles (>1 mm diameter) recovered from Romney ewes during the oestrous cycle. Aromatase assay was performed using  $50 \times 10^3$ - $600 \times 10^3$  granulosa cells + 1  $\mu$ g testosterone in 1 ml medium for 3 h at 37 °C. There was a significant relationship between the health of the follicle and the level of aromatase activity in granulosa cells ( $P < 0.01$ ; contingency table analysis; data from ref. 34).



Table 4. Steroid concentrations in healthy and atretic bovine follicles during the oestrous cycle (geometric means and 95% confidence limits)

Follicle type or diameter (mm)	Steroid (ng/ml)			n
	Androstenedione	Testosterone	Oestradiol	
<b>HEALTHY</b>				
Dominant	11 <sup>a</sup> (9-15)	17 <sup>a</sup> (11-27)	60 <sup>a</sup> (25-105)	32
≥8	10 <sup>a</sup> (8-12)	14 <sup>a</sup> (10-19)	27 <sup>b</sup> (17-42)	56
5-7.5	10 <sup>b</sup> (15-25)	31 <sup>b</sup> (23-42)	7 <sup>c</sup> (5-9)	45
2-4.5	34 <sup>c</sup> (27-43)	59 <sup>c</sup> (47-74)	4 <sup>d</sup> (3-4)	77
<b>ATRETIC</b>				
≥8	9 <sup>a</sup> (5-15)	15 <sup>a</sup> (6-38)	2 <sup>a</sup> (2-2)	17
5-7.5	17 <sup>b</sup> (14-21)	23 <sup>a</sup> (18-30)	3 <sup>a</sup> (2-3)	73
2-4.5	35 <sup>c</sup> (32-37)	42 <sup>b</sup> (38-46)	3 <sup>a</sup> (2-3)	707

Numbers (in columns) with different superscripts are significantly different from one another a,b,c,d =  $p < 0.05$  (ANOVA). A dominant follicle was the follicle with the highest concentration of oestradiol in follicular fluid (ref. 35).

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Table 5.  $^{125}\text{I}$ -hCG binding constants and steroidogenic response to LH of bovine theca interna from healthy and atretic follicles

Follicle status	Kd (nm)	B <sub>(max)</sub> (fmol/mg protein)	Androstenedione (ng/10 mg theca/3 h)	
			-LH	+LH
Healthy	0.10±0.01	14±2	97±13	451±49
	(19)	(19)	(15)	
Atretic	0.12±0.02	12±1	80±12	70±17
	(18)	(18)	(5)	(5)

LH = NIAMDDK-LH-b4 (200 ng/ml) administered for 20 min. The medium was collected for 3 h during and after LH exposure. The perfusion-rate was 1.4 ml/min. Medium was Krebs-Ringer bicarbonate gassed with 50% O<sub>2</sub> in air at 37 °C.

Values are mean ± s.e.m. ( ) determinations (ref. 42).

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## Influence of follicular atresia on LH-induced cAMP and steroid synthesis by bovine thecae interna

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### Summary

The aim of the present study was to examine the interrelationships between the luteinizing hormone (LH) receptor, the LH-induced changes in adenosine cyclic 3', 5'-monophosphate (cAMP) and steroid synthesis in theca interna tissue of large antral follicles ( $\geq 8$  mm diameter) from oestrous cycling cows. Three distinct types of theca interna were identified (types I, II and III), all of which contained an LH receptor: type I was capable of secreting increased amounts of cAMP dehydroepiandrosterone, androstenedione and testosterone when exposed to LH; type II was capable of secreting increased amounts of cAMP and progesterone but not the androgens when exposed to LH; type III was incapable of cAMP or steroid synthesis when exposed to LH. Follicles with type I thecae contained: (a) a full complement of granulosa cells; (b) high intrafollicular concentrations of oestradiol; and (c) granulosa cells with a high capacity to metabolise testosterone to oestradiol. These follicles were considered to be non-atretic structures. Follicles with types III thecae contained: (a) fewer granulosa cells; (b) low intrafollicular concentrations of oestradiol; and (c) granulosa cells with a low capacity to metabolise testosterone to oestradiol. Moreover, follicles with type III thecae contained the highest concentrations of progesterone and the lowest concentrations of androstenedione and testosterone. These follicles were considered to be severely atretic structures. Follicles with type II thecae contained granulosa cell populations and progesterone, and androgen concentrations which were intermediate between those with thecae of types I and III. These follicles were considered to be at an intermediate stage of atresia. Collectively the data suggest that during follicular atresia the LH receptor characteristics in theca interna remain unchanged while the tissue first loses its capacity to respond to LH to synthesize androgens and then loses its ability to respond to LH to synthesize cAMP.

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Androstenedione is the major steroid synthesized by bovine theca interna tissue which is the source of androgen in the bovine ovary (McNatty et al., 1984a). In cattle, as in other species, the synthesis of androstenedione is regulated, at least in part, by luteinizing hormone (LH) (McCracken et al., 1969; Baird et al., 1976; McNatty et al.,

1984a, b, c). In bovine antral follicles, specific receptors for human chorionic gonadotrophin (hCG)/LH are present in the theca interna but not the theca externa or ovarian stroma (Henderson et al., 1984). It is known that hCG binds to the theca of most antral follicles irrespective of whether they are small, large, non-atretic or atretic (bovine,

Merz et al., 1981; rat, Uilenbroek et al., 1980; hamster, Oxberry and Greenwald, 1982; monkey, Zeleznik, 1982). However, not all theca interna have the capacity to synthesize androstenedione when exposed to hCG/LH (Henderson et al., 1984). Thus, the lack of available hCG/LH receptors is unlikely to be the reason why some thecae do not synthesize androstenedione. Although hCG/LH-induced androstenedione biosynthesis is mediated by adenosine cyclic 3', 5'-monophosphate (cAMP) (Erickson and Magoffin, 1983), the interrelationships between the LH receptor and the LH-induced changes in cAMP and steroidogenesis are poorly understood. The aim of the present study was to examine the aforementioned interrelationships in thecae interna of large antral follicles ( $\geq 8$  mm diameter) from oestrous cycling cows.

#### Materials and methods

##### *Bovine ovaries and preparation of thecae*

Ovaries were recovered from parous Angus cows, 2–7 years of age, on days –4 to 0 of the oestrous cycle (day 0 = day of oestrus). The thecae interna were recovered as described in detail elsewhere (McNatty et al., 1984a). Briefly, individual follicles were dissected from ovaries and freed of adhering tissues. The diameter of each follicle was recorded and then each was slit open to release the intrafollicular contents. The follicular fluid was aspirated and the follicle wall washed gently and repeatedly to remove the granulosa cells with medium (Medium A) consisting of Medium 199 with Earle's salts, L-glutamine (2 mM), gentamycin (50 mg/l), Hepes buffer (20 mM) (Gibco, Grand Island, NY, U.S.A.), sodium heparin (50 IU/ml) and 0.1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO, U.S.A.). The follicle wall was washed several more times and the total number of granulosa cells in all the medium washings quantified using a haemocytometer. For the purpose of this study, follicles ( $\geq 8$  mm diameter) with a pink to dark-red theca interna were retained for further investigation, whilst all other follicles were discarded. Previously we had shown that when the internal face of the follicle wall was colourless, the theca interna was either in an advanced stage of degeneration or

absent (McNatty et al., 1984a, b). Thecae interna, which were pink to dark red in colour, were relatively easy to separate from the external layer (i.e. theca externa), using finely pointed curved watchmaker forceps. After separating the theca interna the tissue was washed with several changes of Medium A. Theca interna prepared from such follicles was about 70–90% pure with the major contaminant being theca externa and/or stroma. There was minimal contamination by granulosa cells (5–25 cells/7  $\mu$ m section).

The thecae interna from each follicle ( $n = 22$  in total) were subdivided into fractions ( $\geq 10$  mg wet weight) for LH-induced cAMP and steroid perfusion studies. From 14 of the above 22 theca interna preparations, sufficient tissue was available for [ $^{125}$ I]hCG binding studies. For the binding studies the thecae interna were prepared as described in detail elsewhere (Henderson et al., 1984). Briefly the tissue from each follicle was homogenized by hand with an all-glass Potter-Elvehjem homogenizer in 0.01 M phosphate-buffered saline (pH 7.4) containing 100 mM sucrose and 5 mM  $\text{MgCl}_2$  (PBS). The homogenate was filtered through several layers of sterile gauze and aliquots of the filtrate stored frozen at  $-20^\circ\text{C}$  until the binding studies were performed.

##### *[ $^{125}$ I]hCG binding studies*

The data on [ $^{125}$ I]hCG binding to the thecae interna reported herein were part of a larger study published elsewhere (Henderson et al., 1984). hCG (CR121, 13 450 IU/mg) was iodinated to a specific activity of 30–50  $\mu\text{Ci}/\mu\text{g}$  using lactoperoxidase  $\text{H}_2\text{O}_2$  (Miyachi et al., 1972). Aliquots of the thecal homogenates (100–1000  $\mu\text{g}$  protein) were incubated in disposable plastic tubes with [ $^{125}$ I]hCG (0.5–50 ng) in a final volume of 1 ml PBS containing 0.5% egg albumin (Sigma Chemical Co., St. Louis, MO., U.S.A.). Incubations were performed at  $22^\circ\text{C}$  for 24 h. To separate receptor-bound from free [ $^{125}$ I]hCG, the tubes were centrifuged at  $4000 \times g$  for 30 min at  $4^\circ\text{C}$ . The pellets were washed with ice-cold incubation buffer and the radioactivity in the pellets determined following a second centrifugation. Non-specific binding was determined by co-incubating thecal homogenates with 10 IU/ml of unlabelled gonadotrophin (gonadotrophin LH, Paines & Byrne Ltd., Green-

ford, U.K.). Owing to the limited amounts of theca interna from individual follicles, incubations were performed in duplicate. The coefficient of variation between the replicate incubations was < 8%. The hCG/LH binding characteristics, namely the equilibrium dissociation constant ( $K_d$ ) and the maximum binding capacity ( $B_{max}$ ), were calculated from the Woolf equation (Haldane, 1957; Cressie and Keightley, 1981).

#### *Thecal perfusions*

The bovine thecae interna were perfused *in vitro* as described in detail elsewhere (McNatty et al., 1984a, b). Briefly, 10–25 mg of theca interna was placed into a sintered glass column ( $4 \times 0.7$  cm, Econo-column, Bio-Rad Laboratories, CA, U.S.A.) containing 50 mg of Sephadex G-25 and pre-washed for 1 h with a perfusion medium consisting of sterile Krebs-Ringer bicarbonate buffer containing 1% bovine serum albumin (w/v) which was gassed continuously with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. The theca on the columns was perfused at 37°C with the above perfusion medium at a flow rate of 1.4 ml/min with 12 min fractions being collected, 22 fractions (sample numbers 1–22) being collected over the perfusion period. LH (NIAMDD-bLH-4, 200 ng/ml) was introduced into the perfusion medium for 20 min after the theca had been perfused for 1 h (i.e. after sample number 5). At the end of the perfusion period the tissue was fixed for histological assessment of purity and the medium from each tube divided into aliquots and frozen until assayed for cAMP and steroids. For these studies, samples from tubes numbered 1, 3, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20 and 22 were assayed for cAMP and steroid.

#### *Granulosa cell oestradiol-17 $\beta$ assays*

Granulosa cells from each follicle were collected into Medium A, washed and resuspended in fresh Medium A without sodium heparin but containing sodium bicarbonate (0.85 g/l) so that the final cell concentration was  $1-6 \times 10^5$  cells/ml; 0.5 ml aliquots of these cell suspensions were placed in  $10 \times 75$  mm test tubes containing 0.5 ml of a solution of testosterone (2.7  $\mu$ mol/l) in Medium A with sodium bicarbonate or Medium A plus sodium bicarbonate, gassed with 5% CO<sub>2</sub> in

air, stoppered and then incubated for 3 h in a shaking water bath at 37°C. All assays were performed in duplicate. At the end of the incubation the assay tubes were snap-frozen to  $-70^\circ\text{C}$ . Subsequently the tubes were thawed, centrifuged and the supernatants assayed for oestradiol. Preliminary studies revealed that the rate of oestradiol formation using testosterone substrate was linear for the first 3 h for cells from all atretic and many non-atretic follicles. However, in some of the cells from the non-atretic follicles, the rate of metabolism was not linear after 2 h so that the true rate of oestradiol-17 $\beta$  synthesis was underestimated by up to 20%.

#### *cAMP and steroid assays*

**cAMP.** Ethanol (2 ml) was added to 0.4 ml of sample for cAMP determinations. The precipitated protein after centrifugation at  $2000 \times g$  for 15 min was discarded and the decanted supernatant evaporated to dryness under N<sub>2</sub>. The residue was redissolved in 0.05 M sodium acetate buffer (pH 6.2) and aliquots were assayed for cAMP using the New England Nuclear [<sup>125</sup>I]cAMP radioimmunoassay kit; the acetylation step was included. Tritiated cAMP was used to monitor the recovery of cAMP which following ethanolic extraction and recovery, was always > 90%. The results were normalised as pmoles cAMP/10 mg theca interna/12 min. The inter- and intra-assay coefficients of variation were < 12%.

**Steroids.** These were assayed directly without extraction using the same antisera and published radioimmunoassay (RIA) procedures for pregnenolone (Henderson et al., 1981) and progesterone, androstenedione, testosterone and oestradiol (McNatty et al., 1984a). The antiserum for dehydroepiandrosterone was purchased from Radioassay Systems Laboratories Inc. (Carson, CA, U.S.A.) and the RIA procedure for this steroid was that of Abraham et al. (1977). All antisera were highly specific for the steroid being investigated other than that for testosterone. The testosterone antiserum (WA-36) displayed 75% cross-reaction with 5 $\alpha$ - and 5 $\beta$ -dihydrotestosterone. However, preliminary studies on 10 pools of media from the perfused thecal tissues which had been subjected to celite column chromatography to separate testosterone from dihydrotestosterone revealed that

> 95% of the material was authentic testosterone. All samples for each steroid were measured in the same assay. The intra-assay coefficients of variation for all the above assays were < 10%.

#### Statistical treatment

The differences in the mean steroid concentrations in follicles at different stages of degeneration were examined by analysis of variance after log transformation.

The differences in mean cAMP or steroid levels at discrete time intervals during or after LH stimulation compared to the mean levels before stimulation were analysed by a Student's *t*-test procedure. For each experiment the mean basal level of cAMP or steroid before LH treatment was calculated and subtracted from each experimental observation with respect to time during or after LH stimulation. A *t*-test was then performed on these differences.

#### Results

##### LH-induced cAMP and steroid synthesis in thecae interna

The cAMP and androstenedione responses by thecae interna were separable into three distinct types (I, II and III; Fig. 1). From type I thecae the cAMP output increased significantly ( $p < 0.05$ ) within the first 12 min of LH exposure and peaked 36–48 min later. Thereafter cAMP output returned gradually to basal levels around 3 h later. Androstenedione output from type I thecae increased significantly within 12–24 min of LH exposure and peaked 48–96 min later. Thereafter the androstenedione output declined gradually, although the output was still 3-fold greater ( $p < 0.01$ ) than basal values some 3 h after removal of the LH stimulus.

LH-stimulated thecae of type II responded by producing a significant increase in cAMP 24–36 min after LH exposure ( $p < 0.01$ ) with the peak cAMP output occurring after 36–48 min (Fig. 1). Overall basal cAMP output was 3-fold lower from type II thecae compared to that from type I and the peak cAMP output after LH exposure in type II thecae was also 3-fold lower than from type I. From type II thecae there was no discernible change in androstenedione output following LH

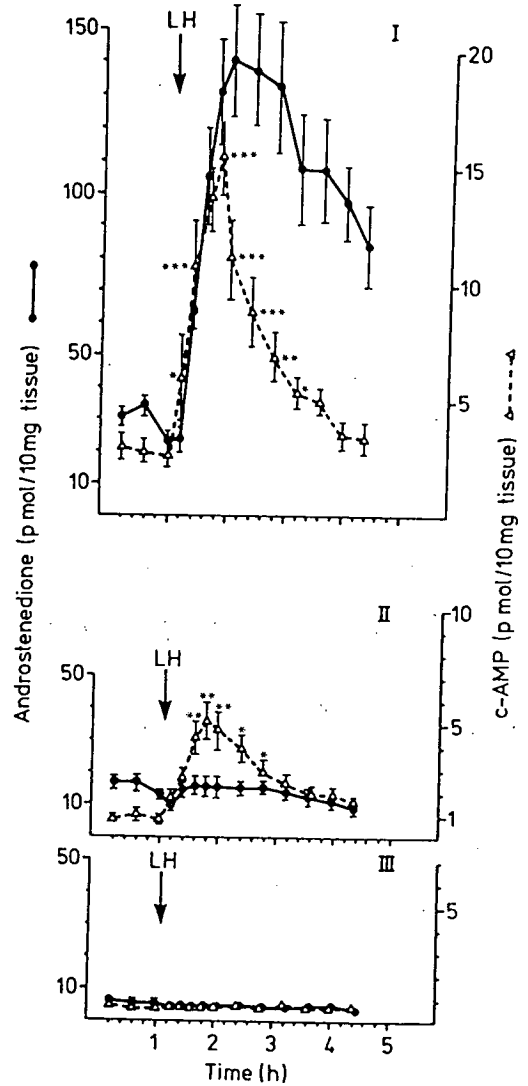


Fig. 1. Changes in the outputs of androstenedione (●—●) and cAMP (Δ—Δ) from perfused theca interna tissue of types I ( $n = 9$  theca), II ( $n = 6$ ) and III ( $n = 7$ ) during and after LH stimulation (NIAMDD-bLH-4, 200 ng/ml for 20 min). Arrow indicates time when LH infusion began. Vertical bars,  $\pm$  SEM. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$  with respect to mean level before LH treatment. All androstenedione outputs from 24 min after the LH treatment were significantly greater ( $p < 0.01$ ) than before treatment. The thecae were perfused at 1.4 ml/min with Krebs-Ringer bicarbonate + 0.1% bovine serum albumin at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>.

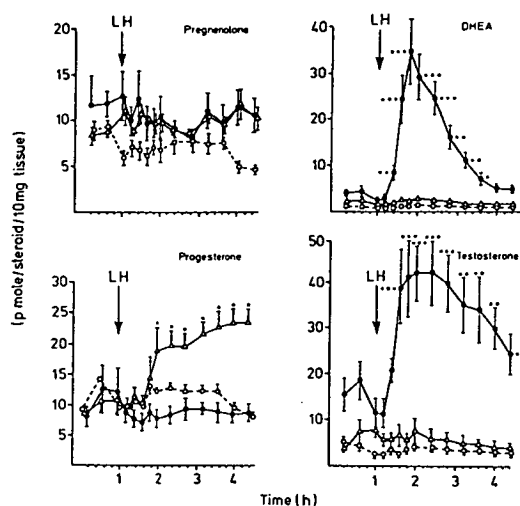


Fig. 2. Changes in the outputs of pregnenolone, progesterone, dehydroepiandrosterone (DHEA) and testosterone from perfused theca interna tissue of type I ( $n = 9$  theca), II ( $n = 6$ ), III ( $n = 7$ ) during and after LH stimulation (NIAMDD-bLH-4, 200 ng/ml for 20 min). Arrow indicates time when LH infusion began. Vertical bars =  $\pm$  SEM.  $*** p < 0.001$ ,  $** p < 0.01$ ,  $* p < 0.05$  with respect to mean level before LH treatment. The thecae were perfused at 1.4 ml/min with Krebs-Ringer bicarbonate + 0.1% bovine serum albumin at 37°C. The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>.

exposure. Thecae of type III did not increase their outputs of cAMP or androstenedione in response to LH. The basal levels of cAMP in type III thecae

were 0.5-fold of those in type II.

The LH-induced outputs of pregnenolone, progesterone, dehydroepiandrosterone and testosterone from thecae interna of types I, II and III are shown in Fig. 2. From type I thecae a significant increase in dehydroepiandrosterone and testosterone but not in pregnenolone or progesterone output was noted following exposure of the thecae interna to LH. From type II thecae a significantly increased output of progesterone, but not of pregnenolone, dehydroepiandrosterone or testosterone, was noted following exposure to LH. From type III thecae no significant change in any of the above steroids was noted after exposure to LH.

#### [<sup>125</sup>I]hCG binding to the theca interna

The equilibrium dissociation constants ( $K_d$ , nM) and maximum binding capacities ( $B_{max}$ , fmoles/mg protein) for [<sup>125</sup>I]hCG binding to thecal types I, II and III were as follows: type I ( $n = 5$  theca pools),  $K_d = 0.11 \pm 0.02$ ,  $B_{max} = 16 \pm 4$ ; type II ( $n = 3$ ),  $K_d = 0.11 \pm 0.02$ ,  $B_{max} = 14 \pm 3$ ; type III ( $n = 6$ ),  $K_d = 0.10 \pm 0.01$ ,  $B_{max} = 11 \pm 1$ . No significant differences in the hCG binding characteristics were noted between any of the thecal types.

#### Relationships between thecal types I, II and III and other follicle characteristics

These data are summarized in Table 1. Follicles with type I thecae contained significantly more

TABLE 1

RELATIONSHIPS BETWEEN THECAL TYPES I, II AND III, FOLLICLE DIAMETER, GRANULOSA CELL NUMBER AND CAPACITY TO SYNTHESIZE OESTRADIOL-17 $\beta$  AND STEROIDS IN FOLLICULAR FLUID

Thecal type	Follicle diameter (mm)	Granulosa cell		Steroids in follicular fluid [results are geometrical means (and 95% confidence limits) in nmoles/l]				n
		Number $\times 10^{-6}$	Rate of oestradiol synthesis (pmoles/ $10^6$ cells/3 h)	Progesterone	Androstenedione	Testosterone	Oestradiol	
I	10.5 $\pm$ 0.5	11.1 $\pm$ 0.6 <sup>a</sup>	338 $\pm$ 68 <sup>a</sup>	57 <sup>a</sup> (44-76)	43 <sup>a</sup> (38-41)	83 <sup>a</sup> (52-139)	268 <sup>a</sup> (136-570)	9
II	10.4 $\pm$ 1.2	3.2 $\pm$ 0.2 <sup>b</sup>	7 $\pm$ 2 <sup>b</sup>	245 <sup>c</sup> (146-438)	39 <sup>a,b</sup> (10-158)	73 <sup>a,b</sup> (21-233)	7 <sup>b</sup> (7-11)	6
III	10.4 $\pm$ 1.2	1.2 $\pm$ 0.4 <sup>c</sup>	5 $\pm$ 12 <sup>b</sup>	695 <sup>d</sup> (323-1488)	18 <sup>b</sup> (10-39)	28 <sup>b</sup> (17-45)	7 <sup>b</sup> (7-11)	7

Values for follicle diameter and granulosa cells are means  $\pm$  SEM. Numbers in columns with a different alphabetical superscript are significantly different from one another: a vs. b,  $p < 0.01$ ; a vs. c,  $p < 0.01$ ; a vs. d,  $p < 0.01$ ; b vs. c,  $p < 0.05$ ; c vs. d,  $p < 0.05$ .



granulosa cells ( $p < 0.01$ ) than follicles with type II thecae, which in turn contained more granulosa cells ( $p < 0.01$ ) than follicles with type III thecae. Follicles with thecal type I contained granulosa cells with significantly greater capacity to metabolise testosterone to oestradiol-17 $\beta$  than was present in cells from follicles with thecal types II ( $p < 0.01$ ) and III ( $p < 0.01$ ). Follicles with type I thecae contained the lowest concentrations of progesterone and the highest concentrations of oestradiol in follicular fluid compared to those with thecal types II and III. In contrast, follicles with thecal type III contained the highest concentrations of progesterone and the lowest concentrations of androstenedione and testosterone.

#### Discussion

These data have identified three distinct types of theca interna (I, II and III) from bovine follicles. Type I theca contained an LH receptor coupled to adenylate cyclase and was capable of synthesizing dehydroepiandrosterone, androstenedione and testosterone (i.e. androgens) in response to LH; type II theca contained an LH receptor coupled to adenylate cyclase and was capable of synthesizing progesterone but not the androgens in response to LH; and type III theca contained an LH receptor which was uncoupled from adenylate cyclase and incapable of synthesizing steroid in response to LH. The evidence suggests that follicles with type I thecae were typically non-atretic structures since they contained: (a) their full complement of granulosa cells; (b) high intrafollicular concentrations of oestradiol; and (c) granulosa cells with a high capacity to metabolise testosterone to oestradiol (McNatty et al., 1984a, b). In contrast, follicles with type III thecae were typical of those undergoing atresia since they contained: (a) low populations of granulosa cells; (b) low concentrations of oestradiol; and (c) granulosa cells with low oestradiol synthetic activity. Follicles with type II thecae appeared to be at an intermediate stage of atresia since they were deficient in granulosa cells (McNatty et al., 1984a, b), although they contained more cells than were present in follicles with type III thecae. Moreover, the progesterone levels in follicular fluid in follicles with type II thecae were intermediate between

those with type I and III thecae while the levels of androstenedione and testosterone varied between those in types I and III. It appears that the three types of theca were present in follicles at progressive stages of atresia from type I to type III. If this is so, then it seems likely that the LH receptor-adenylate cyclase-steroidogenic system in theca interna uncouples during atresia in the reverse sequence so that described for their coupling during follicular maturation (Erickson and Magoffin, 1983).

The basal cAMP output from type I thecae was 3-fold greater than that from type II thecae (Fig. 1). The significant LH-induced outputs of dehydroepiandrosterone, androstenedione and testosterone from type I thecae but not from type II thecae indicate a greater level of C<sub>21</sub> steroid 17 $\alpha$ -hydroxylase and 17-20 desmolase enzyme activity in the former compared to the latter. Perhaps a reduction in basal cAMP synthesis during early atresia contributes initially to a reduction in C<sub>21</sub> steroid 17 $\alpha$ -hydroxylase and 17-20 desmolase but not  $\Delta^5$ -3 $\beta$ -o1 dehydrogenase/ $\Delta^5$ -isomerase enzyme activity, thereby causing an increase in thecal progesterone output as was observed in type II. Perhaps also the latter enzyme activities are reduced when basal cAMP levels fall still further, as was observed in thecal type III compared to those from thecal type II. However, the changes observed in basal and LH-stimulated cAMP secretions from thecal tissue during follicular atresia cannot be attributed to alterations in the hCG/LH receptor, since no changes in the hCG/LH binding characteristics were observed (see also Henderson et al., 1984).

Follicles with types II and III thecae contained significantly higher concentrations of progesterone than those with type I. These findings, together with those of McNatty et al. (1984a, b), suggest that during atresia there is first a loss of granulosa cell oestradiol synthetic activity followed by a reduction in thecal androgen synthesis concomitant with an increased output of progesterone. A similar reduction in androgen synthesis and an increased output of progesterone was also noted by Uilenbroek et al. (1980) and Braw and Tsafiri (1980) in studies of preovulatory follicles in rats which had been pretreated with pentobarbitone to block the preovulatory LH surge. However, in the

rat, in contrast to the cow, the loss of oestradiol synthetic activity was preceded by a reduction in thecal androgen synthesis. What then are the cellular origins of the progesterone in atretic bovine follicles? Our studies show that type II thecae interna, but not types I or III, were capable of contributing to the increase in follicular progesterone concentrations. However, granulosa cells in follicles with types II and III thecae may also synthesize progesterone. These cells synthesize 3- and 5-fold more progesterone respectively in vitro within the first 24 h of explantation, compared to those from follicles with type I thecae (K.M. Henderson, P. Smith and K.P. McNatty, unpublished data).

In conclusion, these data suggest that during follicular atresia the LH receptor characteristics in theca interna remain unchanged while the tissue first loses its capacity to respond to LH to synthesize androgens and then loses its ability to respond to LH to synthesize cAMP.

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## STEROIDOGENESIS *IN VITRO* BY THE THECA INTERNA

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Ovarian thecal tissue is first recognisable as a concentric stromal sheath around growing preantral follicles. During antrum formation, two distinct layers of theca become apparent and these are referred to as the theca interna and externa. The theca interna (i.e., the layer closest to the granulosa cells) contains fibrocytic and steroidogenic cells (Fig. 1), whereas the theca externa contains predominantly fibrocytic and myoid cells and few, if any, steroidogenic cells (22). The theca interna is an extensively vascularized tissue with a particularly dense network of capillaries close to the basal lamina of the membrana granulosa (Fig. 1).

The thecal and/or stromal tissues are thought to be a major source of androstenedione and to a lesser extent of testosterone (7, 15, 20). These two androgens serve, equipotently, as substrates for follicular oestradiol synthesis (11, 14). The synthesis and secretion of androstenedione and testosterone are regulated, at least in part, by luteinizing hormone (LH) (4, 12, 15, 20, 24). Receptors for LH have been localized in stromal and/or thecal tissues as well as in the membrana granulosa and corpora lutea (1, 5, 6, 21, 25). Thus LH stimulation of thecal and/or stromal tissues may ultimately influence oestradiol output by regulating the supply of androgen substrate.

In most species, LH secretion is characterized by discrete episodic discharges of the hormone. Each LH secretory episode (excluding the preovulatory LH surge) results in a temporary increase in the plasma concentrations of LH for between 15 and 60 min. These LH secretory episodes occur at high frequency (i.e., >1 every 2 h) during the follicular phase and at a low frequency (i.e., <1 every 2 h) during much of the prepubertal period, the luteal phase, the early post-partum interval, pregnancy or other periods of anovulation (2, 3, 8, 9, 18, 19, 23). The increased LH secretory frequency during the follicular phase results in a significant (1.5-5 fold) increase in the basal levels of LH compared to those observed during the luteal phase. In sheep, it is known that the aforementioned increase in the plasma concentrations of LH before the preovulatory LH surge is an essential prerequisite for preovulatory follicular

maturation (13, 17). In view of the episodic nature of LH secretion and the extensive blood capillary system in the follicle wall, it would seem reasonable to expect the endocrine milieu in the theca interna to be fluctuant, particular around the time of an LH secretory episode.

The purpose of this review is to summarise some recent observations in the cow and sheep on the characteristics of the LH receptor in the theca interna and the effects of medium flow, LH concentration and follicular health on steroidogenesis by the theca interna *in vitro*.

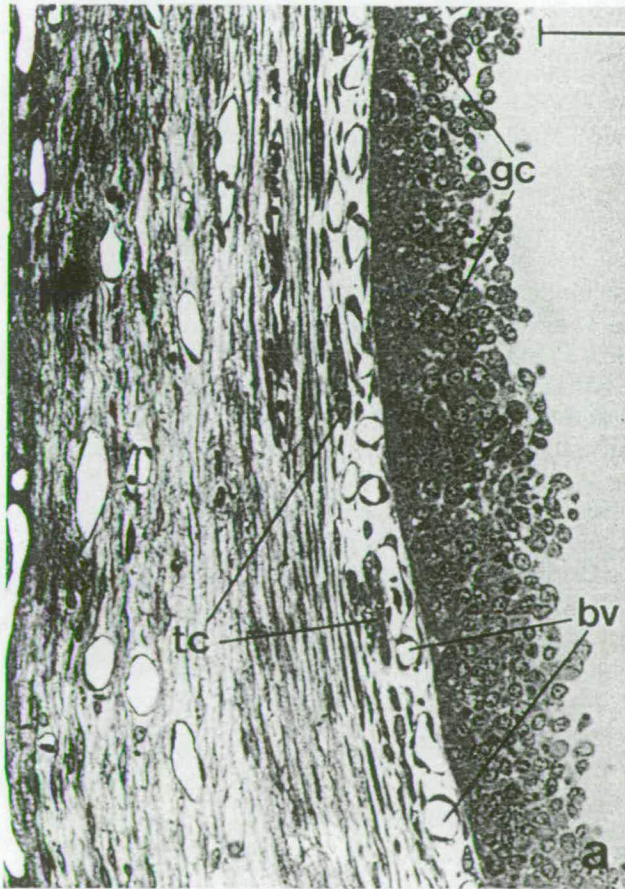
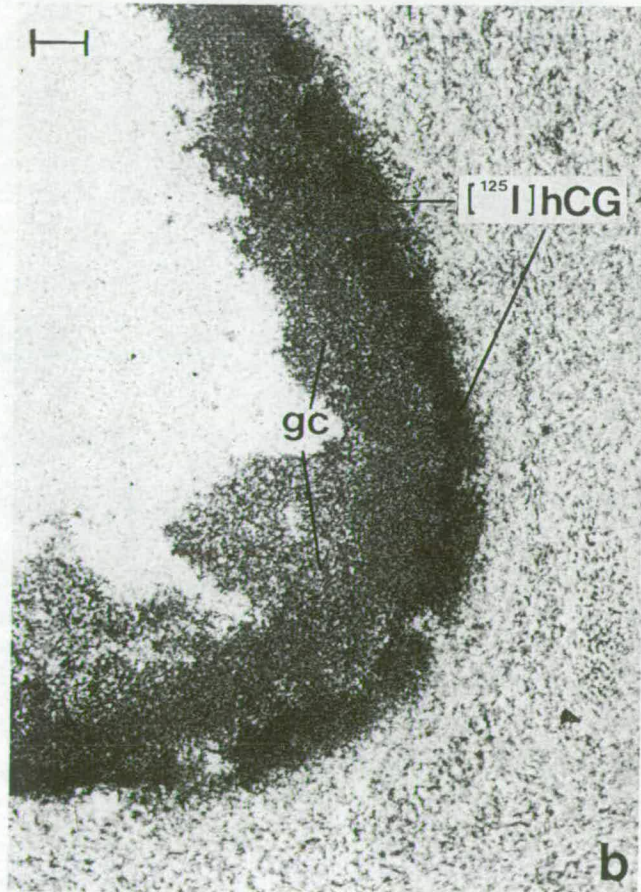


Fig. 1. Cross-sectional views of: (a) an ovine antral follicle highlighting the extensive blood capillary network adjacent to the basement membrane (bar represents 50  $\mu\text{m}$ ); (b) specific [ $^{125}\text{I}$ ]hCG binding in ovine theca interna adjacent to the basement membrane (bar represents 100  $\mu\text{m}$ ) and; (c) a typical sample of the purity of theca interna that was perfused (bar represents 50  $\mu\text{m}$ ). The theca interna tissue was recovered from a  $\geq 10$  mm healthy bovine follicle. gc=granulosa cells; bv=blood vessels; te/s - theca externa/stroma; and tc=theca interna cells.

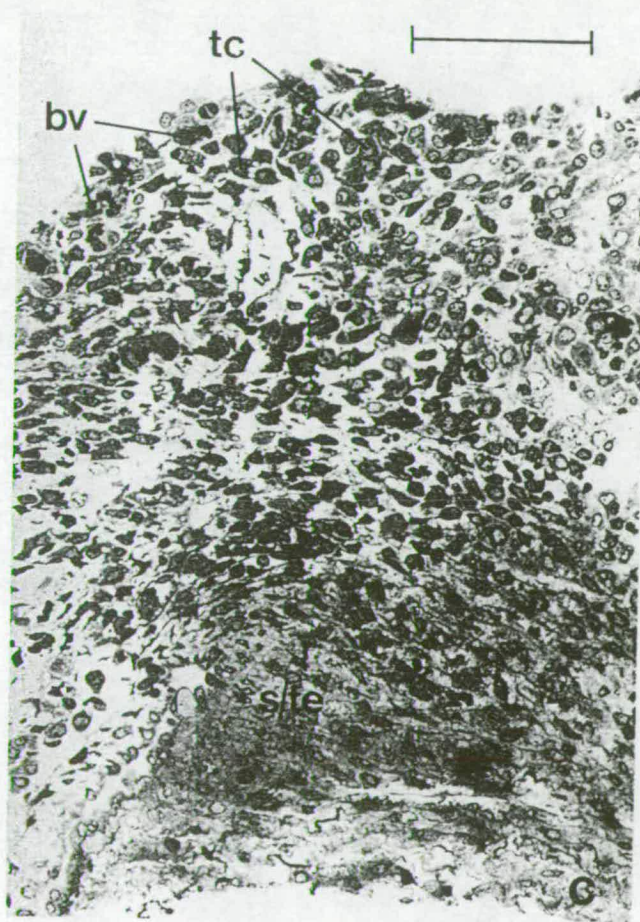
**Recovery and purity of isolated thecal tissue**

The recovery by microdissection of theca interna from bovine and ovine follicles has been described in detail elsewhere (15, 16). The thecae interna described herein were relatively free of granulosa cells (Fig. 1). For example, in each 5-6  $\mu\text{m}$  section of tissue, the geometric mean number (and 95% confidence limits) of contaminating granulosa cells was 2 (0-4) ( $n=41$  follicles) for ovine theca interna and 3 (0-6) ( $n=40$ ) for bovine theca interna. In general, the bovine and ovine thecae interna were estimated to be  $\sim 70\%$  and  $\sim 50\%$  pure respectively with the major contaminant being stroma and/or theca externa (Fig. 1). none of the data reported here were adjusted for purity of theca interna.



## LH receptors in thecal and/or stromal tissue

Autoradiographic analysis of [ $^{125}$ I] human chorionic gonadotrophin (hCG; CR121, 13450 iu/mg) binding to serial frozen sections (10  $\mu$ m) of ovine ovaries indicated that the hCG/LH receptive thecal cells were concentrated in the vascularized region immediately adjacent to the basement membrane (Fig. 1).



The equilibrium dissociation constants ( $K_d$ ) and maximum binding capacities ( $B_{max}$ ) for [ $^{125}$ I]hCG binding to bovine and ovine theca interna were calculated from Woolf plots after incubation of the tissues for 20 h at 22  $^{\circ}$ C to achieve equilibrium (10). The mean  $\pm$  s.e.m.  $K_d$  values for specific [ $^{125}$ I]hCG binding to bovine and ovine theca interna were  $0.09 \pm 0.01$  nM ( $n=12$  pools) and  $0.10 \pm 0.01$  nM ( $n=14$  pools) respectively. The mean  $\pm$  s.e.m.  $B_{max}$  values of [ $^{125}$ I]hCG to bovine and ovine theca interna were  $12 \pm 3$  fmoles/mg protein ( $n=12$  pools) and  $10 \pm 2$  fmoles/mg protein

(n=14) respectively. The binding constants for [ $^{125}$ I]hCG binding to the theca interna from either bovine or ovine follicles were unaffected by follicular diameter (cow, 4-15 mm diameter; ewe, 1-7.5 mm diameter). The binding of [ $^{125}$ I]hCG to theca interna could be displaced by unlabelled hCG and LH whereas follicle stimulating hormone, thyroid stimulating hormone, growth hormone, prolactin and gonadotrophin-releasing hormone were all ineffective (ref. 10; unpublished data). No specific [ $^{125}$ I]hCG binding was observed in thecae externa or stroma in either the cow (n= 6 pools of theca interna or stroma) or sheep (n=6 pools of theca externa or stroma). In these studies the thecal tissue (interna/externa) and stroma were recovered from sheep and cattle during the luteal phase of the oestrous cycle.

The binding constants for specific [ $^{125}$ I]hCG binding to ovine theca interna and corpora lutea during the luteal and follicular phases of the oestrous cycle are shown in Table 1. During the luteal phase, the K<sub>d</sub> and B<sub>max</sub> values for corpora lutea were 4.4 to 4.8 fold higher than those for theca interna. During the follicular phase, the binding constants for the theca interna were similar to those for luteal phase corpora lutea. Also, during the follicular phase, the LH/hCG binding capacity of the theca interna exceeded that of regressing corpora lutea more than 15 fold and that of similar-sized follicles during the luteal phase, more than 5 fold (Table 1).

Table 1. Binding constants for specific [ $^{125}$ I]hCG binding to the ovine theca interna and corpora lutea during the luteal and follicular phases of the oestrous cycle. Values are means  $\pm$  s.e.m.

Tissue	K <sub>d</sub> (nM)		B <sub>max</sub> (fmol/mg protein)	
	Luteal	Follicular	Luteal	Follicular
Theca interna	0.10 $\pm 0.01$	0.37 $\pm 0.11$	8 $\pm 1$	55 $\pm 11$
Corpora lutea	0.44 $\pm 0.05$	0.25 $\pm 0.03$	38 $\pm 5$	3 $\pm 0.5$

K<sub>d</sub>=equilibrium dissociation constant, B<sub>max</sub> = maximum binding capacity. The binding constants were calculated from Woolf plots of specific [ $^{125}$ I]hCG binding to homogenates of corpora lutea (n=3, luteal phase; n=3 follicular phase) and thecal homogenates from  $\geq 3.5$  mm diameter non-atretic follicles (n=6, luteal phase; n=3 follicular phase). Follicular phase tissue were recovered 36 h after an injection of 125  $\mu$ g cloprostenol (a PGF<sub>2 $\alpha$</sub>  analogue; ICI-Tasman Vaccine Laboratory, Upper Hutt, N.Z.) to sheep on Day 8 of the oestrous cycle; luteal phase tissues were recovered on Day 8 of the oestrous cycle. For details of methodology relating to binding studies see ref. 10.

### The site of androstenedione synthesis in the follicle wall

The tissue concentrations and cumulative production rates for isolated bovine and ovine theca interna, theca externa, stroma and granulosa cells



with or without exposure to LH have been compared using an *in vitro* perfusion system (15). The evidence from both species was that the theca interna, with or without exposure to LH, was the major source (>95%) of androstenedione with only minimal amounts originating from the other tissues (Fig. 1; 15, 16).

#### Comparison of steroids synthesized by theca interna *in vitro*

The respective cumulative (3 h) outputs (mean  $\pm$  s.e.m.) of steroids from LH (NIAMDD-bLH-4)-primed theca interna of three preovulatory follicles ( $\geq 10$  mm diameter) recovered from cows at oestrus were as follows: progesterone,  $24 \pm 5$ ; dehydroepiandrosterone,  $121 \pm 5$ ; androstenedione,  $1013 \pm 16$ ; testosterone,  $315 \pm 20$  and oestradiol,  $24 \pm 4$  ng/10 mg theca interna/3 h. The term cumulative outputs, refers to the total amount of steroid produced per 10 mg tissue during and after 20 min exposure to LH. The

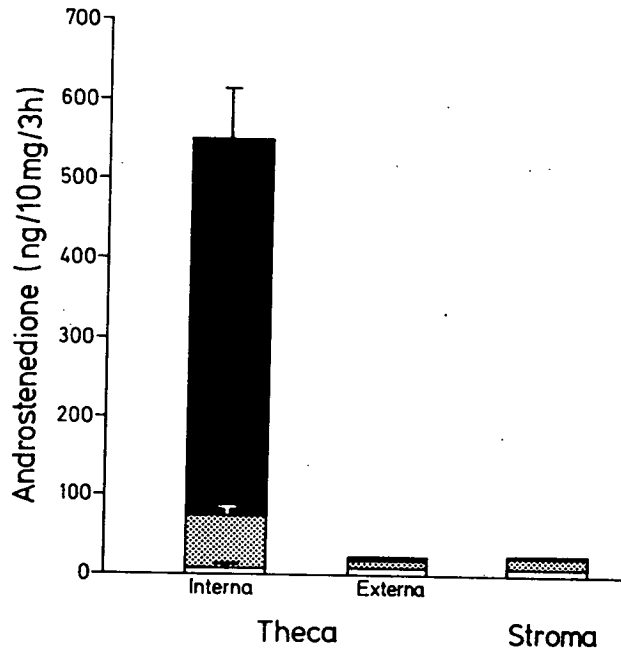


Fig. 2. Cumulative (3 h) production (mean  $\pm$  s.e.m.) of androstenedione by theca interna, theca externa and stroma during and after exposure to LH (NIAMDD-bLH-4; 200 ng/ml) for 20 min (black histograms) or in medium alone (stippled histograms) as well as the amounts (ng/10 mg tissue) present at time 0 (white histograms). For the theca externa and stroma, the s.e.m. for all histograms were  $< 1$  ng/10 mg/3 h. Thecae were recovered from non-atretic follicles 10.0-11.5 mm diameter of 3 cows on Day -1 ( $n=2$ ) or 0 ( $n=1$ ) of the oestrous cycle (Day 0 = day of oestrus). The stromal tissues were recovered from the regions adjacent to the above follicles. The medium which was perfused at 1.4 ml/min was Krebs-Ringer bicarbonate + 0.1% bovine serum albumin (BSA). The medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub> and perfused through the tissue at 37°C. Data from (4) with permission.

thecae interna were perfused at a rate of 1.4 ml/min with Krebs-Ringer bicarbonate + 0.1% BSA at 37°C; the medium was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. Of the 5 steroids measured, the androstenedione, testosterone, dehydroepiandrosterone fractions constituted 68%, 21% and 8% respectively of the total production while progesterone and oestradiol together represented only 3% of the total output.

#### **Effect of medium flow-rate, LH concentration and duration of LH exposure on androstenedione synthesis by thecae interna**

The androstenedione output by bovine thecae interna was enhanced ( $p < 0.001$ ) 45 fold when the rate of medium flow across the LH-primed tissue was increased from 0 to 1.4 ml/min (Fig. 3). Similarly, the androstenedione output from ovine theca increased >20 fold from 5 ng/10 mg theca/3 h in static culture to 103 ng/10 mg/3 h when perfused at 1.4 ml/min. Under the experimental conditions employed (15, 16), the androstenedione outputs were not enhanced when the flow-rates were increased beyond 1.4 ml/min.

When bovine theca interna was perfused at 1.4 ml/min and exposed to LH for 20 min, the peak output of androstenedione was achieved with an LH concentration of 200 ng/ml and the half-maximum output with an LH concentration of 9 ng/ml (Fig. 3). Even at the lowest LH concentration used (i.e., 1 ng/ml), the output of androstenedione was significantly greater than that in the control ( $p < 0.01$ ; Fig. 3). When bovine theca interna was perfused at 1.4 ml/min and exposed to 200 ng/min of LH for varying periods of time, peak androstenedione output occurred after 20 min LH exposure but the half-maximum output was achieved after only 45 seconds (Fig. 3). When a 20 min LH pulse of 1, 10 or 200 ng/ml was delivered to theca interna, an increase in androstenedione secretion was observed within 12 to 24 min, and peak steroid outputs were sustained for 36, 60 and  $\geq 84$  min respectively (Fig. 4).

The sensitivity of theca interna to an LH pulse was influenced by its previous exposure to LH. For example, when theca interna (perfused at 1.4 ml/min with Krebs-Ringer bicarbonate + 0.1% BSA) was exposed to a 20 min LH pulse of 10 ng/ml, 4.8 h after an earlier 20 min LH pulse of 1, 10 or 200 ng/ml, the respective mean  $\pm$  s.e.m. androstenedione output ( $n=3$ ) to the second pulse were  $106 \pm 13$ ,  $36 \pm 4$  and 0% of the output to a control LH pulse of 10 ng/ml administered 4.8 h after exposing thecae to an LH-free medium. The full recovery of thecal LH responsiveness 4.8 h after the low amplitude LH pulse (i.e., 1 ng/ml) was probably due to the fact that the steroidogenic response to the first pulse had been completed some 60-90 min earlier, whereas, when the first LH pulse amplitude was  $\geq 10$  ng/ml the theca was still actively secreting androstenedione to this pulse 5 h later (Fig. 4).

When theca interna was perfused with LH at a constant rate for 4.8 h before receiving a 20 min pulse of 10 ng/ml, a significant negative relationship was found between the LH concentration in the perfusion medium and the

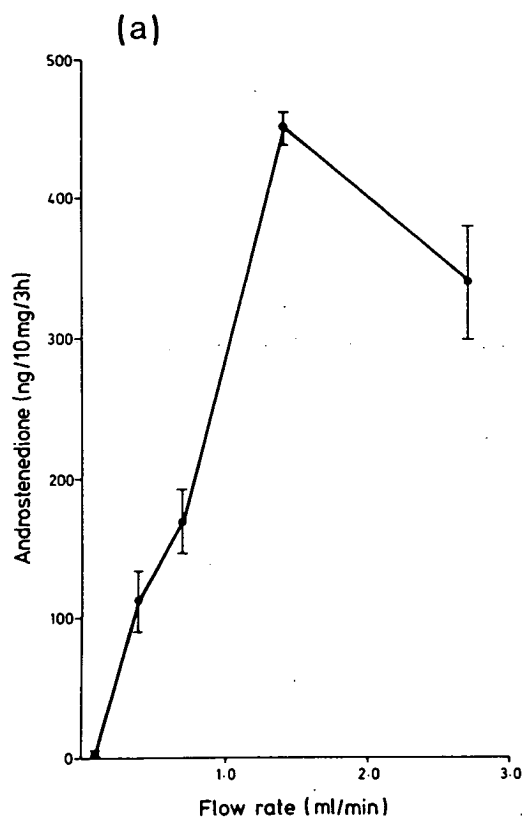
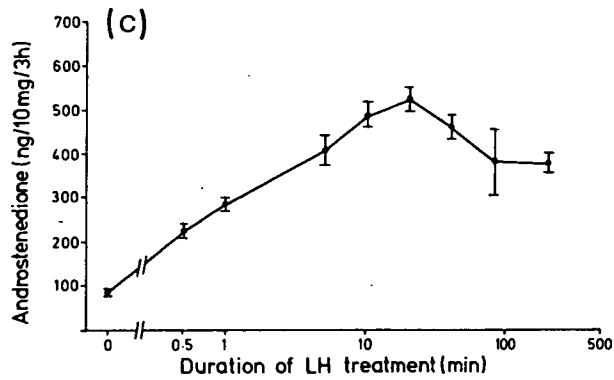
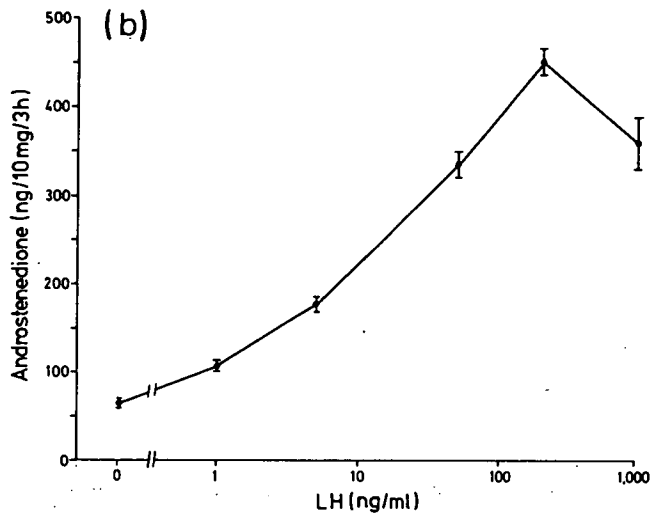


Fig. 3. Effect of medium flow rate (a), LH concentration (b) and duration of LH exposure (c) on the cumulative (3 h) production of androstenedione during and after exposure of the theca interna to LH. In Fig. 3a, the thecae at all flow-rates were exposed to a constant amount of LH (NIAMDD-bLH-4; 5600 ng) which was delivered over a 20 min interval. Results are means  $\pm$  s.e.m. of 4 experiments. In Fig. 3b, all thecae were perfused at 1.4 ml/min and the results are means  $\pm$  s.e.m. from 7 experiments. In Fig. 3c, the LH concentration was 200 ng/ml and the me-



dium was perfused at 1.4 ml/min. The results are means  $\pm$  s.e.m. from 6 experiments. For all studies, the theca interna was from non-atretic preovulatory-sized bovine follicles ( $\geq 10$  mm diameter). The perfusion medium was Krebs-Ringer bicarbonate + 0.1% BSA which was gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub> and delivered to the theca at 37°C. Before LH exposure the theca was prewashed with media for 1 h. Data from (4) with permission.

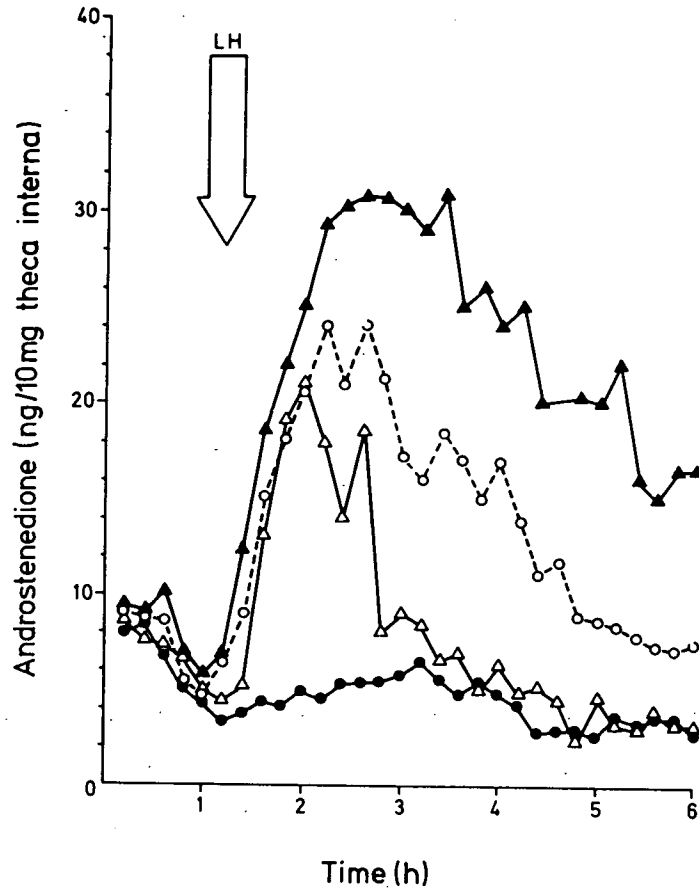


Fig. 4. The output of androstenedione by ovine theca interna every 12 min before, during and after exposure to 0 ng/ml LH (●—●), 1 ng/ml LH (▲—▲), 10 ng/ml LH (○—○) and 200 ng/ml LH (△—△) for 20 min and a medium flow-rate of 1.4 ml/min. The medium was Krebs-Ringer bicarbonate + 0.1% BSA gassed with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. These data were from a single experiment with pooled thecae interna from non-atretic preovulatory-sized bovine follicles (≥ 10 mm diameter).

androstenedione response to the subsequent LH pulse. This relationship could be expressed by the equation  $Y=172-23 \ln X$  ( $r=0.93$ ;  $p<0.01$ ,  $n=5$ ) where  $X$  = the LH concentration in the perfusion medium (pg/ml) and  $Y$  = the androstenedione response to a control LH pulse of 10 ng/ml administered 4.8 h after perfusing theca interna with an LH-free medium. For example, if the LH concentration in the perfusion medium was  $\leq 70$  pg/ml and theca was more than 70% responsive to an LH pulse 4.8 h later, but if the LH concentration was 200 or 2000 pg/ml then the thecal response to a 10 ng/ml LH pulse was 50% and  $<5\%$  respectively of that found in the controls.

### Effect of follicular diameter and phase of oestrous cycle on androstenedione output by theca interna

When bovine or ovine theca interna was perfused at 1.4 ml/min there were no significant effects of follicular diameter on the cumulative output of androstenedione per unit mass of tissue during and after a 20 min LH pulse of 200 ng/ml (bovine LH, NIAMDD-bLH-4; ovine LH, NIH-LH-S23) (15, 16). However, the total mass of theca interna is known to increase with increasing follicular diameter (11). Thus when the cumulative output of androstenedione was expressed per total mass of theca interna per follicle, a 3-5 fold greater output ( $p < 0.01$ ) was noted in large follicles ( $\geq 8$  mm diameter in the cow;  $\geq 3.5$  mm diameter in the ewe) compared to that in small follicles (2-4.5 mm in the cow; 1-1.5 mm in the ewe) (14, 15).

As mentioned earlier (see Table 1), the hCG/LH binding capacity in ovine theca interna was  $>5$  fold greater during the follicular phase compared to the luteal phase. However, the cumulative androstenedione output by LH stimulated ovine theca interna (NIH-LH-S23; 200 ng/ml for 20 min), did not differ between the follicular and luteal phases of the oestrous cycle (luteal phase,  $52 \pm 4$  ng androstenedione/10 mg theca interna/3 h,  $n=16$  pools; follicular phase,  $56 \pm 6$  ng/10 mg/3 h,  $n=18$  pools). The mean  $\pm$  s.e.m. weight of theca interna from the largest non-atretic follicles ( $\geq 3.5$  mm diameter) during the follicular phase did not differ from that in similar sized follicles during the luteal phase (i.e., follicular phase,  $10.2 \pm 1.2$  mg,  $n=15$ ; luteal phase,  $9.7 \pm 0.9$  mg,  $n=4$ ). Thus, under the experimental conditions employed, the increased binding capacity of ovine theca interna during the follicular phase did not result in an increased output of androstenedione either on a per unit mass or a total mass basis. Therefore the significance of the increased LH binding capacity of theca interna during the follicular phase remains to be determined.

### CONCLUSIONS

From these studies in sheep and cattle we can conclude that hCG/LH binding in the follicle wall is localised to the theca interna and specifically in the region immediately adjacent to the basal lamina. During the follicular phase of the oestrous cycle the LH-binding capacity in the theca of preovulatory follicles exceeds that in regressing corpora lutea more than 15 fold, and that in similar sized follicles during the luteal phase more than 5 fold. The theca interna was the major site of LH-induced androstenedione synthesis in the follicle wall; little or no LH-induced androstenedione synthesis was demonstrable in the theca externa, stroma or granulosa cells. In an *in vitro* perfusion system, LH induced androstenedione synthesis could be increased by 20-45 fold in both ovine and bovine theca interna by increasing the flow-rate of medium from 0 to 1.4 ml/min. This finding supports the proposal of Zeleznik *et al.* (25)

that the level of steroidogenesis at localised intraovarian sites might be amplified considerably by increased blood flow. The level of LH-induced steroidogenesis in theca interna was influenced significantly by LH concentration, the duration of LH exposure and the previous exposure of the tissue to LH. Collectively the results from the *in vitro* perfusion studies suggest that thecal tissue is acutely sensitive to low amplitude LH pulses (i.e., 1 ng/ml for 20 min) when the basal LH concentrations and/or LH peak frequencies are low. They also support the notion that the thecal androstenedione output to an LH pulse is diminished when the basal LH concentrations are high or when the rate of blood flow is reduced.

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## Influence of follicular health on the steroidogenic and morphological characteristics of bovine granulosa cells *in vitro*

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**Summary.** In 24-h cultures, steroid production by cells from non-atretic follicles increased with increasing follicular diameter. Cells from atretic follicles, of all sizes, produced low amounts of oestradiol-17 $\beta$ , but very high amounts of progesterone, relative to cells from non-atretic follicles. Increasing the culture period to 72 h caused little change in daily progesterone and oestradiol-17 $\beta$  production by granulosa cells from atretic follicles. In contrast, in cells from non-atretic follicles, daily progesterone production increased and daily oestradiol-17 $\beta$  production decreased to the levels observed with cells from atretic follicles. Dibutyryl cyclic AMP (1.0 mM) significantly stimulated progesterone production by cells from atretic, but not from non-atretic, follicles. Testosterone (1  $\mu$ g/ml) had no effect on progesterone production by cells from atretic follicles, while oestradiol-17 $\beta$ , oestrone, testosterone, androstenedione and 5 $\alpha$ -dihydrotestosterone (0–1000 ng/ml) each significantly suppressed progesterone production by cells from non-atretic follicles in a dose-dependent manner.

Morphometric analysis revealed few subcellular differences between cells from non-atretic and atretic follicles. Mean cell volume was significantly higher for cells from atretic compared to non-atretic follicles, but the mean volumes of the major subcellular components were not influenced by follicle health. The mean surface area of the plasma and nuclear membrane, and granular endoplasmic reticulum was also significantly higher in cells from atretic compared to non-atretic follicles.

### Introduction

Oestradiol-17 $\beta$  and progesterone are the two major steroids produced by bovine granulosa cells. Previous studies have shown that oestradiol-17 $\beta$  production by bovine granulosa cells *in vitro* is related to follicle size and health (Henderson *et al.*, 1984; McNatty *et al.*, 1984a). However, no information is available about how progesterone production by bovine granulosa cells *in vitro* is influenced by these follicular characteristics. Progesterone concentrations in follicular fluid of follicles collected during the follicular phase before the ovulatory LH surge are significantly higher in atretic than in non-atretic follicles, irrespective of follicle size (Bellin & Ax, 1984; McNatty *et al.*, 1984a; Kruip & Dieleman, 1985). As granulosa cells may be a source of progesterone in follicular fluid, it is possible that follicle health may influence progesterone production by granulosa cells. This possibility was examined in the present study which was undertaken to examine the relationship(s) between follicular health and size, and progesterone and oestradiol-17 $\beta$  production by bovine granulosa cells *in vitro*. In addition, morphometric analysis of granulosa cells was performed to determine whether any morphological characteristics of granulosa cells were related to follicle health. Except for one experiment, granulosa cells were obtained from the ovaries of regularly cyclic Angus cows at known days between Days –4 and +1 of the oestrous cycle

(oestrus = Day 0). The days were chosen in an attempt to assess what effects (if any) the period during which final follicular preovulatory maturity is attained has on granulosa cell function *in vitro*. In one experiment, ovaries were obtained from cows (breed and stage of oestrous cycle unknown) slaughtered in a local abattoir.

### Materials and Methods

**Reagents.** Testosterone, androstenedione, 5 $\alpha$ -dihydrotestosterone, oestradiol-17 $\beta$ , oestrone and dibutyl cyclic AMP were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Cyanoketone (2 $\alpha$ -cyano-4,4-17 $\alpha$ -trimethyl-17 $\beta$ -hydroxy-5-androsten-3-one) was a gift from the late Professor G. S. Boyd (Department of Biochemistry, University of Edinburgh, U.K.). Gonadotrophins (NIH-LH-B10 and NIH-FSH-S15) were obtained from the NIADDK, National Institutes of Health, Bethesda, U.S.A.

**Cows and ovaries.** Twenty-three Angus cows were grazed on open pasture with a vasectomized bull and examined twice daily for signs of oestrus. Ovariectomy was performed between Days -4 and +1 of the oestrous cycle (oestrus = Day 0). The day of the cycle was initially calculated from knowledge of the previous mean oestrous cycle length and date of last oestrus. This dating was subsequently confirmed or modified from details of the histology of the corpus luteum (CL), the weight of the CL, the binding characteristics of <sup>125</sup>I-labelled human chorionic gonadotrophin to homogenized luteal tissue and the progesterone secretory characteristics of dispersed luteal cells during 3-h incubation *in vitro*, as described previously (McNatty *et al.*, 1984a). Overall, 2 cows were judged to be at Day -4 of the cycle, 5 at Day -3, 5 at Day -2, 4 at Day -1, 5 at Day 0 and 2 at day +1.

**Ovarian dissections.** Pairs of ovaries from the Angus cows were treated as follows. Under a dissecting microscope ( $\times 10$ -40 magnification) all antral follicles  $\geq 2.0$  mm in diameter and corpora lutea were individually dissected out of each pair of ovaries into sterile Medium 199 with Earle's salts (Eagle, 1959) supplemented with Hepes buffer (20 mM), L-glutamine (2 mM), gentamicin (50  $\mu$ g/ml) (Gibco, Grand Island, NY, U.S.A.), sodium heparin (50 i.u./ml) (Weddel Pharmaceuticals Ltd, London, U.K.) and 0.1% bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, MO, U.S.A.) (Medium A). After recording the follicular diameter to the nearest 0.5 mm and examining the thecal vasculature, the follicle was incised to release the contents. The released follicular fluid was examined for the presence or absence of debris and then aspirated through a fine-bore capillary tube. The internal follicle wall was washed gently and repeatedly with 2 ml Medium A and the released clumps of granulosa cells were dispersed by pipetting several times through a finely drawn Pasteur pipette. The oocyte was isolated and assessed subjectively as being healthy or degenerate as previously described (McNatty *et al.*, 1983). The follicle wall was washed several more times, and the total number of granulosa cells in the pooled washings was counted by using a haemocytometer, and their viability was determined by uptake of nigrosin dye. The colour of the theca interna was noted. Ovaries obtained from the abattoir were treated as above except that only follicles  $\geq 10$  mm in diameter were recovered.

**Follicle classification.** Each follicle was classified as non-atretic, intermediate or atretic on the basis of its morphological appearance. Details of the follicle classification scheme have been described previously (McNatty *et al.*, 1984a). Follicles considered to be non-atretic were those with: visible thecal capillaries when viewed at  $\times 10$  magnification under a dissecting microscope; no debris in the follicular fluid;  $> 75\%$  of the expected maximum number of granulosa cells for a given follicle size; a healthy looking oocyte; and a pink to red theca interna. Intermediate follicles had the same characteristics as non-atretic follicles except that the theca interna was white. All other follicles were considered to be atretic.

**Granulosa cell cultures.** To obtain sufficient granulosa cells from each pair of Angus cow ovaries for replicate cultures, it was often necessary to pool cells from follicles of a similar size and classification, particularly for small and/or atretic follicles. In the experiment using abattoir material, granulosa cells were pooled from non-atretic follicles  $\geq 10$  mm in diameter until a sufficient number of cells had been obtained. After collecting the granulosa cells, and pooling when appropriate, the cells were centrifuged at 200 *g* for 10 min, resuspended in Medium A devoid of sodium heparin, Hepes and bovine serum albumin, but containing 10% newborn calf serum (Gibco) (Medium B) and recounted. Aliquants of approximately 100 to 600  $\times 10^3$  'live' granulosa cells in 0.5 ml Medium B were pipetted into individual wells of multi-welled tissue-culture Petri dishes (Sterilin, Middlesex, U.K.). In some instances the wells contained an 18 mm<sup>2</sup> glass cover-slip for attachment of the cells. A further 0.5 ml Medium B either alone or containing added steroid, dibutyl cyclic AMP or gonadotrophin was added and the cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Steroids were added to the culture medium in ethanol or acetone; the concentration of organic solvent in the culture medium never exceeded 1%. Control cultures received vehicle alone. For each Angus cow 2-4 replicate cultures for each treatment were established. Cultures of cells for 24 h were established for all 23 cows, and when there were sufficient cells (which occurred with about half the cows) 72-h cultures were also established. In the 72-h cultures, the medium was renewed every 24 h with the spent media being stored frozen. At the end of the culture period the cells were washed thoroughly with Medium B devoid of serum, and then either stored frozen until assayed for protein by the method of Lowry *et al.* (1951) as modified by Patterson (1979), or processed for scanning electron microscopy.

**Scanning electron microscopy.** Granulosa cells attached to glass cover-slips were immersion-fixed for 1 h at 4°C in 2% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4), and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M-cacodylate buffer. The cells were then dehydrated in ethanol, critical point-dried using liquid CO<sub>2</sub> and then platinum-coated in a Polaron E5100 coating unit (Polaron Equipment Ltd., Watford, U.K.). The cells were viewed in a Super III-A scanning electron microscope (International Scientific Instruments Inc, Santa Clara, CA, U.S.A.).

**Transmission electron microscopy.** Freshly collected granulosa cells from non-atretic, intermediate and atretic follicles were immersion-fixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4). The cells were then pelleted by centrifugation and encased in 3% agar. After rinsing with 0.1 M-cacodylate buffer, the cells were post-fixed for 1 h in 0.1 M-cacodylate-buffered 1% osmium tetroxide. After further rinsing, the cells were stained en bloc with 2% aqueous uranyl acetate, dehydrated through a graded series of ethanol solutions and embedded in Spurr's resin. Thin (silver) sections were cut at intervals throughout the depth of the cell pellets, stained with uranyl acetate and Reynold's lead citrate and viewed in a Philips 201 transmission electron microscope (Philips, Eindhoven, The Netherlands). Thicker sections (1 µm) were also cut, and stained with methylene blue for determination of cell volumes.

**Morphometric analysis.** Volume density (organelle volume per unit cell volume) and surface density (surface area of the membrane per unit cell volume) were determined from electron micrographs at a final magnification of × 14 500. The cells for study were chosen randomly: 2–3 micrographs of each cell were prepared and over the 3 classes of follicle health 296 cells were studied. Volume density was determined by lineal analysis as described by Williams (1977). Surface density was determined using uni-direction linear probes as described by Weibel (1980a). For both determinations a sufficient number of measurements was made so that the values of the relative standard errors were ≤ 5% (Weibel, 1980a). Determination of cell volume allowed the volume and surface density values to be expressed as organelle volume and membrane surface area per granulosa cell.

Cell volumes were calculated from light photomicrographs of methylene blue-stained sections of freshly isolated granulosa cells, using the equations described by Abercrombie (1946) and Williams & Cope (1981). At least 200 nuclear transections were measured for each determination.

Calculation of the numerical density ( $N_v$ ) of mitochondria was based on the formula described by Weibel (1980b) in which  $N_v = k/\beta \times (N_A)^{3/2}/(V_v)^{1/2}$  where  $V_v$  = volume density of mitochondria;  $N_A$  = number of profiles per unit section area;  $\beta$  = shape coefficient and  $k$  = size distribution coefficient based on the size distribution of the mean tangent diameter.

**Radioimmunoassays.** The concentrations of progesterone, pregnenolone and oestradiol-17β were measured without extraction in aliquants of granulosa cell culture medium using specific radioimmunoassays described previously (Henderson *et al.*, 1983; McNatty *et al.*, 1984b). The limit of sensitivity of the assays (per tube) was 25 pg for progesterone, 50 pg for pregnenolone and 5 pg for oestradiol-17β. The intra- and inter-assay coefficients of variation were < 10%. Steroid concentrations in the culture media were normalized with respect to the protein content of the granulosa cells at the end of the culture period, and the results were expressed as ng steroid/mg protein. For each Angus cow, the coefficient of variation between replicate cultures having the same treatment ranged between 6 and 27%.

**Statistics.** For the Angus cows, average values were calculated for each cow and these values were then used to calculate group means, or geometric means for (N) cows. With the cultures established from a single pool of granulosa cells from abattoir ovaries, mean values were calculated for replicate cultures of each treatment. Further statistical analysis was performed using analysis of variance in conjunction with either the Newman-Keuls multiple range test, when making comparisons between mean values over all groups, or with Dunnett's test when comparing a control (untreated) group mean value with each of the other treatment group means. When heterogeneity of variance was indicated by Bartlett's test, the data were transformed to logarithms to equalize the variances before statistical analysis. In these instances, the data have been presented as geometric means together with 95% confidence limits. The level of significance was set at  $P < 0.05$ .

## Results

Basal production of progesterone was measured in cultures not receiving any exogenous steroid or gonadotrophin. Oestradiol-17β production was measured in cultures receiving testosterone (1 µg/ml). This dose of testosterone allowed production of maximum amounts of oestradiol-17β. No detectable amounts of oestradiol-17β were produced by cultures devoid of exogenous testosterone. In a preliminary study, granulosa cells before and after culture were extracted with ethanol to measure steroid content, but no detectable amounts of progesterone or oestradiol-17β were found.

**Table 1.** Progesterone and oestradiol-17 $\beta$  production by bovine granulosa cells with respect to follicular health and diameter

Steroid	Follicle health	Follicular diameter (mm)		
		< 5	$\geq 5$ –< 10	$\geq 10$
Progesterone (ng/mg protein)	Non-atretic	76 <sup>c</sup> (10) 50–115	175 <sup>b</sup> (23) 131–233	290 <sup>a</sup> (11) 220–381
	Intermediate	113 <sup>c</sup> (13) 60–209	131 <sup>b,c</sup> (8) 79–217	—
	Atretic	1094 <sup>d</sup> (23) 906–1322	1412 <sup>d</sup> (5) 785–2536	1455 <sup>d</sup> (3) 392–5383
Oestradiol-17 $\beta$ (ng/mg protein)	Non-atretic	122 <sup>c</sup> (4) 17–855	209 <sup>c</sup> (17) 90–475	1357 <sup>a</sup> (11) 633–2899
	Intermediate	71 <sup>c</sup> (7) 16–310	116 <sup>c</sup> (4) 19–698	—
	Atretic	31 <sup>c,d</sup> (19) 22–44	14 <sup>d</sup> (4) 8–24	17 <sup>d</sup> (3) 11–24

Cells were cultured for 24 h. Values are geometric means with 95% confidence limits below for (N) cows. Values not sharing a common superscript in the same row or column are significantly different <sup>ab</sup> $P < 0.05$ ; others,  $P < 0.01$ .

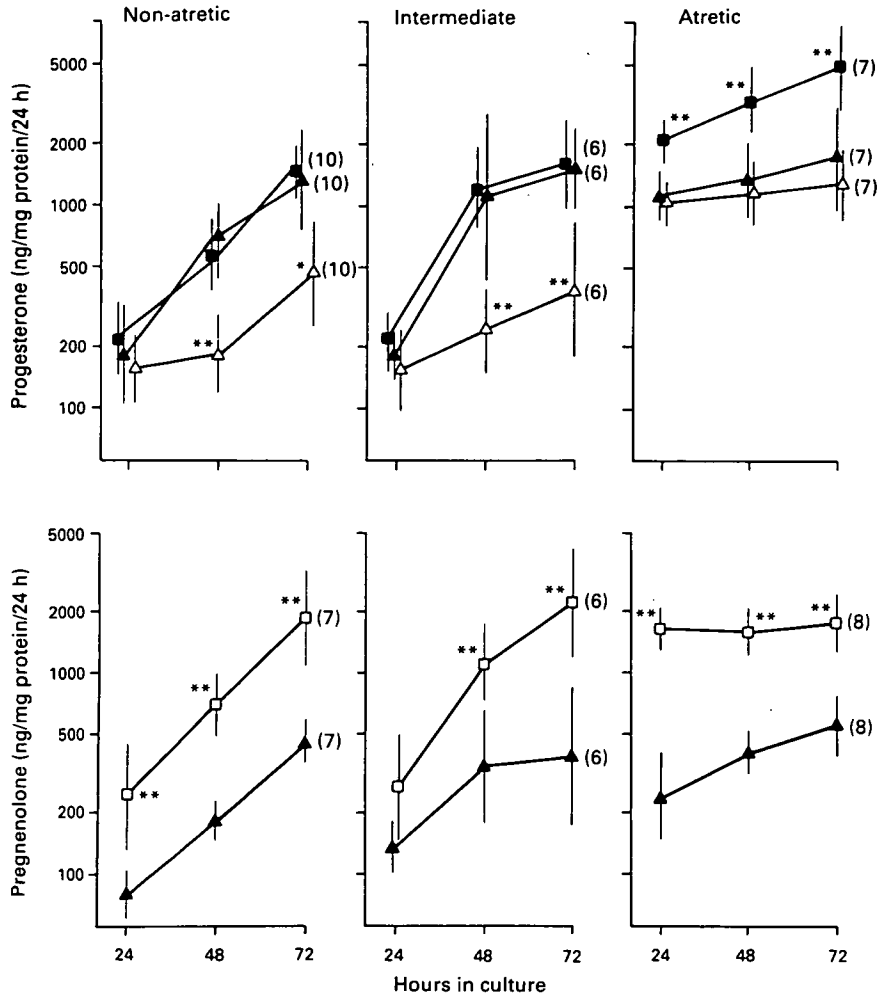
*Influence of follicular health and size on progesterone and oestradiol-17 $\beta$  production by granulosa cells cultured for 24 h*

Mean production of progesterone and oestradiol-17 $\beta$  by granulosa cells from non-atretic follicles increased significantly as follicular diameter increased (Table 1). At all follicle diameters, progesterone production by cells from atretic follicles was significantly higher than that of cells from non-atretic follicles. Oestradiol-17 $\beta$  production by granulosa cells from atretic follicles  $\geq 5$  mm in diameter was significantly less than that of cells from non-atretic follicles of a similar size. These effects of follicle health and size were independent of the days of the oestrous cycle studied. The highest production of progesterone was, however, observed with cells recovered on Day +1 from two haemorrhagic presumptive preovulatory follicles (10 and 11 mm diam.). The cells from these follicles produced 6–7  $\mu\text{g}$  progesterone/mg protein when cultured for 24 h.

*Influence of follicle health on progesterone, pregnenolone and oestradiol-17 $\beta$  production by granulosa cells cultured for 72 h*

During 72 h of culture, there was a  $\sim 5$ -fold increase in the mean daily production of progesterone by granulosa cells from non-atretic follicles (Fig. 1). In contrast, mean daily production of progesterone by granulosa cells from atretic follicles changed little during 72 h of culture. This effect of follicular health was not related to follicle size or day of the cycle (Days –4 to 0). When the effects of LH, FSH, LH + FSH (100 and 1000 ng/ml of each) or dibutyryl cyclic AMP (0.1 and 1.0 mM) or testosterone (1  $\mu\text{g}/\text{ml}$ ) on progesterone production were examined, testosterone and dibutyryl cyclic AMP (1.0 mM) were the only treatments to have a significant effect, relative to untreated cultures. Testosterone significantly inhibited progesterone production by granulosa cells from non-atretic and intermediate follicles after the first 24 h of culture, but had no effect on cells from atretic follicles (Fig. 1). Dibutyryl cyclic AMP stimulated significantly progesterone production by cells from atretic follicles, but had no effect on cells from non-atretic and intermediate follicles (Fig. 1).

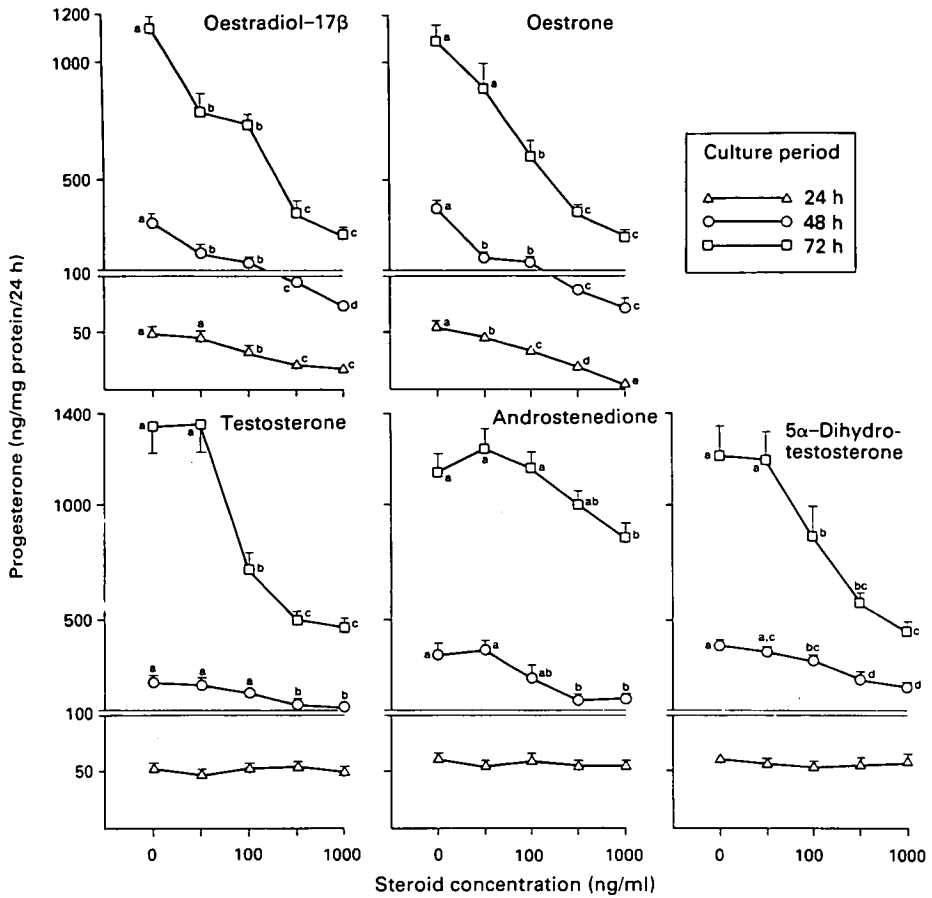
The differences in the amounts of progesterone produced by cells from non-atretic and atretic follicles during the first 24 h of culture (Table 1; Fig. 1) might have arisen from differences in the ability of the cells to metabolize progesterone further, or to synthesize its precursor pregnenolone.



**Fig. 1.** Progesterone and pregnenolone production by granulosa cells from non-atretic, intermediate and atretic follicles cultured for 72 h. Values are geometric means with 95% confidence limits for (N) cows. ▲, △, ■ and □ refer to untreated (control), testosterone (1 µg/ml), dibutyryl cyclic AMP (1.0 mM) and cyanoketone (1 µg/ml)-treated cultures respectively. \**P* < 0.05; \*\**P* < 0.01: geometric mean values significantly different from corresponding untreated (control) values.

To test this possibility cells were cultured in the presence of cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase. Preliminary dose-response studies established that, at a dose of 1 µg cyanoketone/ml, progesterone production was < 12% of that produced by untreated cultures, while pregnenolone production was comparable to that of progesterone produced by untreated cultures. Irrespective of follicle size, the changes in cellular pregnenolone production in cyanoketone and untreated cultures (Fig. 1, lower half), were similar, with respect to follicle health and time in culture, to those observed for progesterone in untreated cultures (Fig. 1, top half).

Mean daily production of oestradiol-17β (in the presence of 1 µg testosterone/ml) by granulosa cells from atretic follicles of all sizes changed little during 72 h of culture, remaining at about 10–20 ng/mg protein/24 h. During the first 24 h of culture, mean production of oestradiol-17β by granulosa cells from non-atretic and intermediate follicles ≥ 5 mm diameter was significantly higher than that of cells from atretic follicles (Table 1). However, over the next 24 h of culture,



**Fig. 2.** Effect of oestrogens and androgens on progesterone production by granulosa cells cultured for 72 h. Values are arithmetic means of 4 replicate cultures. Vertical lines show the s.e.m. when larger than the size of the symbol. Mean values not sharing a common letter superscript over the same culture period are significantly different ( $P < 0.05$ ). Groups of mean values without superscripts are not significantly different.

production of oestradiol-17 $\beta$  by cells from non-atretic and intermediate follicles had fallen to < 10% of their original values, and within 48 h had fallen to levels no different from that of cells from atretic follicles. This decline in daily production of oestradiol-17 $\beta$  could not be prevented by culturing the cells in the presence of 1  $\mu$ g testosterone/ml with FSH (100 or 1000 ng/ml) or with LH (100 ng/ml) or with dibutyryl cyclic AMP (1.0 mM).

#### *Effect of oestrogens and androgens on progesterone production by granulosa cells from non-atretic follicles*

In view of the finding that testosterone could inhibit progesterone production by granulosa cells from non-atretic and intermediate follicles, we decided to test the effects of some other ovarian steroids. Because no more Angus cows were available, and because of the large numbers of cells required, the ovaries for this part of the study were obtained from cows (breed and stage of oestrous cycle unknown) slaughtered at a local abattoir. A single pool of granulosa cells from large ( $\geq 10$  mm diam.), non-atretic follicles was obtained. Figure 2 shows the effect of oestradiol-17 $\beta$ , oestrone, testosterone, androstenedione and 5 $\alpha$ -dihydrotestosterone on progesterone production

**Table 2.** Influence of follicular health on cell volume and surface area of subcellular components, and numerical density of mitochondria of pre-culture granulosa cells

	Follicular health		
	Non-atretic	Intermediate	Atretic
Cell volume ( $\mu\text{m}^3$ )	537 <sup>a</sup> 456–617	745 <sup>b</sup> 681–809	784 <sup>b</sup> 650–918
Subcellular components			
Mitochondria (no./cell)	82 <sup>a</sup> 63–100	91 <sup>a</sup> 65–117	128 <sup>a</sup> 85–171
Plasma membrane ( $\mu\text{m}^2$ /cell)	916 <sup>a</sup> 860–972	1198 <sup>b</sup> 1109–1285	1383 <sup>c</sup> 1296–1469
Nuclear membrane ( $\mu\text{m}^2$ /cell)	548 <sup>a</sup> 498–598	753 <sup>b</sup> 685–821	883 <sup>b</sup> 816–950
Outer mitochondrial membrane ( $\mu\text{m}^2$ /cell)	694 <sup>a</sup> 620–768	674 <sup>a</sup> 598–750	685 <sup>a</sup> 603–768
Agranular endoplasmic reticulum ( $\mu\text{m}^2$ /cell)	256 <sup>a</sup> 220–293	323 <sup>a</sup> 266–380	268 <sup>a</sup> 210–325
Granular endoplasmic reticulum ( $\mu\text{m}^2$ /cell)	638 <sup>a</sup> 545–731	1332 <sup>b</sup> 1117–1547	1269 <sup>b</sup> 1101–1437

Values are means with 95% confidence limits below for 6 cows. Mean values with different letter superscripts in the same row are significantly different ( $P < 0.05$ ).

by these cells over a 72-h culture period. Each steroid caused a dose-related suppression in the mean production of progesterone. The oestrogens inhibited progesterone production from the start of the culture period, whereas the androgens only inhibited after the first 24 h of culture.

#### *Influence of follicular health on morphological characteristics of granulosa cells*

Morphometric analysis was used to quantitate the morphological characteristics of freshly harvested granulosa cells from 3 to 8 mm diameter follicles taken from 6 cows between Days -3 and -1 of the oestrous cycle. Follicular health significantly influenced mean cell volume, and the mean surface area of the plasma and nuclear membranes, and the granular endoplasmic reticulum (Table 2). Follicle health had no significant effect on the mean volumes of the major subcellular components (nucleus, lipid, lysosomes, vacuoles, mitochondria, agranular endoplasmic reticulum and microfilaments), each of which had a mean volume of  $\sim 10$ – $20 \mu\text{m}^3$ /cell, except for the nuclei which had mean values of about  $150$ – $200 \mu\text{m}^3$ /cell.

Irrespective of follicle health freshly harvested granulosa cells appeared spherical with a relatively smooth surface when viewed by scanning electron microscopy. After culture for 24 h, cells from non-atretic follicles were still spherical, but many had surface 'blebs'. In contrast, cells from atretic follicles were generally 'flattened' in appearance, and large amounts of intercellular connective tissue were evident. The appearance of cells from atretic follicles changed little when the culture period was extended beyond 24 h. However, cells from non-atretic follicles gradually acquired the appearance of cells from atretic follicles, and by 72 h were indistinguishable from them. The inclusion of FSH (100 ng/ml) or dibutyryl cyclic AMP (1.0 mM) in the culture medium markedly reduced the appearance of surface 'blebs' on cells from healthy follicles after 24 h of culture, but could not prevent them appearing like cells from atretic follicles over 72 h of culture.

#### Discussion

The present study demonstrates that follicular health has a major influence on the ability of granulosa cells from cow follicles to produce progesterone and oestradiol-17 $\beta$  *in vitro*. In non-atretic



follicles, the production of these steroids by granulosa cells was also related to follicle size. The increasing daily production of progesterone, and the associated morphological changes, by cultured cells from non-atretic follicles are characteristic of cells undergoing luteinization (Channing & Ledwitz-Rigby, 1974), as they start to express the characteristics of granulosa-luteal cells. Irrespective of follicle size, granulosa cells from atretic follicles displayed the characteristics of luteinized cells within 24 h of culture. Therefore, these cells, in contrast to cells from non-atretic follicles, may have already undergone some luteinization *in vivo*. This view would be consistent with the observation that irrespective of follicle size, progesterone concentrations in follicular fluid are significantly higher in atretic than in non-atretic follicles (Bellin & Ax, 1984; McNatty *et al.*, 1984a; Kruip & Dieleman, 1985). The studies with cyanoketone, an inhibitor of  $3\beta$ -hydroxysteroid dehydrogenase, suggest that the differences in progesterone production between cells from non-atretic and atretic follicles may be a consequence of differences in the synthesis of pregnenolone, the precursor for progesterone.

The regulation of progesterone production by granulosa cells has been widely studied in several species, but usually without regard to the health of the follicles from which the cells were obtained. The present study indicates that steroids may have some importance in regulating progesterone production by granulosa cells from follicles of different health. Although testosterone failed to suppress progesterone production by granulosa cells from atretic follicles both androgens and oestrogens suppressed progesterone production by granulosa cells from non-atretic follicles in a dose-dependent fashion. The inhibitory effects of oestrogens have been shown previously for the cow (Fortune & Hansel, 1979) and other species (see review by Hillier, 1985). However, studies with androgens in other species have generally shown a stimulatory effect on progesterone production by granulosa cells (Hillier, 1985), in contrast to the results of the present study. It could be argued that the inhibitory effects of testosterone and androstenedione might arise, at least in part, from their metabolism to oestradiol- $17\beta$ , though by Day 2 of culture the cells have lost most of their aromatase activity. This argument could not however account for the inhibitory effects of  $5\alpha$ -dihydrotestosterone. Perhaps in non-atretic follicles, thecal androgens, produced in response to LH, together with oestrogens produced by granulosa cell aromatization of these androgens, have a physiological role in limiting progesterone production by granulosa cells during follicular development in the cow. In large atretic follicles there is limited thecal androgen production in response to LH and granulosa cells from small and large atretic follicles have little aromatase activity (McNatty *et al.*, 1984a; Table 1).

Although daily progesterone production by granulosa cells from non-atretic follicles increased with time in culture, production of oestradiol- $17\beta$  declined to levels comparable to that of cells from atretic follicles. While this loss in oestradiol- $17\beta$  synthetase activity may be an artefact of the culture system, resulting from differential cell loss, it is perhaps more likely a consequence of the luteinization process. Bovine luteal cells themselves have a very high capacity for progesterone biosynthesis, but only a very limited ability to aromatize androgens to oestradiol- $17\beta$  (Henderson & Moon, 1979). Collectively, the results of this study suggest that, in the presence of adequate amounts of progesterone precursor substrate, granulosa cells may have a natural tendency to produce large amounts of progesterone, but only a limited tendency to metabolize androgens to oestradiol- $17\beta$ . In non-atretic follicles, granulosa cells may be under considerable regulatory pressure which acts to suppress this natural tendency to secrete progesterone, and in larger follicles drives oestradiol- $17\beta$  production. When these regulatory pressures are reduced, e.g. during follicular atresia, or when the granulosa cells are placed in culture, then the cells revert to a more natural state in terms of steroid production. Perhaps part of the luteinization process during corpus luteum formation is the removal of constraints thereby allowing the granulosa cells to display their inherent tendency to secrete progesterone, as suggested by Rothchild (1981).

Although follicular health had a major influence on progesterone and oestradiol- $17\beta$  production by granulosa cells, morphometric analysis revealed relatively few subcellular differences between cells from non-atretic and atretic follicles. In particular, no ultrastructural differences were

evident in the mitochondria or agranular endoplasmic reticulum (the subcellular sites of progesterone and oestradiol-17 $\beta$  biosynthesis respectively). As follicles became atretic significant increases occurred in the surface areas of the nuclear and plasma membranes. This was not always accompanied by increases in the volume of the organelle, which suggests that these membranes were becoming more convoluted. Such changes together with minimal organelle changes are indicative of cellular swelling (Cheville, 1976). Whether this swelling is expressed *in vivo* or is an artefact of the treatment before fixation is unclear. Nevertheless, it does reflect some difference(s) in membrane structure and/or function between granulosa cells from follicles of different health. This in turn could influence the steroidogenic abilities of these cells.

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OVARIAN FOLLICULAR DEVELOPMENT AND THE EFFECTS OF GONADOTROPHINS  
IN THE SHEEP

K P McNatty, K M Henderson and D C Thurley

Introduction

In sheep as with most mammals, the major determinant of prolificacy is the number of follicles which ovulate. The factors which control the growth of follicles to ovulation involve a complex series of hormone and cell interactions involving the brain and the ovary and probably other tissues such as the liver and thyroid. Our understanding of these interactions is far from complete. The purpose of this paper is to review some aspects of follicle development in sheep with particular emphasis on pituitary hormone action on follicle cells during growth and atresia.

Preantral follicle development

Ovarian follicular development is initiated when the oocyte of a small resting follicle (i.e., a primordial follicle; diameter ~0.03 mm) begins to enlarge and its granulosa cells start to proliferate. The entry of primordial follicles into the growth pool starts during foetal life, occurs throughout infancy and adult life and is not interrupted by pregnancy or other periods of anovulation<sup>(1)</sup>. During early (preantral) follicle development, the number of follicles in the growth phase at any moment in time as assessed by mitotic activity in granulosa cells is extremely low (Table 1). With increasing follicular diameter the proportion of preantral follicles with mitotic granulosa cells increases but the incidence of follicular atresia as assessed by morphological criteria (e.g., pycnotic granulosa cells) remains extremely low. It

Table 1. Preantral follicle growth characteristics in an anoestrous Romney ewe

Number of granulosa cells in a cross-section of the follicle <sup>a</sup>	Number of follicles examined (left + right ovaries)	Number of follicles with at least one mitotic cell (%) <sup>b</sup>	Number of follicles with differing mitotic counts		
			1	2-3	4-6
0-15	4079	1 (0.025)	1	-	-
16-100	1163	19 (1.6)	19	-	-
101-500	72	12 (16.7)	9	3	-
501-1000	33	19 (57.5)	14	3	1
1001-1500	15	12 (80.0)	4	6	2
1501-2000	10	9 (90.0)	3	4	2
>2001	3	3 (100.0)	-	2	1

<sup>a</sup> Follicles were sectioned in a random plane which could underestimate but not overestimate the maximum follicular diameter. Follicles >0.1 mm would be sectioned at least twice and from this size upwards, the number of cells counted would be increasingly proportional to follicle size.

<sup>b</sup> The mean number of mitotic cells per 1000 granulosa cells counted was 0.001.

seems reasonable to suggest that the proportion of preantral follicles in the growth phase at any moment in time increases with increasing diameter (see Table 1), as does the speed of follicle growth. However, overall, follicular growth during the preantral growth phase (i.e., from 0.03 to 0.25 mm) is extremely slow and is estimated to be more than 4 months<sup>(2)</sup>. The hormonal factors which promote the entry of non-growing primordial follicles into the growth phase as well as those which control preantral follicular growth are obscure. The controlling factors may include systemically-derived steroids (e.g., oestradiol) and an array of growth-related peptides [e.g., insulin-like growth factor (IGF<sub>1</sub>, IGF<sub>2</sub>)], fibroblast growth factor, vaso-active peptides and the inhibin family of peptides including transforming growth factor (TGF $\beta$ ). It is thought that the gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are also involved in preantral follicular growth but precisely when or how is still unclear. After short (~7 days) or long (~70 days)-term hypophysectomy, granulosa cells continue to proliferate but the rate of mitotic activity after long- but not short-term hypophysectomy is reduced<sup>(2)</sup>. It is by no means certain that preantral follicles have an absolute requirement for gonadotrophins but the evidence suggests that growth-related processes are slowed-down in their absence.

#### Antral follicle development

As follicles develop beyond 0.25 mm in diameter they form a fluid-filled antral cavity and growth thereafter is commonly referred to as antral follicle growth. At ovulation the diameter of a sheep follicle is between 6 and 10 mm. Thus antral follicle growth is associated with the accumulation of 6-10 x 10<sup>6</sup> granulosa cells, 0.25 ml follicular fluid as well as the differentiation or accumulation of an indeterminate number of theca interna cells. The estimated time for a follicle to grow from 0.25 mm in diameter to preovulatory size is <2 months<sup>(2)</sup> with most of this time spent in growing to 2-2.5 mm in diameter; from 2.5 mm in diameter a follicle can grow to preovulatory size in 48-60 hours. Normally, at least one ovarian follicle will be growing to preovulatory size (e.g., 5-7 mm diameter) at any one time irrespective of time of year or stage of the oestrous cycle. The formation of a fluid-filled antrum provides the means by which cells within the avascular region of a follicle (i.e., the granulosa cells and the oocyte) can be exposed to an endocrine microenvironment which is different from that in plasma and in adjacent follicles<sup>(3)</sup>. The hormonal microenvironment within a follicle has a major influence on the maturation and steroidogenic potential of the oocyte and/or granulosa cells<sup>(4)(5)</sup>. Follicular fluid is a transudate of serum and is also enriched with secretory products from follicle cells such as glycosaminoglycans, IGF<sub>1</sub>, inhibin, TGF $\beta$ , prostaglandins and steroids; these follicular derived materials together with systemically derived hormones (e.g., interleukin-1, cachectin, thyroxine, triiodothyronine, prolactin, etc)<sup>(6)(7)(8)</sup> may also augment, amplify or inhibit the actions of FSH and LH, at the level of the follicle cells although most of these putative interactions are poorly understood. Hypophysectomy during the sheep oestrous cycle results in anovulation and blocks antral follicle growth beyond 2.5 mm in diameter. Conversely, gonadotrophin supplementation increases the number of antral follicles >2.5 mm diameter and also the ovulation-rate<sup>(9)(10)</sup>. However, in the presence of physiological concentrations of gonadotrophins, follicular atresia (as assessed by pycnosis or number of granulosa cells per follicle

size), is the fate of most antral follicles. Thus although there is an absolute requirement for gonadotrophins, antral follicles also have temporal threshold requirements with respect to follicular growth, steroidogenesis, and ovulation-rate(10)(11).

#### Relationships between antral follicular development, gonadotrophin secretion, follicular steroidogenesis and stage of the oestrous cycle

On most days, irrespective of the time of the year (i.e., breeding or non-breeding seasons) or day of the oestrous cycle, there are, in each ewe, between 8 and 50 antral follicles  $\geq 1$  mm diameter and between 1 and 3 follicles of preovulatory size (i.e.,  $\geq 5$  mm diameter)(12). The presence of  $\geq 5$  mm diameter follicles is probably due to the plasma FSH concentrations being consistently in excess of 1 ng/ml (i.e., as assessed by the National Institutes of Health homologous radioimmunoassay kit for ovine FSH). Suppression of plasma FSH concentrations to  $< 1$  ng/ml for more than 24 h by infusion of bovine follicular fluid (or inhibin), oestradiol implants, hypophysectomy or hypothalamic-pituitary disconnection blocks the development of antral follicles beyond 2.5 mm diameter(9)(10)(12)(13). Irrespective of time of the year, or presence or absence of a corpus luteum (CL), the ovarian secretions of androstenedione and oestradiol from ovaries with  $\geq 5$  mm diameter follicles are invariably higher than the respective secretions from ovaries with follicles  $< 5$  mm diameter(14)(15). Moreover, significant linear correlations exist between the concentrations of oestradiol in large follicles and the levels in ovarian venous blood from the ovary containing the large follicle ( $r = 0.72$ ;  $p < 0.01$ ) and also between androstenedione and oestradiol in ovarian venous blood ( $r = 0.80$ ;  $p < 0.01$ )(15). Nevertheless, in  $\geq 5$  mm follicles during the luteal phase of the oestrous cycle, the intrafollicular milieu of androstenedione, testosterone and oestradiol and the LH- and FSH-induced responses are extremely variable, suggesting that these large follicles are functionally active for only short-periods of time. Although the CL via its feedback effects on the hypothalamic-pituitary axis doesn't inhibit FSH-dependent events in the ovary, it suppresses markedly the secretions of LH and thus the level of LH-stimulable steroidogenesis in developing follicles. During the luteal phase of the oestrous cycle, the frequency of LH secretory episodes from the pituitary gland is  $< 1$  episode per 2 hours. During anoestrus, follicular steroidogenesis is also impaired by the low frequency of LH release due to increased sensitivity of the brain to steroid feedback at this time(16). After the onset of luteal regression, there is a small (2- to 5-fold) sustained ( $\sim 40$  hour) increase in the mean level of plasma LH resulting from an increased frequency of LH release (e.g., from  $< 1$  episode per 2 hours to 1 episode every 1.5 hours)(11)(15) (Fig. 1).

During the follicular phase, following a prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ )-induced luteolysis, there is a highly significant linear relationship ( $r = 0.98$ ;  $p < 0.001$ ) between the plasma concentrations of LH and the secretion-rate of oestradiol- $17\beta$  from the ovary containing the presumptive preovulatory follicle (Fig. 1). The significance of the follicular phase rise in the concentrations of plasma LH in preovulatory follicular maturation can be demonstrated in anoestrous ewes where the administration of exogenous LH for 20-72 h either as frequent intravenous LH 'pulses' (i.e., once every 20 min, 30 min, 60 min or 120 min) or as a constant infusion stimulated ovulation and normal CL function(11)(17).

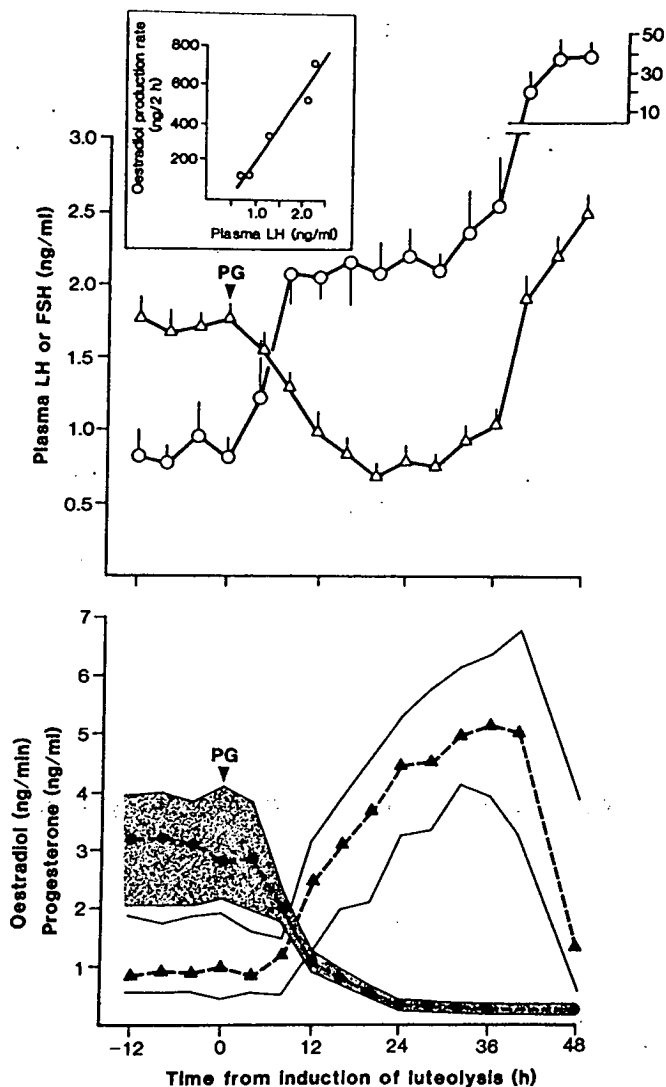


Fig. 1 Temporal relationships in ewes between: plasma LH (o — o); mean  $\pm$  s.e.m., FSH ( $\Delta$ — $\Delta$ ); mean  $\pm$  s.e.m. and progesterone ( $\bullet$  ---  $\bullet$ ; mean and range) concentrations and ovarian oestradiol secretion ( $\blacktriangle$ — $\blacktriangle$ ; mean and range) before and after the induction of luteolysis with cloprostenol (125  $\mu$ g, i.m.; PGF<sub>2 $\alpha$</sub>  analogue). Mean and range of oestradiol output was from the ovary containing the presumptive preovulatory follicle (n = 4-8 ewes per point). Insert refers to relationship between LH and the ovarian production-rate of oestradiol from the active oestrogen-secreting ovary during the first 12 h of a cloprostenol-induced follicular phase. The oestradiol production-rate was calculated after determining the secretion-rate at 2 hourly intervals in ewes under thiopentone anaesthesia at the time of sampling. The average level of plasma LH for each 2 hour interval per ewe was determined from the mean of 13 measurements made at 10 min intervals. All ewes were at day 10 of the oestrous cycle when treated with cloprostenol.

The question is often asked as to which pool of follicles provides the source of the preovulatory follicle? In luteal-phase ewes treated with  $\text{PGF}_{2\alpha}$ , it appears that the presumptive preovulatory follicle may emerge from any one of a number (i.e., 3-15) of healthy follicles  $\geq 2$  mm in diameter. In some animals, a large healthy and 'oestrogenic' follicle ( $\geq 5$  mm diam.) may be present at the onset of luteolysis. However, it seems that this follicle does not always go on to ovulate unless supplementary FSH is administered. Commonly, the largest healthy 'oestrogen-enriched' follicle is 2.5-4 mm in diameter at 6 h after luteolysis, 3-5 mm at 10 h and between 6-10 mm diam at ovulation some 60 h later.

The emergence of a dominant oestrogen-secreting follicle, some 10-24 h after PG-induced luteolysis is accompanied by a widespread increase in atresia (i.e., from 50 to 80%) to most other antral follicles ( $\geq 1$  mm diam). This wave of atresia can be prevented temporarily by injecting PMSG (500 i.u.) simultaneously with  $\text{PGF}_{2\alpha}$ <sup>(18)</sup>. The limitations imposed on the number of preovulatory follicles is determined around the time of luteolysis and can be influenced by the FSH concentrations during the 48 h interval both before and after luteolysis and by the number of follicles with a capacity for oestradiol synthesis and/or a capacity to be stimulated by FSH<sup>(15)(19)</sup>. As will be shown later, granulosa cells from follicles  $\geq 3$  mm diameter are between 2- to 100-fold more sensitive to FSH than follicles  $< 2$  mm in diameter (see section on effects of FSH and LH on ovarian cell function). Between 12 and 24 h after a  $\text{PGF}_{2\alpha}$ -induced luteolysis the plasma FSH concentrations fall to  $\sim 50\%$  of the pretreatment values (Fig. 1): it is likely that this reduction in FSH secretion is the principal cause in limiting the number of follicles that ovulate. The causal link between ovulation-rate and plasma FSH has recently been demonstrated by infusing exogenous FSH for 48 h from luteolysis in order to reduce the level of FSH suppression at that time<sup>(10)(19)</sup>. Infusion of ovine FSH (2.5  $\mu\text{g}$  or 5  $\mu\text{g}/\text{h}$  of NIADDK-oFSH-16) raised the plasma concentrations of FSH<sup>(10)</sup> and led to a significant increase in mean ovulation-rates (Table 2). If infusion of FSH from luteolysis was maintained for only 24 h or commenced 24 h after luteolysis, the ovulation-rate remained similar to that in control ewes. In contrast, supplementing the already elevated plasma LH concentrations (Table 2) with additional LH (NIADDK-oLH-S24; 5  $\mu\text{g}/\text{h}$ ) lowered the mean ovulation-rate. The results of this study show that continual exposure to FSH throughout the first 48 h after luteolysis is essential for multiple ovulation.

Collectively these findings highlight the importance of gonadotrophins on ovarian follicular development and on ovulation-rate. The purpose of the subsequent sections is to review some of the biochemical actions of FSH and LH on ovarian cell function.

### **FSH and LH binding to ovarian cells**

#### **FSH**

All known effects of FSH and LH are expressed via cell-surface receptors in ovarian cells. FSH receptors are detectable in granulosa cells during preantral development<sup>(20)</sup>. In sheep, a single class of FSH receptors has been identified in antral follicles. The maximum number of available FSH binding sites (i.e.,  $B_{\text{max}}$ ) and the equilibrium dissociation constant (Kd) are similar in both small (1-2.5 mm diameter) and large (3-7 mm



Table 2. Effect of treatment with ovine FSH or LH on ovulation rates in Romney ewes. Data from reference 19

Treatment	Ovulation rate					Geometric mean ovulation rate (95% confidence limits)
	0	1	2	3	4 to 14	
Controls	0	10	6	0	0	1.3 (1.1-1.6)
2.5 µg/h FSH	0	1	6	3	0	2.1 (1.7-2.7)
5.0 µg/h FSH	0	0	3	0	5	4.8 (2.4-8.8)
5.0 µg/h LH	2	6	0	0	0	0.7 (0.3-1.2)

diameter) follicles and are not found to differ between healthy or atretic follicles or between sheep breeds with different ovulation-rates (e.g., Romney's or Booroola Merino's with or without the fecundity F gene): the overall  $B_{max} = 17$  fmoles/mg cell protein which is equivalent to 800 receptors per cell and the overall  $Kd = 1.0$  nM<sup>(21)</sup>. There is no evidence for FSH receptors in thecal cells but some, albeit low, binding is detectable in the CL<sup>(21)</sup>. In general there is much more immunoreactive FSH in plasma than FSH receptors in ovarian cells. For example, a preovulatory follicle in the ewe contains 8.5 fmoles of available FSH receptor and overall there is approximately 400 fmoles of available FSH receptor sites per pair of ovaries, whereas in every millilitre of plasma there are 26000 fmoles of FSH. Paradoxically, the amount of FSH in plasma is still limiting since increasing the plasma FSH concentrations by exogenous administration increases the number of non-atretic follicles (>2.5 mm diameter) as well as the ovulation-rate<sup>(9)</sup> <sup>(10)</sup>. Thus, although there may be more hormone than receptor, several factors seem to mitigate against FSH binding to its receptor. For example, FSH binding is inhibited competitively by polyamines, phosphate ions, and both low and high molecular weight proteins in plasma and follicular fluid<sup>(22)</sup><sup>(23)</sup>. Moreover, FSH exists in different molecular forms and several may not bind to the receptor. In vitro, the total binding of <sup>125</sup>I-FSH to its receptor is between 1-30% at equilibrium. Moreover, under ideal in vitro conditions where isolated granulosa cells are washed free of follicular fluid, serum proteins and phosphate ions, FSH binding is rapid with 50% of the total fraction which binds being bound within 3 minutes and equilibrium established within 30 min. However, under in vivo conditions, granulosa cells are bathed in the above inhibitory substances and the time taken for FSH to traverse from the blood supply across the extravascular spaces and basement membrane to the granulosa cells is relatively slow. For example the time taken for FSH to equilibrate between follicular fluid and serum is 3-4 hours.

### LH

A single class of specific receptors for LH have been identified in theca interna cells in all-sized antral follicles irrespective of stage of health. LH receptors are also located in granulosa cells from preovulatory-sized follicles (>5 mm diameter) and in CL (Table 3), but not in granulosa cells from follicles <5 mm diameter, or the ovarian stroma.

During the luteal phase of the cycle, the LH binding  $B_{max}$  is low in the theca interna but high in granulosa cells of preovulatory-sized follicles and CL. In contrast, during the follicular phase when the CL are under-

Table 3. Binding constants for specific [ $^{125}I$ ]hCG binding<sup>+</sup> to ovine theca interna and granulosa cells of preovulatory sized follicles and corpora lutea during the luteal and follicular phases of the oestrous cycle. Values are means  $\pm$  s.e.m. (3 pools of cells/tissue per phase of the oestrous cycle).

Tissue	Kd (nM)		$B_{max}$ (fmoles/mg protein)	
	Luteal	Follicular	Luteal	Follicular
Theca Interna	0.10 <sup>a</sup> $\pm 0.01$	0.37 <sup>b</sup> $\pm 0.11$	8 <sup>a</sup> $\pm 1$	55 <sup>b</sup> $\pm 11$
Granulosa Cells	0.13 <sup>a</sup> $\pm 0.02$	0.10 <sup>a</sup> $\pm 0.01$	55 <sup>b</sup> $\pm 6$	61 <sup>b</sup> $\pm 7$
Corpora Lutea	0.34 <sup>b</sup> $\pm 0.03$	0.25 <sup>b</sup> $\pm 0.04$	52 <sup>b</sup> $\pm 4$	3 <sup>c</sup> $\pm 0.5$

Each value was obtained from pools of granulosa or theca cells from healthy or non-atretic follicles from 10-30 ewes. All luteal phase ewes were at Day 10 of the oestrous cycle (Day 0 = day of oestrus), whereas all follicular phase ewes were at 36 h after a 125  $\mu$ g cloprostenol injection (s.c.) given on Day 10 of the oestrous cycle.

<sup>+</sup> HCG = human chorionic gonadotrophin which is used as a surrogate for ovine LH. Data from reference 24 with permission.

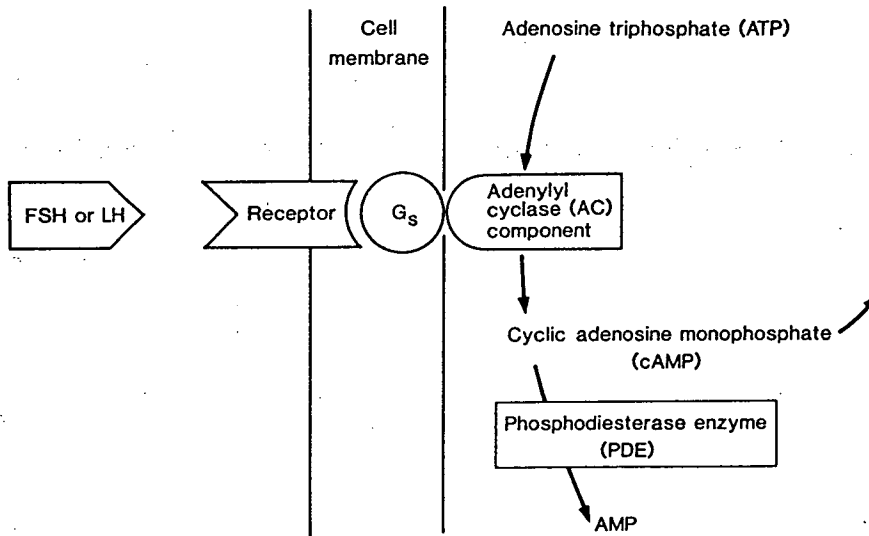
For Kd or  $B_{max}$ , the values which have different alphabetical superscripts in either rows or columns are significantly different from one another ( $p < 0.05$ ).

going regression, few LH receptors are found in CL tissue but high LH receptor binding is found in theca and granulosa cells. The factors which control LH receptor populations in the ovine cell-types are not known. From studies in rats, it is thought that LH receptor induction in granulosa cells is under the control of FSH and oestradiol. The avidity of LH for its receptor, as assessed from the Kd value, is 3-10 fold higher than that of FSH for the FSH receptor. In granulosa cells and CL, the  $B_{max}$  values for the LH receptor during the follicular phase (Table 3) correspond to 3600 receptors per cell. For the theca interna, the relationship between protein concentration and number of theca interna cells is not known, thus the number of receptors per theca cell cannot be estimated. Relatively little is known about potential inhibitors of LH binding to LH receptors. Under *in vitro* conditions, LH/hCG receptor binding is less susceptible to interference by buffers, ions, etc., than is the case for FSH. Although ovine LH is thought to exist in several different forms, the proportion of an LH preparation capable of binding to a receptor is much greater than that for FSH (e.g., 30-50% vs 1-30%). In ovine follicles  $\geq 5$  mm diameter, there are more receptor binding sites for LH than for FSH with LH binding more avidly. As will be shown later,

LH is much more potent on a molar basis than FSH in stimulating cellular metabolism for reasons outlined above although it may also be due, in part, to the relative purities of currently available standards of FSH and LH. In contrast to FSH which is always measurable in plasma at concentrations between 0.5 and 4 ng/ml, the immunoreactive concentrations of LH fluctuate between undetectable values (i.e., <0.1 ng/ml) and 10 ng/ml. When present at >1 ng/ml, the amount of LH per ml exceeds the number of follicular or CL receptors. The dynamics of LH interaction with its ovarian receptors are probably more closely related to the pulsatile pattern of LH concentrations in plasma than to putative inhibitory agents in plasma or follicular fluid.

### Effects of FSH and LH on ovarian cell function

After binding to ovarian cells, LH and/or FSH initiate cytodifferentiation, mitotic activity, follicular fluid accumulation or steroidogenesis. All of these cellular responses are mediated, at least in part, via the synthesis of cyclic 3',5'-adenosine monophosphate (cAMP). The level of cAMP which is synthesized is dependent, upon the FSH or LH signal being transmitted from the receptor across the cell membrane via regulatory (G) and catalytic (AC, adenylate cyclase enzyme) components (Fig. 2).

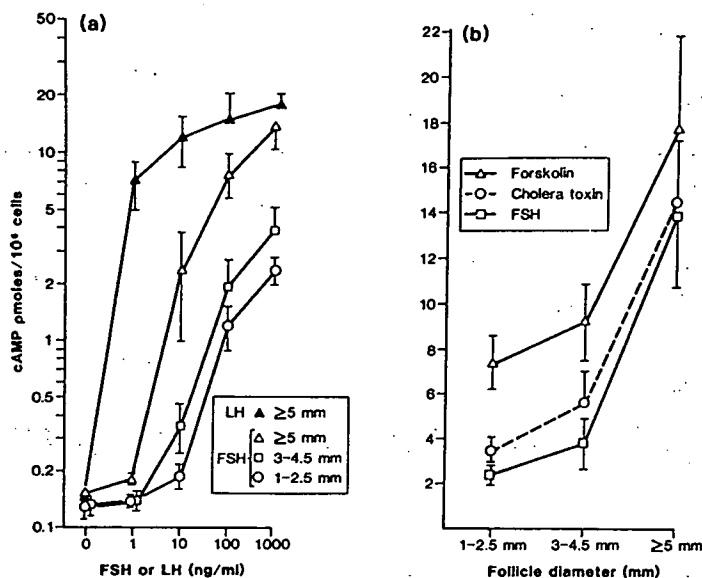


**Fig. 2** G<sub>s</sub> = the stimulatory G-protein complex which transmits the receptor encoded message to the catalytic (AC, adenylate cyclase enzyme) component. G<sub>s</sub> induction of cAMP can be stimulated with cholera toxin, AC stimulation of cAMP can be activated with forskolin which is a plant diterpene that stimulates adenylate cyclase in many mammalian tissues (see ref. 25 for review).

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When produced, the intracellular level of cAMP is influenced by the activity of a phosphodiesterase enzyme (PDE) which can degrade cAMP to AMP.

Several of these membrane and enzyme characteristics have been examined in ovine granulosa cells from antral follicles at different stages of development. These data with respect to cAMP synthesis and metabolism are summarized in Fig. 3 and Table 4. The results show that granulosa cells become increasingly sensitive to FSH as follicle size increases (i.e., the same amount of cAMP can be produced in response to smaller amounts of FSH with respect to follicle size). The increase in cell sensitivity to FSH with increasing follicle size is not due to any change in FSH receptor number(21) or to any difference in cAMP-PDE activity in healthy follicles (Table 4). However, the increase in cell sensitivity to FSH can be mimicked with cholera toxin and forskolin (Fig. 3). The finding that the level of forskolin-stimulable cAMP activity increases



**Fig 3(a).** Effects of different doses of FSH (NIADDK-oFSH-S16) or LH (NIADDK-oLH-S24) on cAMP synthesis by ovine granulosa cells over 45 minutes *in vitro*. The cells were recovered from non-atretic follicles and incubated in Dulbecco's phosphate buffered saline (PBS) with 0.1% (w/w) bovine serum albumin (BSA) and a phosphodiesterase inhibitor methylisobutyl xanthine (MIX; 0.2 mM).

**(b).** Effects of forskolin ( $10^{-4}$  M), cholera toxin (500 ng/ml) or FSH (1000 ng/ml) on cAMP synthesis by ovine granulosa cells from different-sized non-atretic follicles incubated in PBS + 0.1% BSA + 0.2 mM MIX. Values are means  $\pm$  s.e.m. from 4-6 separate experiments.

2-3 fold between small and large follicles (Fig. 3) suggests that the level of FSH-induced cAMP synthesis with follicle size is, at least in part, directly related to either the level of adenylate cyclase (AC) enzyme activity or to the total amount of AC per se. The factors which regulate the AC component in ovine cells are not known.

In granulosa cells from large (>5 mm diameter) follicles relatively low doses of LH (compared to FSH) will stimulate peak cAMP synthesis (Fig. 3a). This ability of low doses of LH to generate peak cAMP is probably related in part to the high LH receptor number (e.g., LH receptor numbers 3600/cell vs FSH receptor numbers 800/cell) as well as the high avidity of LH receptor binding.

During follicular atresia, there seems to be no major loss in the number or affinity of the FSH or LH receptors but there is a significant reduction in the level of FSH or LH-stimulable cAMP and this in part is due to an increase in the level of cAMP-PDE activity (Table 4).

**Table 4.** Cyclic AMP-phosphodiesterase activity (cAMP-PDE) in ovine granulosa cells with respect to follicle size and health. Values are means  $\pm$  s.e.m. (n = 15 separate experiments per test).

Source of granulosa cells	Cyclic AMP-PDE activity (pmoles cAMP metabolized per min)
Small healthy follicles (1-2.5 mm diam.)	0.19 $\pm$ 0.04 <sup>a</sup>
Small atretic follicles (1-2.5 mm diam.)	0.34 $\pm$ 0.03 <sup>b</sup>
Large healthy follicles (>3 mm diam.)	0.14 $\pm$ 0.02 <sup>a</sup>
Large atretic follicles (>3 mm diam.)	0.26 $\pm$ 0.03 <sup>b</sup>

a v b; p<0.01

Compared to the pig, cow, human or rat, granulosa cells from the sheep are relatively inactive in synthesizing steroids *de novo* in response to FSH, LH or cAMP. This difficulty in measuring steroidogenesis in ovine cells has presented problems in establishing the interrelationships among gonadotrophins, cAMP and the levels of steroid production with respect to follicle growth and atresia. Nevertheless, as healthy but not atretic ovine follicles increase in diameter, they develop an increasingly greater capacity to metabolise (i.e., aromatise) androstenedione or testosterone to oestradiol-17 $\beta$ . For example, as follicles enlarge from 1-2.5 mm to 3-4.5 mm to >5 mm in diameter, the respective geometric mean (95% confidence limits), levels of aromatase activity increase significantly (p<0.05; between each follicle size range) from 0.2 (0.2,0.3) to 1.4 (0.6,2.6) to 3.9 (2.1,6.6) ng oestradiol per 10<sup>6</sup> cells per 3 hours. Also, a significant positive relationship between the level of FSH or LH induced cAMP synthesis and the ability of ovine granulosa cells to aromatise testosterone to oestradiol can be demonstrated (Table 5).

However, under *in vitro* conditions, it has not been possible to influence directly the level of aromatase enzyme activity with FSH, LH or cAMP

suggesting that other factors may also be involved in the control of oestradiol synthesis. For example, it is possible that second messenger systems other than cAMP which mediate the actions of certain growth factors (e.g., IGF<sub>1</sub>, basic fibroblast growth factor, bFGF; TGF $\beta$ ) may also influence aromatase enzyme induction/activation.

### Thecal Cells

Unlike granulosa cells, ovine or bovine thecal interna cells are capable of active de novo synthesis of steroids<sup>(24)</sup>. Thecal steroidogenesis is LH dependent and is mediated by cAMP. The in vitro level of production is influenced by the rate of medium perfused across the tissue which is in contrast to granulosa cells whose steroidogenic capacity is not quantitatively different between static and perfusion culture systems. The significance of medium flow-rates on thecal steroidogenesis may infer that the extent of the capillary blood-flow through the theca interna is an important regulatory factor. At present little is known about LH-receptor transmission signal across the thecal cell membrane or the levels of adenylate cyclase or cAMP-PDE activity during follicular maturation. However, in thecae from healthy follicles, the major steroids that are synthesized are the androgens, androstenedione, testosterone and dehydroepiandrosterone. The level of LH-induced steroidogenesis is influenced significantly by the LH concentration, the duration of LH exposure and previous exposure of the tissue to LH. In vitro, the thecal tissue is also acutely sensitive to low amplitude LH pulses<sup>(24)</sup>.

Table 5. Frequency table showing the number of follicles with granulosa cells having a certain level of aromatase activity and FSH (NIADDK-oFSH-S16, 100 ng/ml)- or LH (NIADDK-oLH-S24, 100 ng/ml)-induced cAMP

FSH- or LH-induced cAMP synthesis (pmol/10 <sup>6</sup> cells per h)	Aromatase activity (ng oestradiol-17 $\beta$ /10 <sup>6</sup> cells per 3 h)		
	<1	1-5	>5
FSH <1	22	5	0
1-5	8	3	2
>5	0	2	6
LH <1	27	0	0
1-5	10	1	2
>5	0	2	6

For both FSH and LH treatments there was a significant positive relationship between the ability of granulosa to synthesize oestradiol from testosterone and the level of cAMP the cells could produce in response to FSH or LH (contingency table analysis; p<0.01). Data from reference 26.

During follicular atresia, there is no loss of LH receptor in thecal tissue but there is a marked reduction in the level of cAMP and androgen synthesis with a corresponding increase in progesterone synthesis<sup>(27)</sup>. During the final stages of atresia, the LH receptor is retained but there is no cAMP or steroid synthesized. A question that remains unanswered

is whether the level of AC activity declines during atresia and/or whether there is a marked increase in cAMP-PDE activity. From studies in rats, the ontogeny of LH-induced thecal steroidogenesis is suggested to be in the reverse order to that described for atresia<sup>(28)</sup>.

Thus thecal cell function differs from that in granulosa cells in several important aspects. Thecal cells at all stages of antral follicle growth are acutely sensitive to pulsatile changes in LH concentrations. In contrast, granulosa cells only acquire LH receptors in preovulatory-sized follicles and by being in an avascular environment they are less likely to respond rapidly to short-term changes in LH secretion. Thecal cells synthesize androgens which can serve as substrates for oestradiol-17 $\beta$  formation by granulosa cells whereas the latter cell-type is incapable of synthesizing androgens de novo. In vitro thecal- but not granulosa-cell steroidogenesis is influenced markedly by the flow-rate of the medium: this in turn suggests that locally-produced vaso-active agents may be important in regulating thecal steroidogenesis during both follicle growth and atresia.

### Summary

Ovarian follicular development from 2.5 mm in diameter to ovulation is critically dependent upon FSH and LH but the role of these hormones during preantral and early antral development remain unclear. FSH and LH exert their effects via specific membrane receptors on granulosa or thecal cells: these effects are transmitted across the respective cell membranes via regulatory (G) and catalytic components (i.e., the adenylate cyclase enzyme) and lead to an increase in the intracellular levels of cAMP. FSH-induced cAMP synthesis in granulosa cells together with another factor(s), yet to be defined, lead to the induction or activation of aromatase enzyme activity and eventually the formation of LH receptors. In thecal cells, LH-induced cAMP synthesis leads to the de novo synthesis of androstenedione and testosterone and these are the substrates utilized by granulosa cells for the synthesis of oestradiol. The evidence suggests that FSH determines the number of healthy follicles that develop beyond 2.5 mm in diameter and that the concentrations of FSH around the time of luteolysis have a major influence on the ovulation-rate. In contrast it is the frequency (as well as the amplitude) of the LH secretory episodes which ultimately determines the level of follicular oestradiol synthesis. In the ewe, the pattern of LH secretion capable of stimulating preovulatory oestradiol synthesis to a level which initiates oestrous behaviour, the preovulatory LH surge and ovulation, occurs only during the follicular phase of the oestrous cycle: at this time LH is released at a frequency of 1 LH secretory episode every 60 min for at least 20 hours.

**Acknowledgements:** Most of the data in this review was obtained with the assistance of N.L. Hudson, S. Lun, D.A. Heath, K. Ball, P. Smith and J. Fannin at the Wallaceville Animal Research Centre and their skilled technical work is gratefully acknowledged. We also wish to thank the National Hormone and Pituitary Program and the National Institute of Diabetes and Digestive and Kidney Diseases, Maryland, U.S.A. for supplying the ovine pituitary hormones, and Mrs P. Cattermole for typing the manuscript.

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**Section 3: Papers related to studies of inhibin**

Paper no.: 27

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M.T. Hazeer-Hagelstein, M.T., C. Charlet-Renard, A.  
Demoulin, J.P. Bourguignon & M.J. Lecomte-Yerna

Title: Inhibin: mechanisms of action and secretion.

Reference: Intraovular Regulation of Reproduction (1981) pp.  
167-191. Eds P. Franchimont and C.P. Channing,  
Academic Press

CHAPTER IX  
INHIBIN: MECHANISMS OF ACTION AND SECRETION

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Since 1972, numerous investigations have been directed towards the demonstration of the existence of inhibin, a gonadal peptide which specifically or preferentially decreases the secretion of follicle stimulating hormone (FSH).

Inhibin has been detected in and partially purified from human seminal fluid (Franchimont, 1972; Franchimont *et al.*, 1975b; Scott and Burger, 1980), bovine seminal fluid (Franchimont *et al.*, 1975a; Sairam *et al.*, 1978; Chari *et al.*, 1978), ram rete testis fluid (RTF) (Setchell and Sirinathsingji, 1972; Setchell and Jacks, 1974; Baker *et al.*, 1976; Davies *et al.*, 1979), extracts of spermatozoa (Lugaro *et al.*, 1974; Setchell and Main, 1974), testicular extracts (Lee *et al.*, 1974; Keogh *et al.*, 1976; Baker *et al.*, 1976; Moodbidri *et al.*, 1976), ovarian extracts (Hopkinson *et al.*, 1975, 1977a; Chappel *et al.*, 1978), bovine follicular fluid (De Jong and Sharpe, 1976; Welschen *et al.*, 1977), porcine follicular fluid (Welschen *et al.*, 1977; Marder *et al.*, 1977; Lorenzen *et al.*, 1978), human follicular fluid (Chari *et al.*, 1979), as well as the culture medium of Sertoli cells (Steinberger and Steinberger, 1976) and granulosa cells (Erickson and Hsueh, 1978).

A number of investigators have attempted to purify this hormone (see De Jong *et al.* and Sairam in the present volume). At present, there are few, however, who believe that a completely pure preparation is available; its chemical structure and physico-chemical properties are still highly controversial (De Jong *et al.*, this volume; Sairam, this volume).

The physiological role of this hormone, its actions on the pituitary, the hypothalamus and the gonads, its possible synergism with the sex steroids and the mechanism which control its secretion have also been studied extensively and are considered in the following chapters.

Our own contribution will be to review the sites and modes of action of inhibin and to show that certain mechanisms controlling its secretion by granulosa and Sertoli cells are identical.

### Sites and Mechanisms of Action of Inhibin

Inhibin appears to act at various sites: the pituitary, the hypothalamus and the gonads themselves.

#### *Pituitary Action*

Inhibin certainly acts on the pituitary. Whether it is extracted from human seminal plasma, RTF (Setchell *et al.*, 1977; Baker *et al.*, 1976; Franchimont *et al.*, 1978, 1979; Lee *et al.*, 1979), ram testicular lymph (Baker *et al.*, 1978), Sertoli cell culture medium (Steinberger and Steinberger, 1965; Lagace *et al.*, 1979; De Jong *et al.*, 1978), or follicular fluid (De Jong *et al.*, 1978; Shander *et al.*, 1979), inhibin exerts an effect on basal FSH levels and on the FSH response to LH-RH in isolated cultured pituitary cells (Fig. 1). The inhibitory effect is highly specific for FSH in basal conditions and corresponds to a decrease in FSH synthesis. Thus, *in vitro*, various preparations of inhibin (extract of RTF, follicular fluid, etc.) lead to a concomitant reduction of the quantity of FSH in the culture medium and within the cells after 72 hours of incubation in the absence of LH-RH (Fig. 2). This action is more marked on the cell content than on the quantities of FSH released into the culture medium. Under our chosen experimental conditions, no effect was observed on the quantities of LH present in either the culture medium or the cells. These actions on the quantities of FSH in the two compartments show that the inhibin preparations tested have an effect on FSH synthesis under basal conditions. In fact, if the actions were limited to an inhibition of FSH release, the level of FSH would be reduced in the culture medium, whereas the quantities of FSH in the cells would remain the same or be even greater than in the control cells (Franchimont *et al.*, 1978).

Convincing evidence of the action of inhibin on FSH synthesis has been provided by the experiments of Chowdhury *et al.* (1978). These authors studied the incorporation of (<sup>3</sup>H)-leucine into FSH and LH produced by organ cultures of rat anterior pituitaries cultured in a

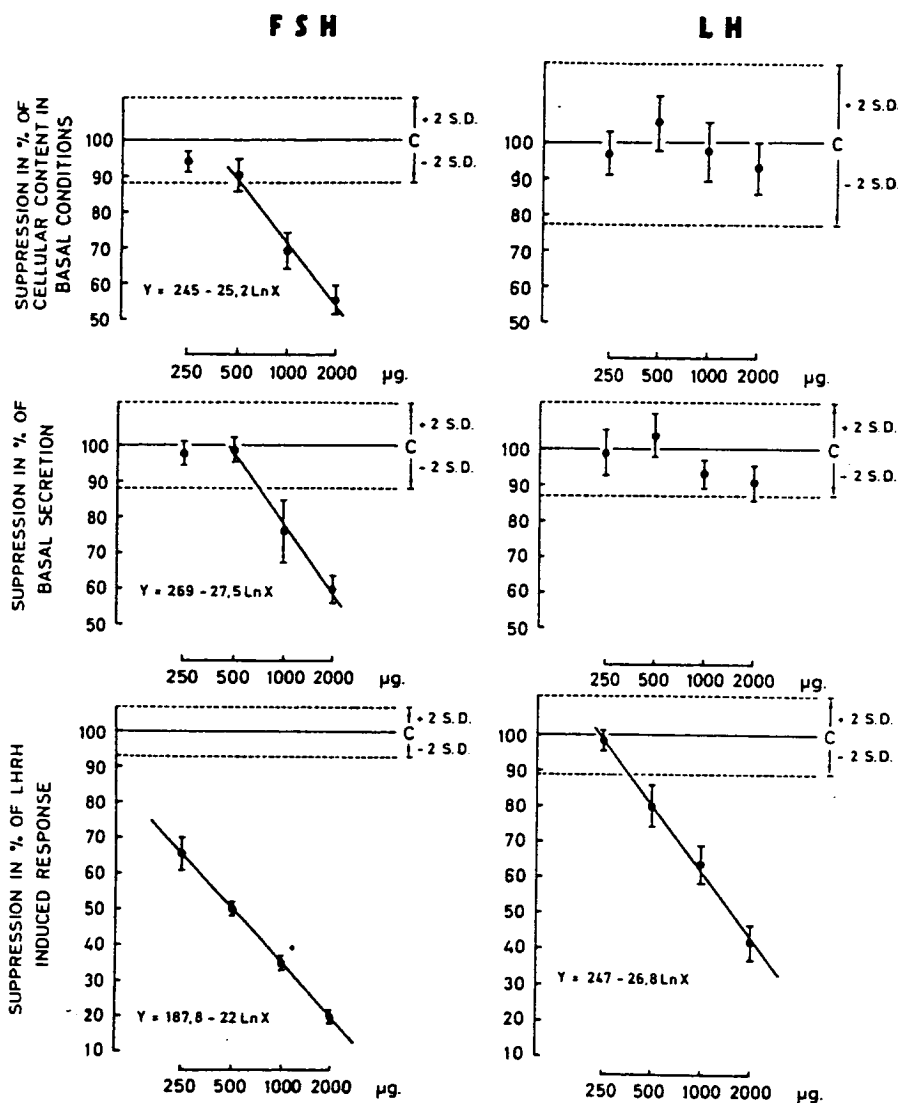


Fig. 1: Effect of an inhibin preparation extracted from ovine testicular lymph on FSH (left panel) and LH (right panel) production by dispersed pituitary cells: effect on intracellular gonadotrophin content (upper part) and release in culture medium (Middle part) in basal condition. This preparation was also tested on LH-RH-induced gonadotrophin response (lower part). Results are expressed in percent of the control value (C) i.e. in absence of inhibin, representing 100 per cent. Ovine testicular lymph (OTL S) is, in fact, the reference preparation used in the assay of inhibin. These inhibin curves represent the mean curves calculated from 17 individual experiments.

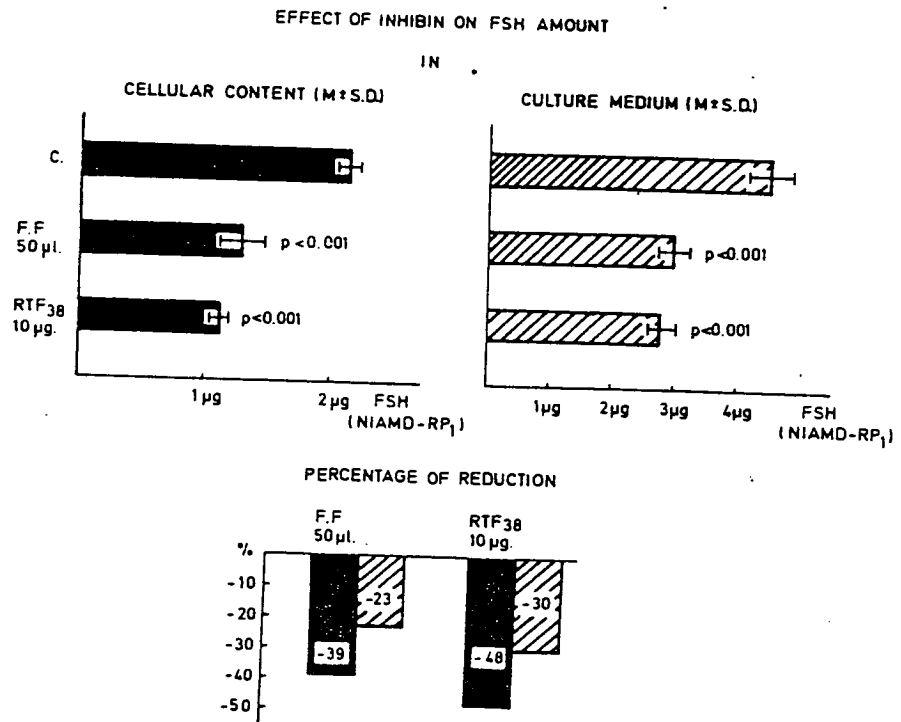


Fig. 2: Effect of 50 µl of steroid-free bovine follicular fluid (F.F.) and 10 µg of ram rete testis fluid extract (RTF<sub>38</sub>) on amounts of follicle-stimulating hormone (FSH) cell content and in culture medium after 3 days of incubation. Bar C: Control values observed in the absence of inhibin preparations. The upper part of the graph indicates absolute FSH amounts ± SD, and the lower part represents the reduction of FSH amounts in cellular content (black columns) and in culture medium (hatched columns) expressed as percentage, the control values representing 0 per cent. Under these experimental conditions, no effect was observed on luteinizing hormone intracellular and culture medium contents (from Franchimont *et al.*, 1979, with permission).

medium previously used for 2-5 days to culture Sertoli cells. The Sertoli cell factor present in the culture medium selectively reduced the incorporation of labelled leucine into immunoprecipitable FSH without decreasing its incorporation into LH.

As both these experiments were performed *in vitro* in the absence

of LH-RH, it is clear that the observed effect was caused by a direct inhibin-like action on FSH synthesis by the gonadotroph. When gonadotrophin release is stimulated by LH-RH *in vitro*, inhibin exerts an effect not only on FSH, but also on LH release induced by LH-RH. Nevertheless, its action is more selective on FSH secretion. In fact, the minimum dose required to lower FSH levels significantly is much less than the dose that reduced LH levels. In Fig. 1, it is clear that a significant inhibition of LH-RH-induced FSH release was obtained with 250  $\mu\text{g}$  OTL S, whereas 1000  $\mu\text{g}$  of OTL S were needed to produce a significant reduction of LH levels under the same conditions.

The degree of inhibitory activity is quantitatively and temporally cumulative, since an increase both in the dose and length of exposure resulted in an increased degree of inhibition of pituitary release of FSH both in the presence and in the absence of LH-RH.

Inhibition by inhibin preparations is a reversible process. When pituitary cells are exposed to porcine follicular fluid for an initial 24 hour period, basal secretion is suppressed but this effect disappears when the fluid is removed during the second 24 hour incubation interval (Shander *et al.*, 1980).

Inhibin, whether it be an extract of RTF or of bovine or human seminal fluid, has no effect on TSH, prolactin, or growth hormone either *in vivo* or *in vitro* (Franchimont *et al.*, 1975b, 1978).

#### *Hypothalamic Action*

The data of Lugaro *et al.* (1974) suggested an action of inhibin at the level of the hypothalamus. These investigators injected their native extract (100 ng) prepared from bull spermatozoa into the third ventricle and observed a reduction in the levels of FSH. In contrast, no effect on LH was seen.

*In vitro*, we have shown that inhibin preparations extracted from HSP and RTF decrease the endogenous LH-RH content of isolated hypothalami of rats after short-term incubation with several concentrations of inhibin (Demoulin *et al.*, 1979a).

More recently, Lumpkin *et al.* (1981) demonstrated that inhibin preparations purified from ram rete testis fluid preferentially inhibit FSH secretion in the adult male rat by hypothalamic mechanism. Thus, when inhibin was injected into the third ventricle in castrated rats, plasma FSH levels decreased throughout the 24 hour post-injection period, whereas FSH levels increased steadily in controls. Plasma LH levels were not significantly different at any time com-

pared with controls. FSH and LH releases induced by a LH-RH challenge given 6 hours post-injection were similar in inhibin and control groups, thus, arguing against a pituitary site of action of the inhibin injected into the third ventricle.

#### *Direct Action on the Gonad*

As shown in chapter XV (Demoulin *et al.*, 1981), the incorporation of tritiated thymidine into testicular DNA was studied *in vitro* in normal pubertal rats, aged 42 days, weighing 150 g, and showing spermatozoa and/or spermatids from stage 12 in 40 per cent seminiferous tubules. Certain inhibin preparations exert a direct inhibitory effect on the synthesis of DNA by actively dividing spermatogonia at the beginning of spermatogenesis.

Furthermore, certain inhibin preparations (e.g. two fractions extracted from rete testis fluid, RTF<sub>38</sub>, M.W. > 10,000 and RTF<sub>3</sub>, M.W. < 5,000) decrease progesterone secretion from cultured granulosa cells (Henderson and Franchimont, 1981). Granulosa cells from large bovine antral follicles were cultured using the method of Henderson and Moon (1979). The addition of inhibin preparations derived from RTF to the culture medium decreased progesterone production after 48 and 72 hours incubation (Fig. 3). Denaturation of the inhibin preparations with heat (80° for 60 min) and with trypsin lead to loss of this direct inhibitory effect.

The lack of absolutely pure inhibin preparations, however, makes it impossible to decide whether these direct effects on gonadal cells are due to the inhibin itself, or to contamination of the preparations which have been tested with biologically active protein factors. Substances in follicular fluid are known to reduce progesterone secretion *in vitro* e.g. oocyte maturation inhibitor (Channing *et al.*, 1977), luteinization inhibitor (Ledwitz-Rigby *et al.*, 1979), LH receptors binding inhibitor (Yang *et al.*, 1979), the gonadocrinins (Ying and Guillemin, 1979). Whether rete testis fluid and seminal plasma contain these same factors is currently unknown.

#### **Follicular Inhibin Production**

In several animal species, inhibin activity has been found in follicular fluid pre-treated with charcoal to remove sex steroids (De Jong and Sharpe, 1976; Welschen *et al.*, 1977; Schwartz and Channing, 1977; Marder *et al.*, 1977; Franchimont *et al.*, 1979b). It appears to be produced by granulosa cells. Erickson and Hsueh (1978) and Channing *et al.* (1980) showed that cultured granulosa cells secrete a



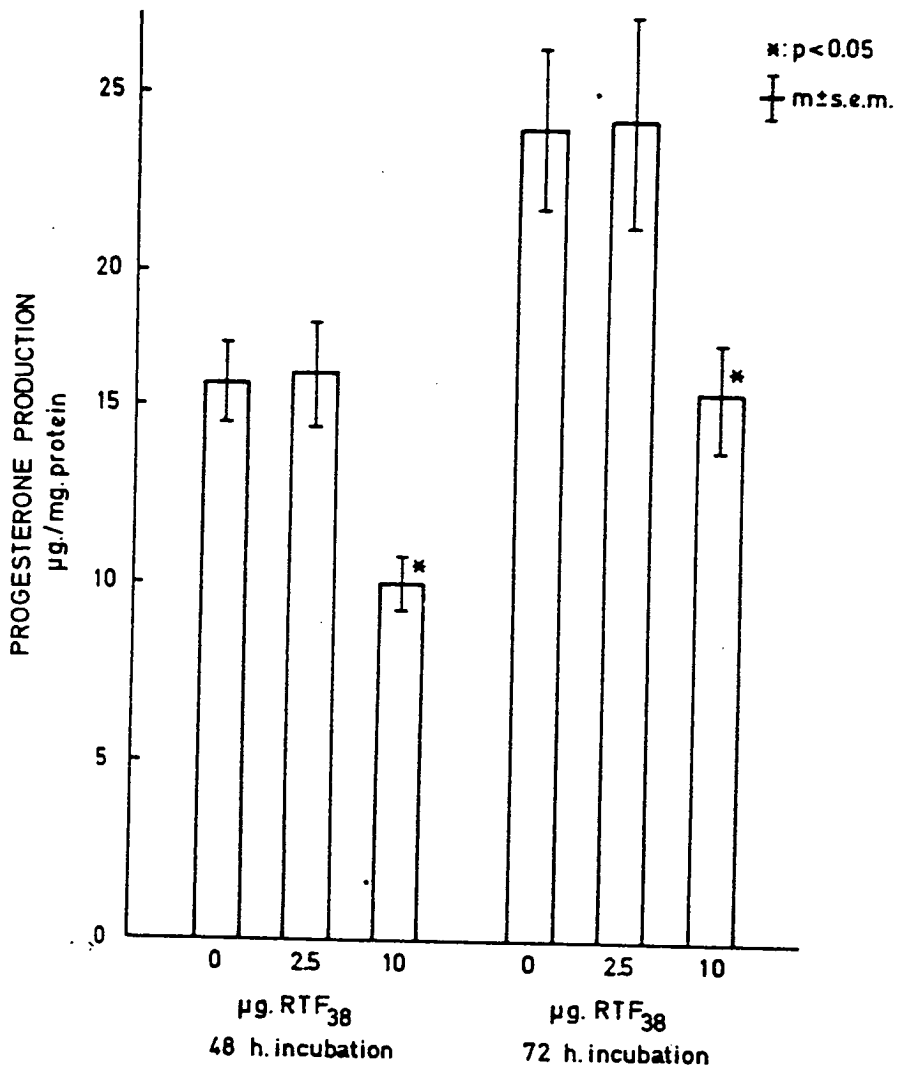


Fig. 3: Inhibin-induced reduction of progesterone secretion by granulosa cell *in vitro* after 48 and 72 hours of incubation.

substance that acts directly on cultured pituitary cells and preferentially suppresses FSH secretion.

We shall examine the mechanisms which control the secretion of inhibin by granulosa and luteal cells *in vitro*, and the relationship between steroid and inhibin concentrations in bovine follicular fluid.

#### *Mechanisms of Inhibin Secretion from Granulosa and Luteal Cells*

To investigate the mechanisms of inhibin secretion, monolayer cultures of granulosa and luteal cells were established according to the method of Henderson and Moon (1979), in the presence of 10 per cent calf serum. Granulosa cells were obtained from large antral follicles (7-15 mm) and luteal cells from bovine corpora lutea (Henderson and Franchimont, 1981). Inhibin was assayed in the culture media on the basis of the inhibition of LH-RH-induced FSH secretion (Fig. 1) by dispersed pituitary cells (Lee *et al.*, 1979; Franchimont *et al.*, 1979b). The reference preparation was derived from ovine testicular lymph (OTL S) given an arbitrary potency 1 U/mg (Hudson *et al.*, 1979). Follicular fluids and culture medium were always pretreated with charcoal (1 per cent) dextran (0.1 per cent) at 4° for 16 hours in order to remove sex steroids.

Granulosa cells produced inhibin and progesterone on the first day of culture. On subsequent days, progesterone secretion increased presumably as a result of luteinization, whilst inhibin production fell. The results of several experiments showed that there was an inverse relationship between progesterone secretion and inhibin production by granulosa cells in monolayer culture (Fig. 4, Henderson and Franchimont, 1981).

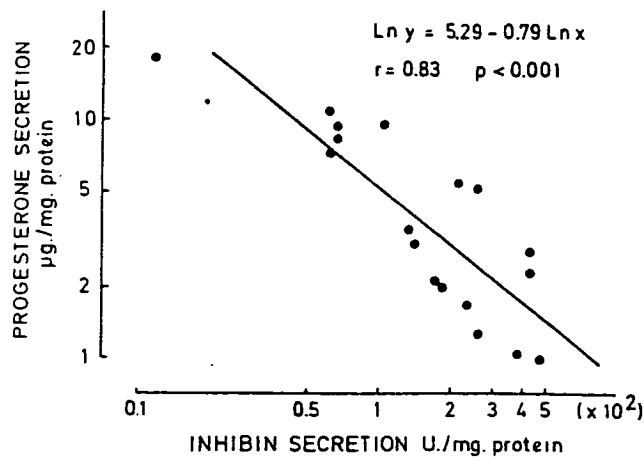


Fig. 4: Inverse relationship between progesterone and inhibin production by granulosa cells after the first and the second day of culture.

Neither purified porcine follicle stimulating hormone (FSH) nor luteinizing hormone (LH) (UCB, Brussels, Belgium, 100-1,000 ng/ml) increased inhibin secretion, although progesterone production was increased. The lack of gonadotrophin effect on inhibin secretion was confirmed by Croze and Franchimont, (1981). Human chorionic gonadotrophin (LH-like gonadotrophin, 5-100 mIU/ml) and pregnant mare serum gonadotrophin (PMSG, predominantly FSH-like gonadotrophin, 0.02 to 1 U/ml) failed to increase inhibin production by immature rat granulosa cells.

The addition of both aromatisable (androstenedione and testosterone) and non-aromatisable (dihydrotestosterone) androgens to the culture medium of granulosa cells led to a marked increase in inhibin production without any change in progesterone secretion (Fig. 5). Oestradiol-17  $\beta$  secretion was increased on the first day of culture in the presence of testosterone and androstenedione, but was subsequently unchanged. Dihydrotestosterone, which cannot be aromatised, did not lead to any significant change in oestradiol-17  $\beta$  secretion. Oestrogens (oestrone and oestradiol-17  $\beta$ ) in doses of 1 to 5  $\mu$ g/ml of culture medium did not change inhibin secretion from granulosa cells. Progesterone, on the other hand, led to a decrease (Fig. 5).

Luteal cells produce large amounts of progesterone in culture. However, inhibin is not detectable. The addition of testosterone (1 and 5  $\mu$ g/ml) has no effect on luteal cells, in contrast to its stimulatory effect on inhibin secretion by granulosa cells.

It may be concluded that inhibin production is decreased when the granulosa cell undergoes luteinization and increases its secretion of progesterone. Luteal cells fail to produce detectable quantities of inhibin. The addition of exogenous progesterone significantly decreases inhibin secretion by cultured granulosa cells. Both aromatisable and non-aromatisable androgens stimulate inhibin secretion by these cells. They appear to act directly and not by being aromatised (as can occur with testosterone and androstenedione). In fact, oestrogens have no effect on inhibin production, while dihydrotestosterone, which cannot undergo aromatisation, is the most potent stimulus of inhibin secretion. Finally, under the conditions of our experiments, the gonadotrophins do not appear to have any direct effect on inhibin secretion by granulosa cells.

#### *Inhibin Concentration in Follicular Fluid. Relationship with Sex Steroid Concentration*

In several animal species, inhibin activity has been found by *in vivo*

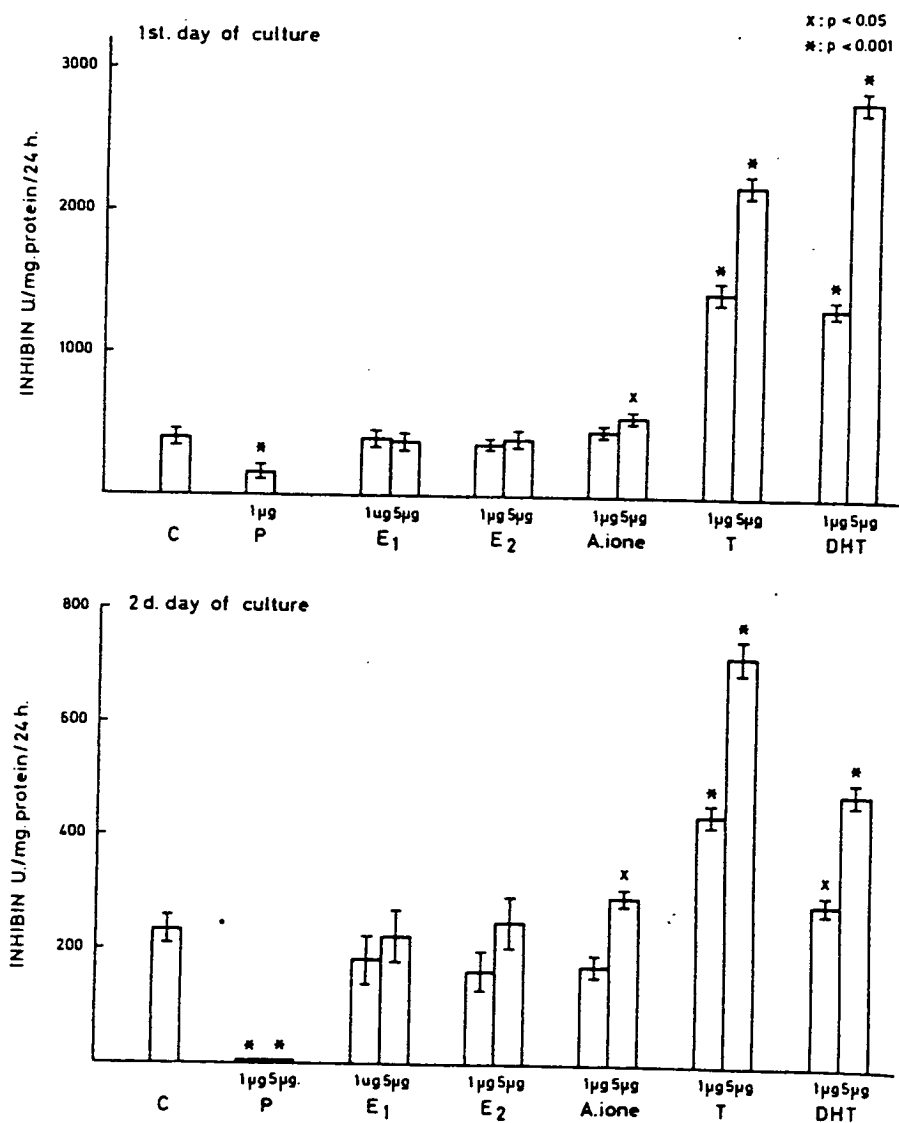


Fig. 5: Effect of several sex steroids at the concentrations of 1 µg/ml and 5 µg/ml on inhibin production (expressed in units per mg protein) by cultured granulosa cells. P = progesterone; E<sub>1</sub> = estrone; E<sub>2</sub> = oestradiol; A.ione = androstenedione; T = testosterone, DHT = dihydrotestosterone. Results are given for the first (upper part of the graph) and second (lower part of the graph) day of culture. Each bar represents the mean value ± S.E.M. calculated from five individual cultures. The stimulatory effect of T and DHT is dose-dependent. C = control.

assays in follicular fluid pretreated with charcoal to remove sex steroids (De Jong and Sharpe, 1976; Welschen *et al.*, 1977; Marder *et al.*, 1977; Lorenzen *et al.*, 1978).

Data from the literature are contradictory concerning the levels of ovarian inhibin during follicular development. According to Lorenzen *et al.* (1978), the concentration of inhibin diminishes with the growth of the follicle in the pig. In contrast, Welschen *et al.* (1977) found inhibin in small bovine follicles (5-10 mm in diameter) and maximum concentrations were reached in medium and large (11-20 mm in diameter) follicles. By assaying inhibin *in vitro*, we have found that the concentration of inhibin is higher in small follicles from which a small volume of fluid is collected. In contrast, when the volume of follicular fluid increases, the inhibin concentrations decrease (Fig. 6).

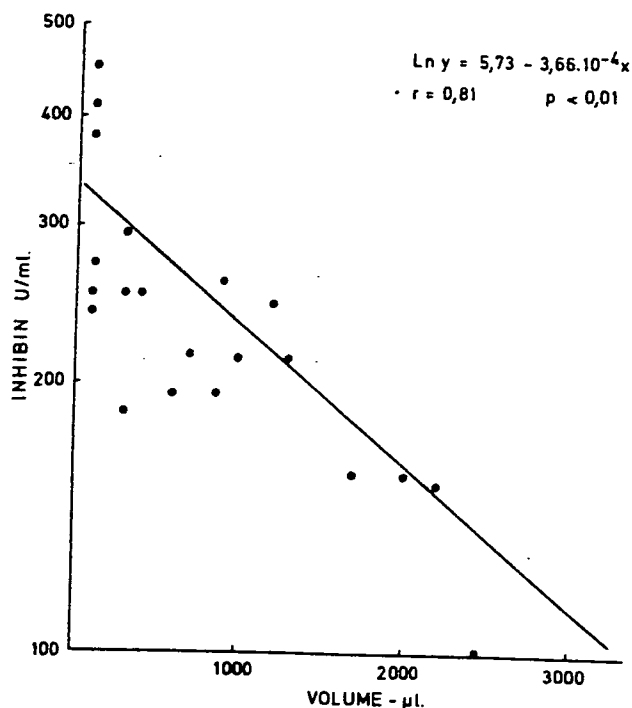


Fig. 6: Inverse relationship between volumes collected from follicles and inhibin concentration.

There is, in addition, an inverse relationship between the concentrations of inhibin and of oestradiol-17  $\beta$  (Fig. 7) and a direct relation-

ship between those of inhibin, testosterone and androstenedione, all three of which decrease with follicular development (Table 1). There is no relationship between progesterone and inhibin concentrations in follicular fluid.

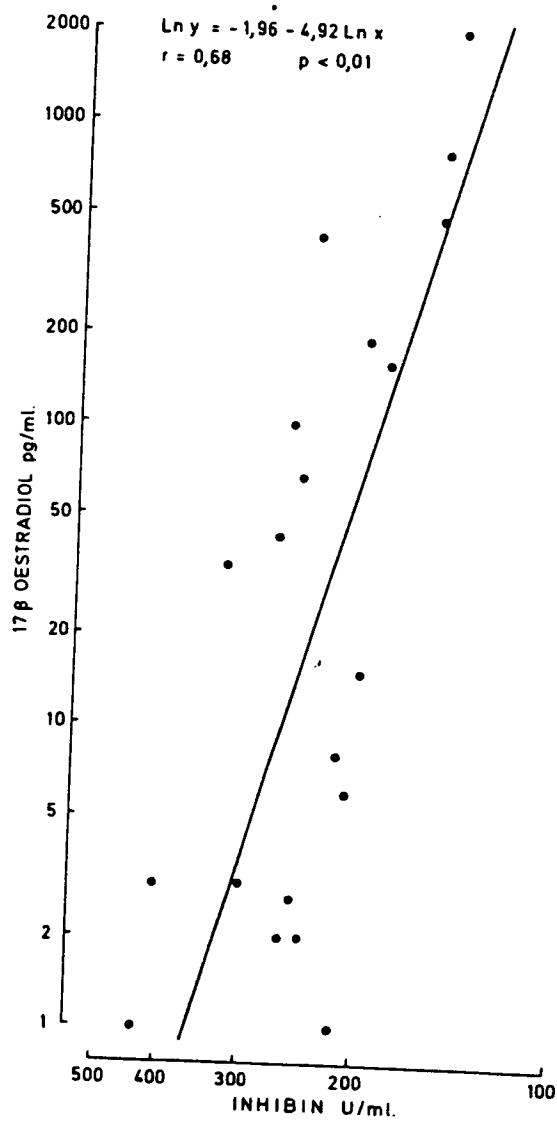


Fig. 7: Inverse relationship between oestradiol-17β and inhibin concentration in the same follicular fluid.

TABLE 1  
Relationship between follicle size and the inhibin and steroid concentrations in bovine follicular fluid

Compound measured		Follicle size (mls antral fluid/follicle)		
		< 0.3 *(46,17)	≥ 0.3 - < 0.8 *(35,25)	> 0.8 *(34,21)
Inhibin	(U/ml)	308 (248 - 380)	180 (157 - 217)	156 (134 - 186)
Estradiol-17 β	(ng/ml)	5 (2 - 8)	23 (15 - 97)	69 (38 - 142)
Testosterone	(ng/ml)	30 (21 - 49)	5 (2 - 4)	3 (2 - 6)
Androstenedione	(ng/ml)	32 (25 - 41)	18 (6 - 30)	10 (5 - 15)
Progesterone	(ng/ml)	35 (24 - 50)	37 (21 - 49)	39 (28 - 55)

\* (x, y) x = minimum number of samples assayed for any of the steroids; y = number of samples assayed for inhibin activity. Results expressed as medians with 95 per cent confidence limit.

The Kruskal-Wallis test indicated that there was a significant relationship between follicle size and FF concentration of inhibin, estradiol-17 β, testosterone and androstenedione (p < 0.01). There was no significant relationship between follicle size and FF concentrations of progesterone (p > 0.05).

#### Physiological Interpretation

Measurement of steroids and inhibin concentrations in bovine FF indicated that as follicle size increased there was a significant fall in FF concentrations of androgens and inhibin. The fall in androgens may be due to their increased aromatization by follicular granulosa cells since the FF concentrations of oestradiol-17 β rose as those of the androgen fell. Taking together the observation that FF concentrations of androgen and inhibin both fell as follicle size increased, and the finding that androgens stimulated granulosa cell inhibin production *in vitro*, it is tempting to speculate that ovarian inhibin production by granulosa cells *in vivo* may be regulated, at least in part, by androgens. There was no relationship between follicle size and FF concentrations of progesterone. Thus, the inhibitory effect of progesterone on granulosa cell inhibin production may be more important in regulating ovarian inhibin production during the period of granulosa cell luteinization and corpus luteum function, when progesterone levels become elevated (Fig. 8).

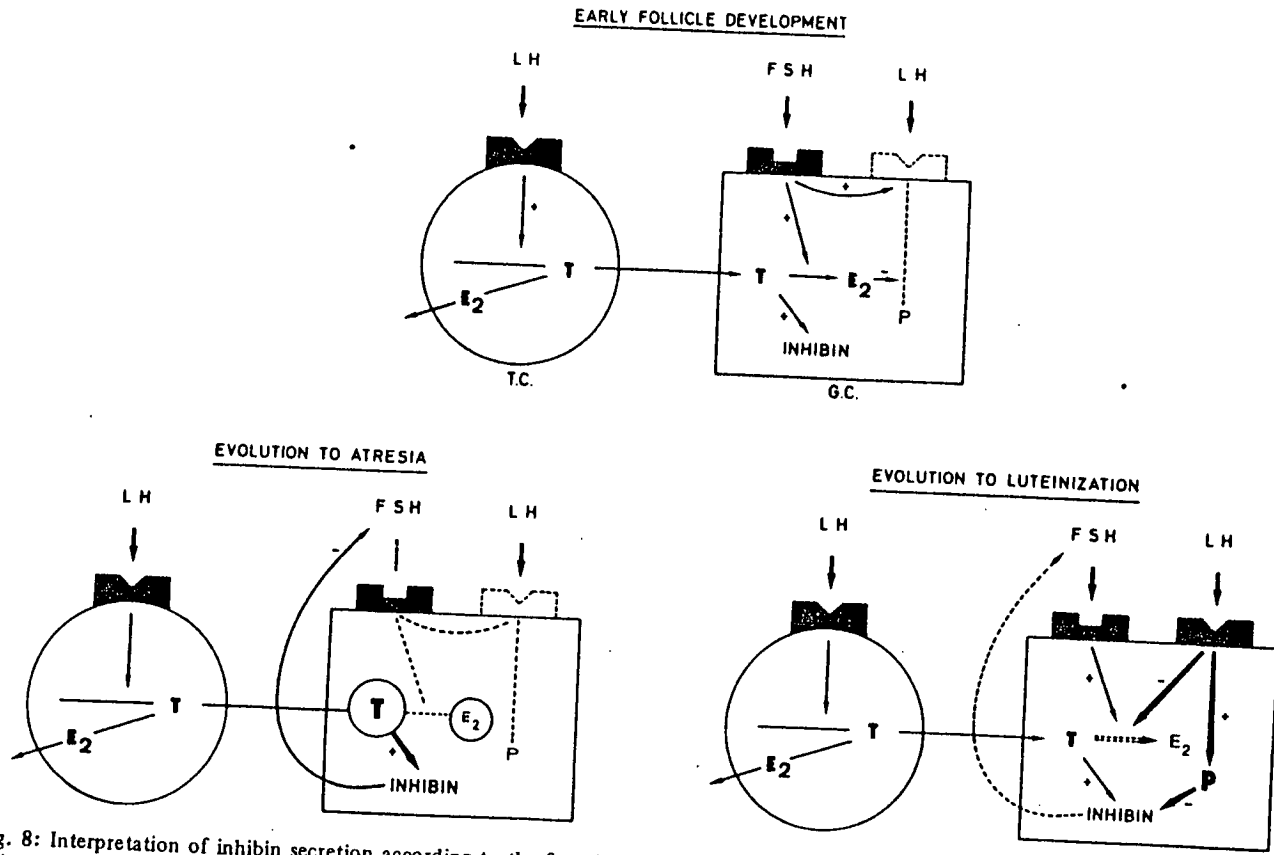


Fig. 8: Interpretation of inhibin secretion according to the functional differentiation of granulosa cells. T.C.: theca cells; G.C.: granulosa cells.



Androgens acting locally within the ovary may be involved in the process of follicular atresia (Louvet *et al.*, 1975; Hillier and Ross, 1979). The present findings raise the possibility that androgens may also promote follicular atresia through stimulating granulosa cell inhibin production which would act to inhibit pituitary FSH production. FSH stimulates ovarian androgen aromatization (Moon *et al.*, 1975; Erickson and Hsueh, 1978b; Armstrong and Papkoff, 1976), and so a decrease in FSH levels would produce an accumulation of follicular androgen from LH action on the theca (Leung and Armstrong, 1979). A closed cycle would thus be formed with the excess androgen driving the follicle further into atresia (Fig. 8).

#### Inhibin Production by the Testis

##### *Origin of Male Inhibin*

The testis is the source of inhibin, as shown by its presence in testicular extracts. The site of production appears to be the seminiferous tubules because large quantities of the hormone are found in the rete testis, where the secretion of the seminiferous tubules accumulates (see review of Franchimont *et al.*, 1979b). Eddie *et al.* (1978) have also identified a substance produced by cultures of rat seminiferous tubules that suppressed the LH-RH-induced secretion of FSH by pituitary cell cultures and inhibited the secretion of LH to a lesser extent.

The experiments of Steinberger and Steinberger (1976) have shown unequivocally that the Sertoli cell is directly involved in the synthesis and secretion of inhibin, called for this reason Sertoli cell factor. In fact, pituitary cells cocultured with isolated Sertoli cells consistently released significantly less FSH than pituitary cells grown alone or cultured with spleen or kidney cells. In contrast, the LH levels in the control and coculture were similar. Furthermore, the culture medium of viable Sertoli cells alone inhibited the spontaneous and LH-RH-induced FSH release in dispersed cultured pituitary cells. Since only minimum or no inhibition of FSH release was caused by ruptured Sertoli cells, the inhibin appears to be synthesized by Sertoli cells *in vitro*. The presence of inhibin in culture medium of Sertoli cells was confirmed by De Jong *et al.* (1978) and by Labrie *et al.* (1978). On the other hand, isolated germ cells or peritubular cells show no secretion of inhibin-like substance (Steinberger, 1980).

Other evidence for the role of the Sertoli cells in the secretion of inhibin has been provided by Demoulin *et al.* (1979b). Mouse testes were maintained in organ culture, and after 4 days the culture

medium was removed and placed on dispersed rat pituitary cells. When the testes were cultured at 37°C, spermatogenesis was greatly altered by day 4, whereas the Sertoli cells maintained their normal light microscopic appearance. This medium depressed LH-RH-induced FSH release without affecting LH release. After 8 days of culture, Sertoli cells were also affected and the inhibitory effect on FSH secretion disappeared.

When testis was cultured at 31°C, spermatogenesis was altered histologically at 8 whereas Sertoli cells remained in good condition for 20 days. The culture medium maintained its inhibin effect for the 20 days of the experiment. Thus, there was a relationship between the histological appearance of the Sertoli cells and the inhibitory potency of the culture medium. In contrast, alteration of gametogenesis did not have any effect on the inhibin activity of the culture medium.

Sources of inhibin other than the seminiferous tubules have not been excluded. Thus, the experiments of Peek and Watkins (1979) demonstrated that bull seminal plasma contains gonadotrophin-inhibiting activity which is not, at least completely, of testicular origin. In fact, they have shown that gonadotrophin-inhibiting activity is also present in the seminal plasma from vasectomized bulls and has the same characteristics as the material extracted from seminal plasma of intact animals. Similarly, Scott and Burger (1980) detected inhibin activity in human seminal plasma from vasectomized subjects although its concentration was lower than that in seminal plasma from intact men. These observations could lead to the conclusion that inhibin is produced from a source other than the testis. However, an alternative interpretation is possible. The presence of inhibin in seminal plasma is frequently interpreted as that which remains of inhibin secretion by the tubules into the epididymis but not reabsorbed at that site (Franchimont *et al.*, 1975b). It could be postulated, however, that inhibin is totally reabsorbed in the epididymis and into the peritubular lymphatics and that certain tissues or organs, such as the prostate and seminal vesicles concentrate circulating inhibin and release it into the seminal plasma.

#### *Mechanisms of Regulation of Inhibin Secretion*

Little data is available concerning the mechanisms regulating inhibin secretion in males.

Steinberger (1980) demonstrated that Sertoli cells obtained from rats which were hypophysectomized for 10 days show little or no

inhibin secretion. Daily treatment of hypophysectomized animals for 10 days with either FSH, LH or testosterone restores inhibin secretion to intact control levels. Treatment with FSH and LH or FSH plus testosterone has a synergistic effect on inhibin secretion compared to treatment with each hormone alone.

We have investigated inhibin secretion by isolated Sertoli cells (Franchimont *et al.*, 1980; Verhoeven and Franchimont, 1981).

A Sertoli cell enriched fraction was prepared according to the method of Verhoeven *et al.* (1979). This method consists of successive treatments with collagenase and pancreatin as initially proposed by Welsch and Wiebe (1975).

Inhibin production and oestradiol-17  $\beta$  secretion have been studied at days 7 and 9 as aromatization of testosterone to oestradiol-17  $\beta$  is maximum at that time (Verhoeven *et al.*, 1979) and as morphological investigation by phase contrast microscopy reveals homogenous monolayers of Sertoli cells. No germ cells were observed. The absence of Leydig cells is indicated by the low or undetectable basal concentrations of testosterone and lack of stimulation of its secretion by HCG.

Inhibin was assayed by the inhibition of LH-RH-induced FSH release and was expressed in units of an ovine testicular lymph preparation (OTL S).

Without any treatment, the production of oestradiol-17  $\beta$  and of inhibin was low or undetectable on days 7 and 9 of Sertoli cell cultures. The addition of HCG in doses of 5, 25 and 45 mIU/ml induced no change in the production of either substance on these days. Pregnant mare serum gonadotrophin (PMSG), with predominantly FSH-like activity (in doses of 0.02 to 10 IU/ml), and purified ovine FSH (0.1 to 1  $\mu$ g/ml for 4 hours) also failed to produce any change in inhibin secretion over the subsequent 24 hours (Verhoeven and Franchimont, 1981).

Testosterone alone (0.5  $\mu$ M) led to a small but significant increase in oestradiol-17  $\beta$  production. This aromatization was much more marked when PMSG (0.02 U/ml) was added to the culture medium with testosterone (Fig. 9). Testosterone alone, or with added PMSG, produced the same degree of increase in inhibin production. The effect was not due to oestradiol-17  $\beta$  production by the Sertoli cells, however, as the oestrogen (0.5  $\mu$ M) had no effect on inhibin production. In contrast, dihydrotestosterone, which cannot be aromatised, stimulated Sertoli cell inhibin secretion (Fig. 10).

#### *Physiological Interpretation*

Testosterone has an important role in the Sertoli cell (Fig. 11).

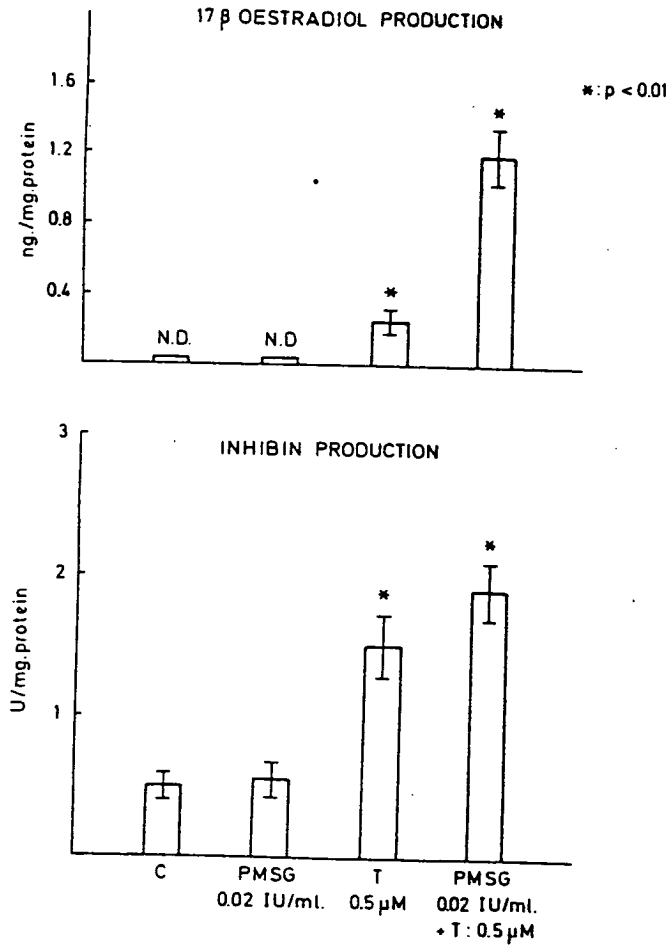


Fig. 9: Effect of pregnant mare serum gonadotrophin (PMSG: 0.02 IU/ml), testosterone (T = 0.5 μM), T (0.5 μM) + PMSG (0.02 IU/ml) on oestradiol-17 β and inhibin production by isolated Sertoli cells. C = control.

Secreted by Leydig cells under the influence of LH, testosterone passes through the walls of the seminiferous tubules and reaches the Sertoli cells. There, in concert with FSH, it induces the synthesis of androgen binding protein (ABP) which carries testosterone and dihydrotestosterone into the tubule, where these steroids are required for spermatogenesis (Steinberger *et al.*, 1978). Furthermore, testosterone can be aromatised to oestradiol-17 β, by immature rat Sertoli cells, in the presence of FSH (Dorrington *et al.*, 1978). By a short

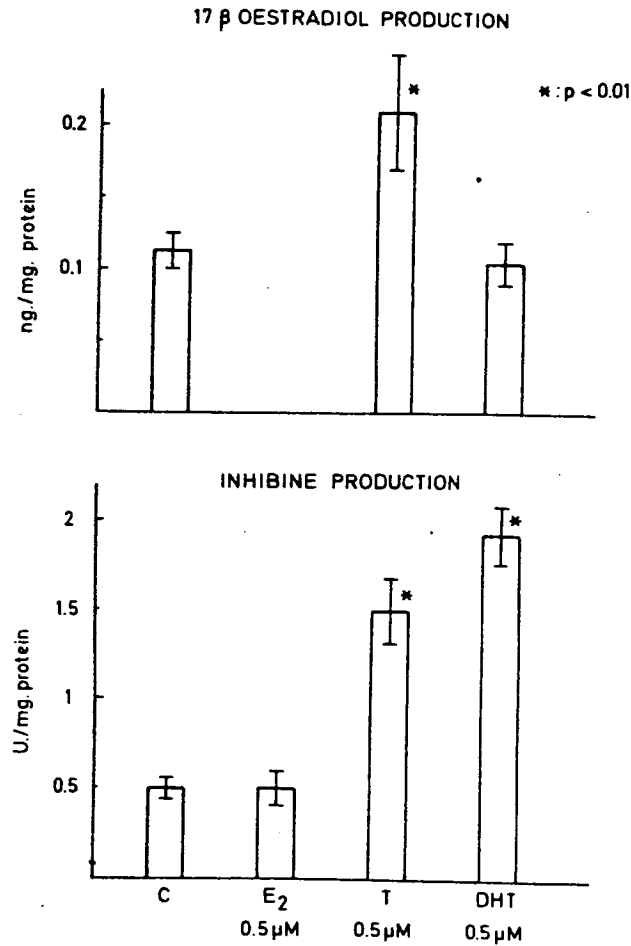


Fig. 10: Effect of testosterone (0.5 μM), oestradiol-17 β (0.5 μM) and dihydrotestosterone (DHT = 0.5 μM) on oestradiol-17 β and inhibin production by isolated Sertoli cells. C = control.

feedback mechanism, oestradiol-17 β can decrease Leydig cell testosterone synthesis (Bartke *et al.*, 1977; Tcholakian *et al.*, 1974; Oshima *et al.*, 1967). In addition, the androgens themselves, without being aromatised, stimulate inhibin production. The latter is absorbed into the circulation and decreases pituitary FSH secretion by a classical negative feedback mechanism. Inhibin also appears to be able to decrease spermatogonial multiplication in the seminiferous tubule directly, as shown by the effect of inhibin preparations on

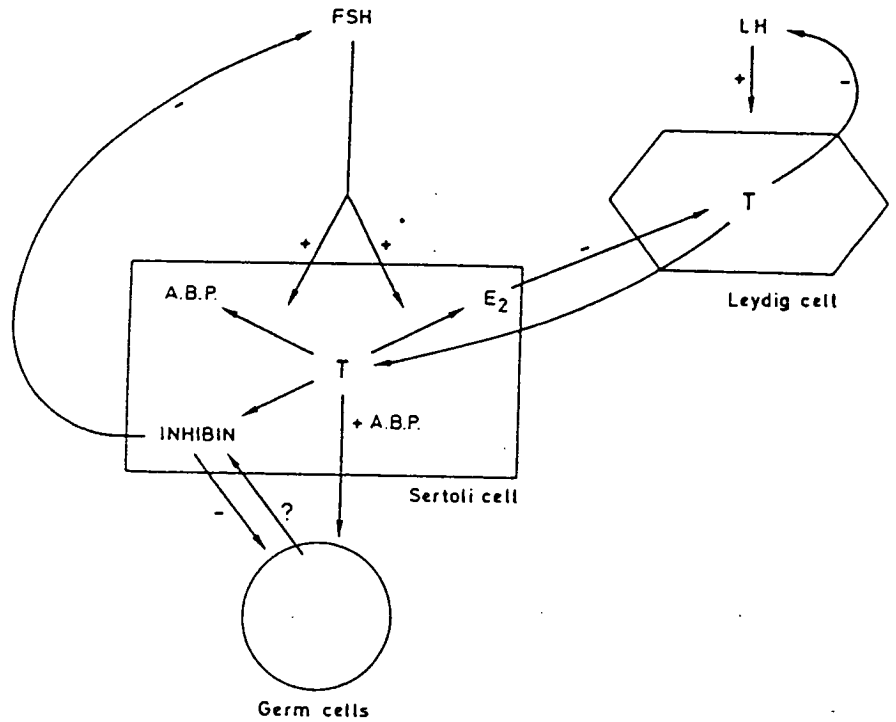


Fig. 11: Some effect of testosterone at Sertoli cell level and possible interrelationships between Leydig, Sertoli and germinal cells.

tritiated thymidine incorporation *in vitro* (see chapter XV, Effect of inhibin in testicular function).

In our current state of knowledge, no direct argument can be put forward for a germ cell signal which would inform the Sertoli cell regarding the state of spermatogenesis and which would stimulate inhibin secretion. On the other hand, it is certain that the androgens, testosterone and dihydrotestosterone are at the same time essential for spermatogenesis and for inhibin production.

#### *Identity of the Mechanisms of Inhibin Secretion by Granulosa and Sertoli Cells*

Our *in vitro* experiments show that inhibin secretion by granulosa and Sertoli cells is stimulated by both aromatisable and non-aromatisable androgens (testosterone and androstenedione and dihydrotestosterone, respectively). The oestrogens do not influence inhibin secretion by both cell types.

Progesterone decreases inhibin secretion by granulosa cells. Its effect on Sertoli cells are still unknown.

The direct role of the gonadotrophins is unclear. Under the conditions of our experiments, no direct effects were seen on Sertoli or on granulosa cells. In contrast, the results of Steinberger (chapter XIII) appear to give to FSH a direct stimulatory role on Sertoli cell inhibin secretion.

### Conclusions

The existence of inhibin may be accepted on the basis of numerous experiments. It appears to exert its effects at different sites: pituitary, hypothalamic and gonadal. Its true actions remain open to dispute in the absence of an absolutely pure preparation. Contamination by other cyberbins such as OMI, L.I., LH-RBI, the gonadocrinins cannot be excluded.

At the pituitary level, inhibin has a specific inhibitory effect on FSH synthesis under basal conditions. LH-RH stimulated gonadotrophin release is inhibited by inhibin, with a preferential effect on FSH: at very low doses, inhibin reduces only LH-RH-induced FSH release, whilst at higher doses, it reduces the release of both FSH and LH.

Some of the mechanisms regulating the secretion of inhibin by granulosa and Sertoli cells are the same: stimulation by aromatisable and non-aromatisable androgens (testosterone and androstenedione and dihydrotestosterone, respectively) and lack of an oestrogen effect. The gonadotrophins, FSH and LH do not appear to have a direct effect *in vitro*. There is an inverse relationship between progesterone and inhibin production by granulosa cells. Thus, when bovine granulosa cells undergo luteinization and differentiate into luteal cells as indicated by histological changes and steadily increasing progesterone production, they progressively lose their capacity to make inhibin. Luteal cells collected from bovine corpus luteum tissue no longer have the capacity to produce inhibin. Furthermore, progesterone reduced inhibin production by granulosa cells. Measurement of steroids and inhibin in individual bovine follicular fluids (FF) indicated that as follicle size increased, FF concentrations of oestradiol-17  $\beta$  increased, those of testosterone, androstenedione and inhibin decreased while progesterone concentrations remained unchanged. The stimulatory effect of androgen on inhibin production *in vitro* together with the parallel changes in FF concentrations of androgen and inhibin suggest that ovarian inhibin production *in vivo* may be controlled, at least in part, through androgens modifying

granulosa cell inhibin production. The inhibitory effect of progesterone on granulosa cell inhibin production may be more important in regulating ovarian inhibin production at the time of granulosa cell luteinization and corpus luteum formation.

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**Reference:** Journal of Reproduction and Fertility (1981) 63, 431-442

## Regulation of inhibin production by bovine ovarian cells *in vitro*

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**Summary.** The regulation of ovarian inhibin production was investigated using a rat pituitary cell culture system as a bioassay for inhibin activity. Bovine follicular granulosa cells produced inhibin *in vitro* provided that the culture medium contained serum. The stimulatory factor(s) present in serum is unlikely to be gonadotrophins, because bovine LH and/or FSH failed to stimulate inhibin production when added to medium devoid of serum. Luteinization of granulosa cells in culture was accompanied by a reduction in their inhibin production and an inverse relationship existed between inhibin and progesterone production by granulosa cells. Bovine corpus luteum cells in culture failed to produce detectable amounts of inhibin.

Androgens stimulated granulosa cell inhibin production with testosterone and 5 $\alpha$ -dihydrotestosterone being more potent than androstenedione. The androgens did not stimulate inhibin production by luteal cells. Progesterone inhibited granulosa cell inhibin production but oestrogens had no effect. Measurement of steroids and inhibin in fluid from individual follicles indicated that as follicle size increased, concentrations of oestradiol-17 $\beta$  increased, testosterone and inhibin decreased and progesterone remained unchanged. The stimulatory effect of testosterone on inhibin production *in vitro* together with the parallel changes in follicular fluid concentrations of testosterone and inhibin suggest that ovarian inhibin production *in vivo* may be controlled, at least in part, through androgens modifying granulosa cell inhibin production. The inhibitory effect of progesterone on granulosa cell inhibin production may be more important in regulating ovarian inhibin production at the time of granulosa cell luteinization and CL formation. The stimulatory effect of androgens on granulosa cell inhibin production might also be a means by which androgens promote follicular atresia.

### Introduction

The ovary, like the testis, may produce a non-steroidal compound, inhibin, which is capable of inhibiting pituitary FSH secretion (Franchimont *et al.*, 1979b). Steroid free bovine (de Jong, Welschen, Hermans, Smith & van der Molen, 1978), equine (Miller, Wesson & Ginther, 1979), human (Chappel, Holt & Spies, 1980), monkey (Channing, Anderson & Hodgen, 1980) and porcine (de Paolo, Wise, Anderson, Barraclough & Channing, 1979b; Schander, Anderson, Barraclough & Channing, 1980) follicular fluid selectively reduce FSH levels *in vivo* and/or selectively inhibit FSH production by anterior pituitary cells *in vitro*. Erickson & Hsueh (1978) indicated that granulosa cells are the probable source of this follicular inhibin, while studies of the rat (de Paolo, Shander, Wise, Barraclough & Channing, 1979a) and monkey (Channing *et*

*al.*, 1980) have shown that inhibin is secreted into the ovarian vein, thus strengthening the concept that ovarian inhibin may be physiologically important in the regulation of FSH secretion. Inhibin activity in follicular fluid is related to follicle size (Welschen, Hermans, Dullart & de Jong, 1977) and ovarian inhibin production changes throughout the oestrous/menstrual cycle (de Paolo *et al.*, 1979a; Chappel *et al.*, 1980). However, there is little information about the regulation of ovarian inhibin production. The aim of the present study was to investigate (a) the regulation of inhibin production by bovine ovarian cells *in vitro* and (b) the relationship between the inhibin concentration in individual bovine follicles to their hormonal environment.

### Materials and Methods

#### Reagents

Purified bovine gonadotrophins were provided by Bioproducts, Peptide Department (UCB, Brussels, Belgium). The bovine FSH had a potency 60 times that of NIH-FSH-B1 (Steelman-Pohley assay) and LH contamination of < 3% (RIA) or < 0.45% (RRA). The bovine LH had a biological activity of 2.3 i.u./mg (Parlow assay) with FSH contamination of < 0.05% (by bioassay with NIH-FSH-S9 as standard). Steroids were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset, U.K.

#### Bovine follicular fluid

Ovaries were obtained from healthy adult cows within 1 h of their slaughter at a local abattoir. Follicular fluid was aspirated from all visible antral follicles and that from follicles containing  $\leq 0.1$  ml fluid was pooled for follicles from the same ovary or each pair of ovaries. Aliquots taken for inhibin determination were freeze-dried while the remaining follicular fluid was stored frozen for subsequent steroid determination.

#### Ovarian cell cultures

**Granulosa cells.** Granulosa cells harvested and pooled from antral follicles containing  $\geq 0.3$  ml follicular fluid were cultured as described previously (Henderson & Moon, 1979). Briefly, a minimum of  $10^5$  'live' cells were cultured in multi-welled tissue culture Petri dishes (Sterilin, Middlesex, U.K.) at 37°C in 1 ml culture medium consisting of 10% donor calf serum and 90% Eagle's Minimum Essential Medium (Modified) with Earle's Salts (Eagle, 1959) and supplemented with Hepes buffer (20 mM), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 µg/ml), amphotericin B (0.625 µg/ml) and non-essential amino acids (Eagle, 1959) (all reagents obtained from Flow Laboratories, Irvine, Scotland). The gas phase was air. The medium was replaced at selected times and aliquots taken and freeze-dried for subsequent inhibin determination while the remainder was stored frozen until assayed for steroids. At the end of the culture period the cells were washed thoroughly with culture medium devoid of calf serum and assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Patterson (1979). In some preliminary studies, granulosa cells were cultured in the above medium devoid of calf serum. Steroids (see below) were added to the culture media in ethanol, with control cultures receiving ethanol alone. The ethanol concentration in the culture media never exceeded 1%.

**Luteal cells.** Dispersed cells were obtained as described previously (Henderson & Moon, 1979). Briefly, corpora lutea were freed of adherent connective tissue, cut into small pieces approximately 2 mm in diameter and washed in Hanks' Balanced Salt Solution (Flow Laboratories) devoid of magnesium and calcium but supplemented with Hepes, glutamine and antibiotics as described above (HBS-HGA). Tissue dissociation was achieved by stirring at

37°C in HBS-HGA containing 0.2% collagenase (Type II, Sigma), the released cells being collected after incubation for 20 and 40 min. Final dispersal of remaining tissue fragments was achieved by drawing through a syringe tip and a series of needles (18–22 gauge). The freed cells were pooled, filtered through sterile gauze, washed 4 times and their viability determined using nigrosin dye. The procedure for the culture of the luteal cells was exactly as described above for the granulosa cells.

#### *Inhibin assay*

The assay of inhibin activity was based on the selective inhibition of basal and LH-RH-induced FSH secretion by cultured anterior pituitary cells as described by Franchimont, Demoulin, Verstraelen-Proyard, Hazez-Hagelstein & Tunbridge (1979a). Dispersed anterior pituitary cells from adult male Wistar rats were obtained by enzymic digestion using the technique described by Hopkins & Farquhar (1973). The dispersed cells ( $1 \times 10^6$ ) were cultured at 37°C on Petri dishes (Falcon Plastics) in 3 ml Dulbecco's Modified Eagles Medium (Dulbecco & Freeman, 1959) supplemented with 5% horse serum, 2.5% fetal calf serum, 1% glutamine and 1% non-essential amino acids (Flow Laboratories). The gas phase was a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub>. After 3 days of culture, the medium was discarded and replaced with 1.6 ml of medium containing the test material or a reference standard preparation of inhibin. Incubation was continued for a further 3 days and then the medium was removed and stored frozen until assayed for gonadotrophins. The culture plates were washed and incubation was continued with 1.6 ml medium containing standards or test material and LH-RH at a final concentration of  $10^{-8}$  M. After incubation for 6 h the medium was removed and stored frozen until assayed for gonadotrophins.

The freeze-dried aliquots of follicular fluid and cell culture medium taken for inhibin assay were reconstituted with distilled water. Steroids were removed by mixing for 16 h at 4°C with activated charcoal (Norit A, 1%) pretreated with dextran (0.1%) followed by centrifugation at 3000 g. The supernatants were filter-sterilized by passing through a 0.45 µm cellulose millipore filter before use. Such dextran-charcoal treatment removed >99.8% of exogenous steroids added to culture medium while endogenous steroids present in follicular fluid were undetectable after this treatment. In the pituitary cell culture medium, the concentration of follicular fluid ranged from 0.5 to 5% (v/v) and that of granulosa or luteal cell culture medium ranged from 3 to 60% (v/v). Culture medium with and without added steroids incubated in the absence of granulosa or luteal cells served as control medium for adding to the pituitary cell culture medium. The standard reference preparation of inhibin, kindly supplied by Professor B. Hudson (Howard Florey Institute of Experimental Physiology and Medicine, Melbourne, Australia) was derived from ovine testicular lymph (OTLP6) and has been given an arbitrary potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979). This inhibin standard was added to the pituitary cell cultures at doses of 250 to 2000 µg/ml. Each standard and aliquot of test material was added to 5 replicate pituitary cell cultures. Inhibin activity in follicular fluid or culture media was expressed relative to the activity of the OTLP6 standard.

#### *Radioimmunoassays*

The gonadotrophin concentrations in media from the pituitary cell cultures were measured in duplicate by double-antibody methods using NIAMDD rat pituitary gonadotrophin reagents supplied by the pituitary agency of NIH. The concentrations are expressed in terms of NIAMDD rat-FSH-RP1 for FSH and rat-LH-RP1 for LH. The sensitivities of the assays were 5 ng FSH/ml and 10 ng LH/ml. The inter- and intra-assay coefficients of variation were 3 and 8% for the FSH assay and 5 and 12% for the LH assay respectively.

Steroid concentrations in follicular fluid and granulosa and luteal cell culture media were

determined using specific radioimmunoassays described previously (Neal, Baker, McNatty & Scaramuzzi, 1975; Van Look, Hunter, Corker & Baird, 1977; Corker & Davidson, 1978). The limits of sensitivity of the assays (per tube) were 25 pg for progesterone, 5 pg for oestradiol-17 $\beta$  and 10 pg for testosterone. The intra- and inter-assay coefficients of variation of all the steroid assays were each < 10% and < 16% respectively.

### Statistics

Unless otherwise stated, data were subjected to analyses by paired or unpaired Student's *t* test.

## Results

### *Specificity and validity of inhibin assay*

As shown in Text-fig. 1, each preparation caused a dose-dependent inhibition of FSH secretion in basal and LH-RH stimulated conditions. The significance of regression (Finney's *Q*) was always higher than 0.01. Variations in the slopes were not significant ( $G^2$ ,  $P > 0.05$ ; Finney, 1964) and the curves were therefore considered to run parallel. LH secretion was reduced only in LH-RH treated cultures and only by much higher amounts of the test substances than those required to impair FSH secretion. The mean lowest detectable doses of inhibin capable of decreasing LH-RH-induced FSH and LH secretion, calculated at 95% confidence limit of gonadotrophin secretion in the absence of inhibin, were  $86 \pm 25 \mu\text{g}$  and  $337 \pm 59 \mu\text{g}$  respectively. In basal conditions, the FSH inhibition curves were less sensitive (lowest detectable dose of inhibin  $315 \pm 62 \mu\text{g}$ ) and less precise (index of precision,  $\lambda$ , range 0.15–0.41) than when the secretion was stimulated by LH-RH addition to cultures ( $86 \pm 25 \mu\text{g}$  inhibin and  $\lambda$  0.06–0.19). The inhibition of LH-RH-induced FSH secretion by pituitary cells was therefore considered to be the better method for determining inhibin activity and was used for comparison to the standard.

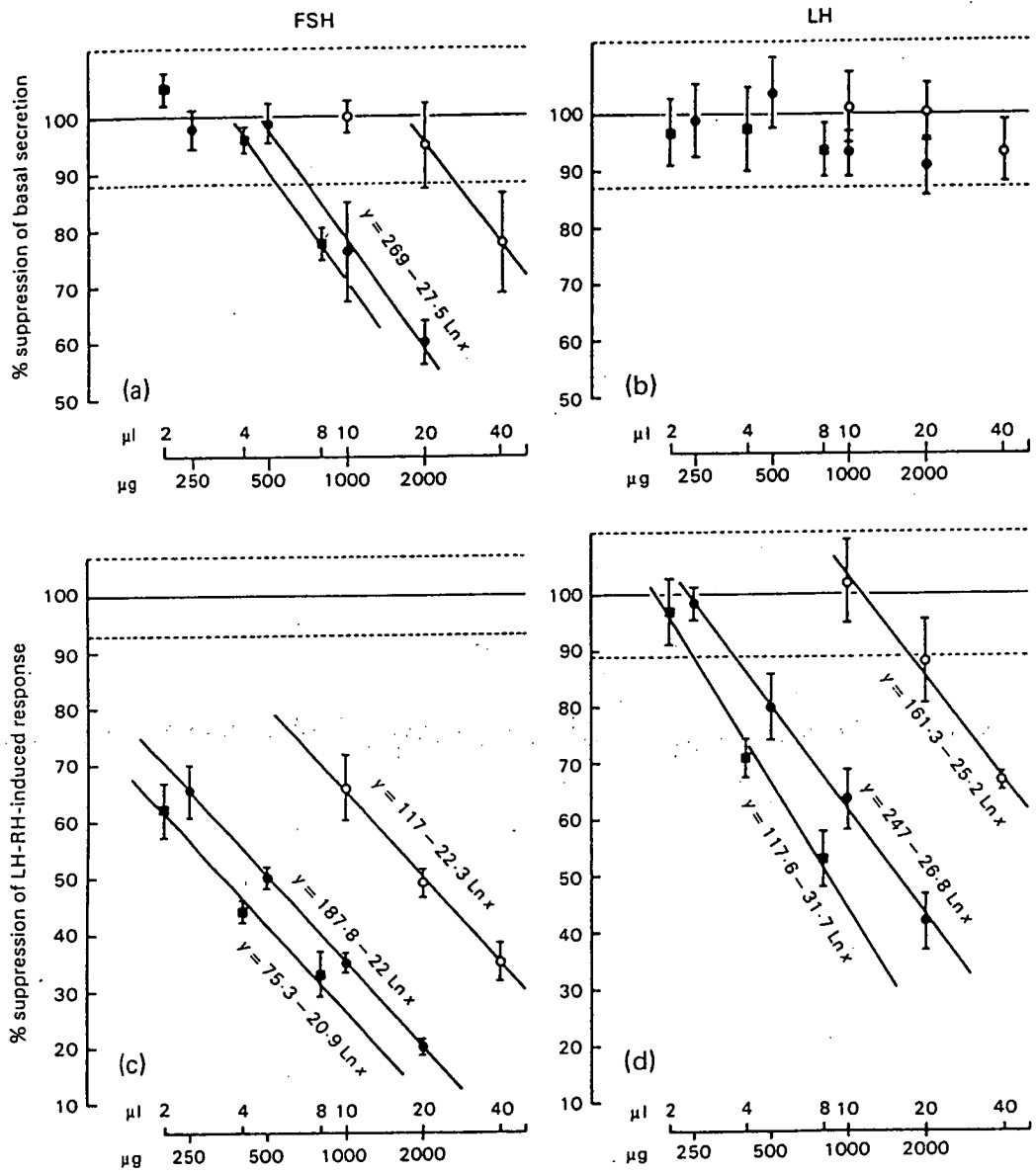
### *Influence of culture conditions on inhibin production by granulosa cells*

Granulosa cells cultured in medium devoid of calf serum failed to secrete inhibin. Only medium from granulosa cells cultured in medium with 10% calf serum selectively inhibited basal FSH production (as demonstrated in Text-fig. 1) and preferentially inhibited LH-RH stimulated FSH production (Table 1) by pituitary cells. Granulosa cells cultured in medium with 10% calf serum underwent morphological and functional changes indicative of luteinization, i.e. cellular hyperplasia and hypertrophy and increased progesterone production (Table 1). Addition of bovine gonadotrophins to the culture medium mimicked the effect of serum in stimulating granulosa cell progesterone production but not the effect of serum in stimulating inhibin production (Table 1).

### *Relative production of inhibin by granulosa and luteal cells*

To test the possibility that inhibin production by granulosa cells might be related to their luteinization, inhibin production by luteinizing granulosa cells and luteal cells was compared. Results in Text-fig. 2 show that as granulosa cell progesterone production increased, inhibin production decreased. Although dispersed luteal cells do not survive well in culture (Henderson & Moon, 1979), as indicated by a steady decline in their production of progesterone, substantial amounts of progesterone were produced during the 2 days of culture, indicative of some functional viability. No detectable amounts of inhibin, however, were produced by the luteal cells.





Text-fig. 1. Effect of progressive amount of the inhibin standard (●) and volumes of bovine follicular fluid (■) and granulosa cell culture medium (○) on basal FSH (a) and LH (b) secretion and on LH-RH-induced release of FSH (c) and LH (d). FSH and LH concentrations are expressed as % of control (100%, no added inhibin preparations). Each is the mean curve of 17 individual curves. Each point represents the mean  $\pm$  s.d. Broken lines represent 2 s.d. from the 100% control value.

**Relationship between progesterone and inhibin production by granulosa cells**

The analysis of several granulosa cell culture experiments revealed an inverse relationship between progesterone and inhibin production (Text-fig. 3).

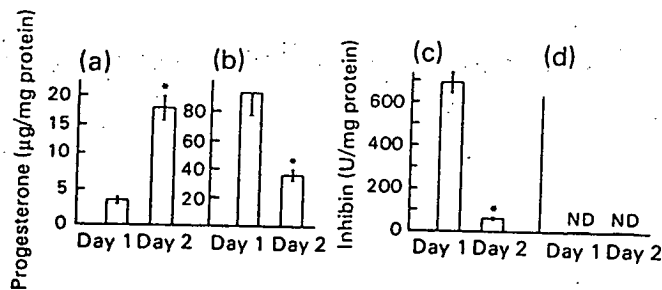
Table 1. Relative efficiency of calf serum and bovine gonadotrophins in stimulating inhibin and progesterone production by bovine granulosa cells cultured for 24 h

Material added to pituitary cell cultures (50 µl/ml culture medium)	Granulosa cells present	LH-RH-stimulated gonadotrophin production by pituitary cells (ng/ml)		Progesterone production by granulosa cells (µg/mg protein)	
		FSH	LH		
Granulosa cell culture medium with 10% calf serum after incubation	-	2047 ± 51	5236 ± 161	—	
	+	1303 ± 54**	4430 ± 102*	3.0 ± 0.4	
Granulosa cell culture medium without 10% calf serum after incubation	Alone	—	—	—	
	—	1845 ± 120	4794 ± 43	—	
	+	1930 ± 119	4896 ± 130	1.1 ± 0.3†	
	+ FSH (500 ng)	—	1799 ± 65	—	
	—	1772 ± 103	4843 ± 83	—	
	+ LH (500 ng)	—	1806 ± 21	4980 ± 22	2.2 ± 0.3
	—	1831 ± 48	4748 ± 36	—	
	+ FSH + LH (500 ng of each)	—	1858 ± 91	4878 ± 80	3.5 ± 0.6
+	1975 ± 67	4645 ± 80	—		
			4958 ± 213	3.3 ± 0.4	

Values are mean ± s.e.m. for 5 replicate cultures.

Values significantly different from effect of culture medium with 10% calf serum but no granulosa cells; \* $P < 0.01$ , \*\* $P < 0.005$ .

† Significantly different from other values in same column:  $P < 0.05$ .

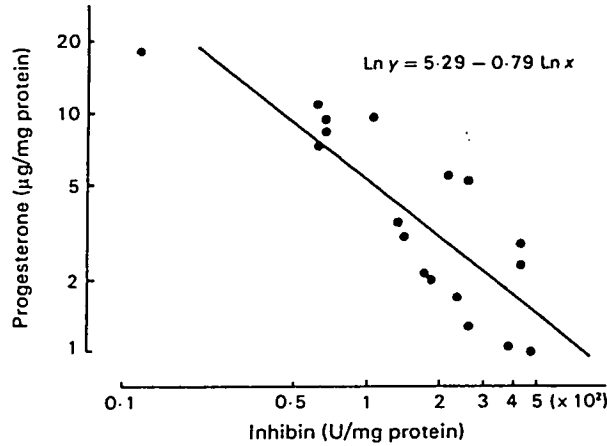


Text-fig. 2. Progesterone (a, b) and inhibin (c, d) production by  $10^6$  granulosa (a, c) and  $10^6$  luteal (b, d) cells on Days 1 and 2 of culture. Values are means ± s.d. for 6 replicate cultures; ND = not detectable. \*Significantly different from Day 1 value ( $P < 0.01$ ).

#### Effect of exogenous steroids on inhibin production and steroidogenesis by granulosa and luteal cells

The results in Table 2 demonstrate that androstenedione at 5 µg/ml and testosterone at 1 and 5 µg/ml significantly stimulated granulosa cell inhibin production. In contrast, progesterone at 1 and 5 µg/ml significantly inhibited inhibin production. Oestrogens had no significant effect ( $P > 0.05$ ) on inhibin production. All the granulosa cell cultures produced significantly less inhibin during the 2nd day of culture than during the 1st day ( $P < 0.01$ ). Progesterone production by granulosa cells was significantly inhibited by oestrogens and androgens during the 2nd day, but not during the 1st day of culture. Oestradiol-17β production by the granulosa cells increased during the 1st day of culture in response to the exogenous androgens and then decreased 5–10-fold during the 2nd day of culture, relative to Day 1.

The effects of the same doses of an aromatizable and non-aromatizable androgen (testosterone and 5α-dihydrotestosterone (DHT) respectively) on inhibin production by



Text-fig. 3. Relationship between progesterone and inhibin production by bovine granulosa cells in culture. The coefficient of linear correlation ( $r$ ) = 0.83 with  $P < 0.001$  ( $n = 18$ ).

Table 2. Effect of steroids on inhibin production and steroidogenesis by bovine granulosa cells in culture

Additions to culture medium (dose/ml)	Inhibin production (U/mg protein)		Steroid production (µg/mg protein)			
			Progesterone		Oestradiol-17β	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
None	375 ± 16	205 ± 7	1.0 ± 0.08	5.5 ± 0.28	0.3 ± 0.08	0.3 ± 0.09
Oestrone						
1 µg	386 ± 23	194 ± 7	0.9 ± 0.02	2.1 ± 0.07 <sup>d</sup>	33 ± 3 <sup>d</sup>	79 ± 8 <sup>d</sup>
5 µg	391 ± 16	222 ± 10	0.8 ± 0.06	1.1 ± 0.08 <sup>d</sup>	166 ± 12 <sup>d</sup>	152 ± 10 <sup>d</sup>
Oestradiol-17β						
1 µg	386 ± 9	204 ± 11	0.9 ± 0.04	2.2 ± 0.04 <sup>d</sup>	—	—
5 µg	360 ± 13	218 ± 8	0.8 ± 0.07	1.1 ± 0.09 <sup>d</sup>	—	—
Androstenedione						
1 µg	437 ± 24	211 ± 5	1.1 ± 0.07	3.4 ± 0.17 <sup>d</sup>	3.0 ± 0.3 <sup>d</sup>	0.3 ± 0.01
5 µg	477 ± 13 <sup>c</sup>	267 ± 17 <sup>c</sup>	1.0 ± 0.05	1.3 ± 0.11 <sup>d</sup>	5.5 ± 0.8 <sup>d</sup>	1.3 ± 0.3 <sup>c</sup>
Testosterone						
1 µg	597 ± 22 <sup>d</sup>	249 ± 7 <sup>c</sup>	1.2 ± 0.09	3.5 ± 0.42 <sup>c</sup>	4.9 ± 0.4 <sup>d</sup>	0.6 ± 0.06 <sup>a</sup>
5 µg	681 ± 37 <sup>d</sup>	277 ± 11 <sup>d</sup>	1.0 ± 0.04	2.3 ± 0.34 <sup>d</sup>	6.4 ± 0.3 <sup>d</sup>	0.5 ± 0.04
Progesterone						
1 µg	170 ± 9 <sup>d</sup>	ND	—	—	ND	ND
5 µg	284 ± 33 <sup>a</sup>	ND	—	—	ND	ND

Values are mean ± s.e.m. for 5 replicate cultures. ND, not detectable.

Values significantly different from those for cultures with no additions: <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.02$ ; <sup>c</sup> $P < 0.01$ ; <sup>d</sup> $P < 0.001$ .

granulosa cells in a separate experiment is shown in Table 3. Both androgens caused a significant (except for 1 µg DHT/ml during the 2nd day of culture) and dose-related increase in inhibin production during both days of culture. Consistent with the results in Table 2, both androgens significantly inhibited progesterone production. Oestradiol-17β production was again stimulated by testosterone with less oestradiol-17β being produced during the 2nd than during the 1st day of culture. A small, but significant, amount of oestradiol-17β was also produced in response to exogenous DHT. This may be the result of slight (< 1%) testosterone contamination of the commercial preparation of DHT which was not repurified before use.

Table 3. Effect of androgens on inhibin and steroidogenesis by bovine granulosa cells in culture

Addition to culture medium (dose/ml)	Inhibin production (U/mg protein)		Steroid production ( $\mu\text{g}/\text{mg}$ protein)			
			Progesterone		Oestradiol-17 $\beta$	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
None	427 $\pm$ 19	260 $\pm$ 10	2.9 $\pm$ 0.3	5.4 $\pm$ 0.3	ND	ND
Testosterone						
1 $\mu\text{g}$	1436 $\pm$ 51 <sup>d</sup>	435 $\pm$ 21 <sup>d</sup>	2.4 $\pm$ 0.2	3.3 $\pm$ 0.3 <sup>c</sup>	23 $\pm$ 3	2.0 $\pm$ 0.3
5 $\mu\text{g}$	2205 $\pm$ 68 <sup>d*</sup>	742 $\pm$ 28 <sup>d*</sup>	1.6 $\pm$ 0.4 <sup>a</sup>	2.4 $\pm$ 0.4 <sup>d</sup>	21 $\pm$ 3	2.0 $\pm$ 0.1
5 $\alpha$ -Dihydrotestosterone						
1 $\mu\text{g}$	1362 $\pm$ 41 <sup>d</sup>	285 $\pm$ 8	2.2 $\pm$ 0.4	3.5 $\pm$ 0.7 <sup>a</sup>	0.3 $\pm$ 0.06	0.1 $\pm$ 0.01
5 $\mu\text{g}$	2848 $\pm$ 89 <sup>d*</sup>	478 $\pm$ 15 <sup>d*</sup>	1.7 $\pm$ 0.2 <sup>b</sup>	3.0 $\pm$ 0.4 <sup>c</sup>	0.2 $\pm$ 0.04	0.1 $\pm$ 0.03

Values are mean  $\pm$  s.e.m. for 6 replicate cultures. ND, not detectable.

Values significantly different from those for cultures with no additions: <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.02$ ; <sup>c</sup> $P < 0.01$ ; <sup>d</sup> $P < 0.001$ .

\* Significantly different from the lower concentration value:  $P < 0.001$ .

Table 4. Effect of testosterone on inhibin production and steroidogenesis by bovine granulosa and luteal cells in culture

Addition to culture media	Inhibin production (U/mg protein)		Steroid production ( $\mu\text{g}/\text{mg}$ protein)			
			Progesterone		Oestradiol-17 $\beta$	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Granulosa cells						
None	470 $\pm$ 21	293 $\pm$ 11	0.9 $\pm$ 0.1	2.1 $\pm$ 0.1	ND	ND
Testosterone, 1 $\mu\text{g}/\text{ml}$	850 $\pm$ 36 <sup>*</sup>	483 $\pm$ 13 <sup>*</sup>	0.7 $\pm$ 0.2	0.9 $\pm$ 0.3 <sup>*</sup>	15 $\pm$ 2	1.0 $\pm$ 0.3
Luteal cells						
None	ND	ND	160 $\pm$ 29	17 $\pm$ 2	ND	ND
Testosterone, 1 $\mu\text{g}/\text{ml}$	ND	ND	115 $\pm$ 15	20 $\pm$ 1	ND	ND

Values are mean  $\pm$  s.e.m. for 6 replicate cultures. ND, not detectable.

\* Significantly different from value for culture with no addition:  $P < 0.001$ .

While testosterone stimulated inhibin production by granulosa cells, it had no effect on luteal cells which failed to produce any detectable amounts of inhibin in the presence or absence of testosterone (Table 4).

Although the granulosa cell culture media were treated with dextran-charcoal before being assayed for inhibin, it could be argued that the effects of the steroids on granulosa cell inhibin production might be artefacts caused by steroids, still present following dextran-charcoal treatment of the media, modifying pituitary FSH production. Addition to pituitary cell cultures of equivalent volumes (50  $\mu\text{l}$ ) of dextran-charcoal treated culture medium containing added steroids but not exposed to granulosa cells revealed, however, that only at the higher concentration tested (5  $\mu\text{g}/\text{ml}$ ) did any of the steroids themselves significantly modify FSH production. Testosterone and progesterone significantly increased ( $P < 0.01$ ) FSH production (mean  $\pm$  s.e.m. for  $n = 5$ ) from 1597  $\pm$  38 to 1790  $\pm$  30 and 1839  $\pm$  33 ng/ml respectively, and oestrone significantly reduced ( $P < 0.05$ ) FSH production from 1597  $\pm$  38 to 1459  $\pm$  41 ng/ml. The other steroids had no effect on FSH production.

*Inhibin and steroid concentrations in follicular fluid*

Inhibin and testosterone concentrations in follicular fluid fell significantly ( $P < 0.01$ ) as follicle size increased while the concentration of oestradiol-17 $\beta$  rose significantly ( $P < 0.01$ ) (Table 5). There was no significant relationship between follicle size and progesterone concentrations ( $P > 0.05$ ).

Table 5. Relationship between follicle size and the inhibin and steroid concentrations in bovine follicular fluid

	Follicle size (ml antral fluid/follicle)		
	< 0.3 (17)*	$\geq 0.3$ –< 0.8 (25)*	$\geq 0.8$ (21)*
Inhibin (U/ml)	308 $\pm$ 32	181 $\pm$ 17	155 $\pm$ 15
Oestradiol-17 $\beta$ (ng/ml)	9 $\pm$ 2	133 $\pm$ 39	181 $\pm$ 45
Testosterone (ng/ml)	43 $\pm$ 5	29 $\pm$ 10	16 $\pm$ 5
Progesterone (ng/ml)	44 $\pm$ 4	55 $\pm$ 11	63 $\pm$ 14

\* Minimum number of determinations in each group. Values are mean  $\pm$  s.e.m.

There was a significant relationship between follicle size and concentrations of inhibin, oestradiol-17 $\beta$  and testosterone ( $P < 0.01$ ; Kruskal-Wallis test).

### Discussion

Dispersed pituitary cell culture has already been used as a method for assaying inhibin (de Jong *et al.*, 1978; Eddie *et al.*, 1979; Franchimont *et al.*, 1979a, b; Scott, Burger & Quigg, 1980). In our experimental conditions, inhibin preparations extracted from rete testis (Franchimont *et al.*, 1979a), from testicular lymph and produced by granulosa cells (Text-fig. 1) specifically decrease basal FSH secretion, and they preferentially inhibit LH-RH-induced FSH secretion. Low doses decrease only LH-RH-induced FSH release whereas higher doses reduce both FSH and LH release under the influence of LH-RH. This preferential effect is due to the fact that inhibin specifically reduces FSH synthesis, but reduces FSH and LH release, although LH release is reduced to a lesser extent (Franchimont *et al.*, 1979b). As inhibition of LH-RH-induced FSH secretion is more precise and sensitive than the specific inhibition of basal FSH secretion, this response was chosen as the end point of the inhibin bioassay (Text-fig. 1). The criteria for the quality of a parallel line assay are fulfilled (Borth, 1976). The specificity of the inhibin assay was ascertained by the absence of an effect on prolactin and thyrotrophin secretion in basal and LH-RH-stimulated conditions, by the absence of inhibition of basal LH secretion and by a more marked inhibition of LH-RH-induced FSH secretion than that of LH-RH-stimulated LH release (Franchimont *et al.*, 1979a).

Granulosa cells cultured in medium devoid of calf serum failed to produce inhibin. The nature of the factor(s) present in serum and responsible for stimulating granulosa cell inhibin production is unknown but is unlikely to be endogenous gonadotrophins. Bovine LH and/or FSH added to medium devoid of serum, although mimicking the effect of serum in stimulating granulosa cell progesterone production, failed to stimulate inhibin production. No effect on inhibin production by rat granulosa cells cultured in medium containing 10% calf serum was observed when PMSG, a predominantly FSH-like gonadotrophin, was added at increasing doses (0.02 to 1 i.u.) to the culture medium (F. Crooze & P. Franchimont, unpublished observations).

Luteinization of granulosa cells, as indicated in culture by cellular hyperplasia and

hypertrophy and increased progesterone production, was accompanied by a reduction in inhibin production. There was an inverse relationship between granulosa cell progesterone and inhibin production. This is unlikely to be an artefact of the culture system because bovine luteal tissue, of which luteinized granulosa cells constitute a major portion, failed to produce any detectable amounts of inhibin *in vitro*. Thus, luteinization of granulosa cells and corpus luteum formation *in vivo* may be accompanied by a reduction in ovarian inhibin production. The finding that exogenous progesterone inhibited inhibin production by granulosa cells suggests that the reduction in inhibin production accompanying granulosa cell luteinization may be due to the increased progesterone produced having a negative feedback effect on cellular inhibin production.

Whatever the dose and the day of culture, exogenous oestrogens failed to modify granulosa cell inhibin production. They did, however, significantly reduce progesterone production by the cells as previously demonstrated (Fortune & Hansel, 1979). A stimulatory effect of androstenedione, testosterone and 5 $\alpha$ -dihydrotestosterone on inhibin production by granulosa cells was evident and was dose-dependent for testosterone and dihydrotestosterone. This stimulatory effect was less marked during the 2nd day of culture and may be due to an inhibitory action of the increased progesterone produced by the granulosa cells over-riding the stimulatory effect of the androgens. The extent by which inhibin production was stimulated by testosterone (1  $\mu$ g/ml) during the 1st day of culture varied between experiments from 1.6- to 3.4-fold (Tables 2, 3 and 4). This probably reflects different sensitivities to testosterone of the pools of granulosa cells used in each study. Further studies to compare the responsiveness to androgens of granulosa cells from individual follicles therefore seem worthwhile. The stimulatory effect of androstenedione and testosterone on inhibin production was associated with their aromatization to oestradiol-17 $\beta$  during the 1st day of culture. During the 2nd day of culture, luteinization of the granulosa cells was accompanied by a reduction in their capacity to aromatize androgens (Henderson & Moon, 1979). The stimulatory effect of the androgens on inhibin production is a direct one, however, and not secondary to their capacity to be aromatized to oestrogens. Exogenous oestrogens failed to modify granulosa cell inhibin production, and 5 $\alpha$ -dihydrotestosterone, essentially a non-aromatizable androgen, stimulated inhibin production. Although stimulating inhibin production by granulosa cells, testosterone did not induce inhibin production by luteal cells. In addition to their effect on inhibin production, the androgens also inhibited progesterone production by granulosa cells.

Measurement of steroids and inhibin concentrations in bovine follicular fluid indicated that as follicle size increased there was a significant fall in fluid concentrations of testosterone and inhibin. The fall in testosterone may be due to its increased aromatization by follicular granulosa cells because the follicular fluid concentrations of oestradiol-17 $\beta$  rose as those of the testosterone fell. Taking together the observation that follicular fluid concentrations of testosterone and inhibin both fell as follicle size increased, and the finding that androgens stimulated granulosa cell inhibin production *in vitro*, androgens may modify, at least in part, granulosa cell inhibin production *in vivo*. There was no relationship between follicle size and follicular fluid concentrations of progesterone. The inhibitory effect of progesterone on granulosa cell inhibin production may therefore be more important in regulating ovarian inhibin production during the period of granulosa cell luteinization and corpus luteum formation, when progesterone levels become elevated.

Androgens acting locally within the ovary may be involved in the process of follicular atresia (Louvet, Harman, Schreiber & Ross, 1975; Hillier & Ross, 1979). Androgens may also promote follicular atresia through stimulating granulosa cell inhibin production which would act to inhibit pituitary FSH production. FSH stimulates ovarian androgen aromatization (Armstrong & Papkoff, 1976) and so a decrease in FSH levels would produce an accumulation of follicular androgen from LH action on the theca (Leung & Armstrong, 1979). A closed cycle would thus be formed with the excess androgen driving the follicle further into atresia.

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## Differences and Similarities Between Male and Female Inhibin

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### ABSTRACT

FSH secretion is modulated in the male as well as in the female by both steroid and protein hormones. The protein hormone named inhibin preferentially reduces FSH secretion by a direct action on the pituitary gland and perhaps also through a hypothalamic regulation. In the male, inhibin is secreted by Sertoli cells and in the female by granula cells. It exists in two forms, a large molecular weight (M.W.) and a small M.W. form. As inhibin has not yet been isolated as a pure molecule, it is premature to conclude about its biochemical similarities. Inhibin secretion by both Sertoli cells and granulosa cells in culture is not influenced by the presence of LH and estradiol in the medium but is stimulated by testosterone and dihydrotestosterone and, in certain circumstances, by FSH. Inhibin preparations extracted from seminal plasma, ovine rete testis fluid, human and mare follicular fluid induce parallel curves of reduction of FSH release by pituitary cells in culture. They also reduce FSH serum levels in normal and castrated rats of both sexes. Biological estimation of inhibin concentrations show that it is comparable throughout the life in the normal male, but decreases in severe oligozoospermia and azoospermia. In the female, the amount of inhibin found in follicular and peritoneal fluid is variable during the follicular phase of the menstrual cycle and is absent or highly reduced in the luteal phase and during menopause.

We conclude that inhibin originating from male or female gonadal fluids has major similarities in its nature and mechanisms of action. However differences exist in its physiological role in the two sexes.

### KEYWORDS

Male inhibin ; female inhibin.

### INTRODUCTION

In both male and female, the levels of FSH and LH can change independently in either physiological, pathological or experimental conditions (reviewed by Franchimont and others, 1979 ; Grady and Schwartz, 1981). Several feedback mechanisms are involved in this regulation. If LH regulation appears to depend on steroids, on the other hand, FSH regulation seems to depend upon both steroid and pro-

tein hormones. In the male, the existence of a testicular hormone distinct from androgens was postulated as early as 1923 by Mottram and Cramer. This hormone has been named inhibin (Mc Cullagh, 1932), Sertoli cell factor (Steinberger and Steinberger, 1976), folliculostatin (Schwartz and Channing, 1977) FSH suppressing principle (Sairam and others, 1978) gonadostatin (Ying and Guillemin, 1979). The presence of a protein hormone regulating FSH secretion in the female has been more recently demonstrated (Hopkinson and others, 1975). The aim of the work reported here is to compare the properties of these male and female hormones which will be indifferently referred to as inhibin.

#### SOURCES OF INHIBIN

In various mammalian species, inhibin activity is detected in the gonads (testicular and ovarian extracts) and in the fluids secreted by these organs (rete testis and follicular fluids, seminal plasma and peritoneal fluids). Inhibin is synthesized in the testis by Sertoli cells and in the ovary by granulosa cells as demonstrated by its presence in the medium of monolayer cultures of these cells. Inhibin-like activity has also been observed in spermatozoa (Lugaro and others, 1974). Similar observations for oocytes have not so far been described.

#### NATURE OF INHIBIN

The purification of inhibin from these different sources is in progress in various laboratories (review by de Jong and others, 1981, Chapter in this book by Chari, de Jong and Baker). Unanimity about its protein nature is obtained. Several methods are used in the purification of inhibin but conflicting results are reported concerning its physicochemical characteristics. Its molecular weight (M.W.) ranges between less than 1000 and 160,000 Daltons. The assigned M.W. usually lies between 10,000 and 30,000 Daltons. However, a low M.W. inhibin (< 5,000 Daltons) is also well characterized. Moreover the presence or absence of a carbohydrate moiety on the molecule and its isoelectric point are still controversial.

The detection of inhibin activity by the classical *in vivo* or *in vitro* bioassays may become hazardous since impure inhibin preparations may be contaminated by other substances normally secreted by the same tissues (e.g. gonadocrinin (Ying and Guillemin, 1979), FSH receptor binding inhibitor (FSHRBI), or stimulator (FSHRBS) (Reichert and others, 1981), testicular chalone (Clermont and Mauger, 1974) etc...). Inhibin, FSHRBI, FSHRBS and gonadocrinin activities have been assessed in ovine rete testis fluid fractions obtained by molecular sieve chromatography (Fig. 1). Presence of inhibin is established in the pituitary cell culture model by a reduction of FSH secretion in basal and LHRH stimulated conditions. When basal LH release is increased in the same assay, gonadocrinin activity is suspected. FSHRBI and FSHRBS activities are detected in the fractions by a respective decrease or increase of labelled FSH binding to isolated bovine testis receptors as described by Reichert and Abou-Issa (1977). As shown in Fig. 1 large M.W. inhibin, FSHRBS and FSHRBI activities are present in isolated fractions. However low M.W. inhibin and gonadocrinin require further purification in order to be separated from each other (Jaspard and others, in preparation). Prior to this study, FSHRBI and low M.W. inhibin have never been separated. Indeed fractions isolated by Daume and others (1979) and Moodbidri and others, (1980) had simultaneously both inhibin and FSHRBI activities.

Because presently available inhibin preparations are not chemically pure, it is premature to conclude about the similarities or the differences in the nature of male and female inhibin.

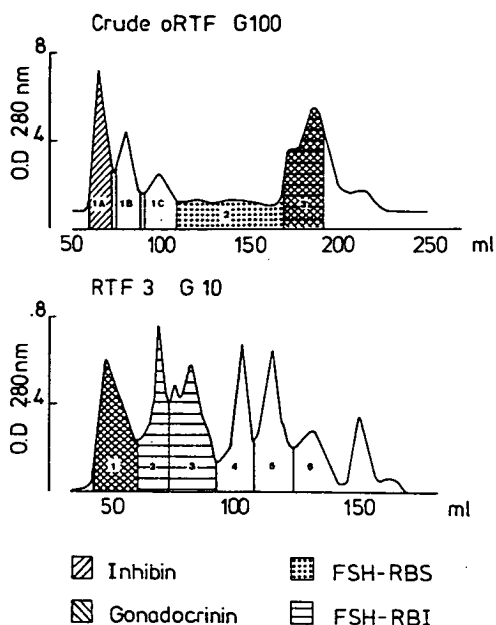


Fig. 1 Localization of inhibin, gonadocrinin, FSH-RBS and FSH-RBI activity after molecular sieve chromatography of ovine rete testis fluid.

The biological activity of inhibin is neither species nor sex specific as preparations of different origins from both sexes reduce FSH secretion in a parallel fashion (Franchimont and others, 1979). Moreover, a radioimmunoassay developed for ovine inhibin corroborates these results since complete cross reactions between ovine rete testis fluid fraction, bovine testicular extract, human seminal plasma fraction and bovine follicular fluid were established (Franchimont and others, 1979). The amounts of inhibin in crude biological fluids from various species are however different. Large M.W. inhibin is well characterized in bull seminal plasma (Chari and others, 1978) but in our hands is still undetectable in human seminal plasma. Follicular fluids (FF) from all size follicles have been collected in women, cows and mares. After charcoal adsorption, protein concentrations have been estimated. The amounts of protein necessary to produce 25% of inhibition of LHRH induced FSH secretion by pituitary cells in culture are variable depending upon the species: equine FF: 2.5 $\mu$ g; bovine FF: 10  $\mu$ g; human FF: > 1000  $\mu$ g.

#### MODE OF ACTION OF INHIBIN

Inhibin acts at various levels: pituitary gland, hypothalamus and gonads.

#### Pituitary Action

As recently reviewed by Baker and others (1981), detection of inhibin activity is based upon the modifications of endogenous FSH release in various biological models using small and large animals or *in vitro* techniques. Male or female inhi-

bin preparations inhibit the post castrational rise of FSH in both sexes and reduce the HCG induced increase of uterine and ovarian weight. Administration of antibodies against male inhibin to male and female rats results in a selective rise of FSH levels by a neutralization of endogenous inhibin (Franchimont and others, 1975, 1977). Pituitary cells, isolated from male or female rats synthesize and release less FSH when inhibin containing materials are present in the culture medium. No modification of LH, GH, PRL and TSH secretions is observed in these basal conditions. The simultaneous presence of inhibin and LHRH reduces the secretion of both gonadotropins with a preferential effect on FSH (Franchimont and others, 1979). Until now, no sex specificity has been described concerning the action of inhibin at the pituitary level.

#### Hypothalamic Action

The endogenous LHRH content of isolated hypothalamus is reduced when incubated in the presence of increasing concentrations of male inhibin (Demoulin and others, 1980). Another argument for the action of inhibin at hypothalamic level is provided by the *in vivo* experiments of Lumpkin and others (1981). Unfortunately female inhibin has not been tested in the same experimental conditions.

#### Gonadal Action

Administration of male inhibin preparations to pubertal rats reduces the incorporation of tritiated thymidine into DNA of type B spermatogonia. The effect must be considered as a direct action on the gonads since DNA synthesis is also reduced in testicular fragments incubated in the presence of inhibin. This phenomenon is however limited to the period of initiation of spermatogenesis (Demoulin and others, 1981). Nevertheless, as the inhibin preparations are incompletely purified, we cannot exclude a contamination by a testicular chalone. Furthermore, equine follicular fluid tested in the same experimental *in vitro* conditions is unable to reduce DNA synthesis (Croze and others, 1980). Similar actions on the ovaries have never been studied.

On the other hand, male inhibin preparations decrease progesterone secretion by cultured granulosa cells (Franchimont and others, 1981, Henderson and Franchimont, 1981). However a contamination of the preparation by gonadocrinin or male equivalents of oocyte maturation inhibitor (Tsafiriri and others, 1976), luteinization inhibitor (Ledwitz-Rigby and others, 1977) or LH receptor binding inhibitor (Yang and others, 1976) can not be excluded. Indeed these ovarian factors reduce progesterone secretion *in vitro*. Evidence of a similar direct action of inhibin on the production of steroids by Sertoli cells in culture is not available.

### REGULATION OF INHIBIN SECRETION

The mechanisms regulating inhibin secretion have been investigated in both male and female by using monolayer cultures of granulosa, luteal and Sertoli cells. The influence of gonadal steroids and gonadotropins on inhibin secretion by the cells has been assessed by the ability of these culture media to reduce FSH secretion in dispersed pituitary cell cultures.

#### Hormonal Regulation in the Male

After one week of incubation, Sertoli cells in culture are treated for 24 hours with HCG, PMSG or FSH. These treatments do not modify the inhibin activity in the culture media. In contrast, incubation with testosterone alone or in combination with PMSG induces a significant increase of inhibin activity with a concomitant

aromatization of testosterone into estradiol-17  $\beta$ . However this aromatization is not necessary in order to modify inhibin secretion since: (1) - the addition of dihydrotestosterone, a non-aromatizable androgen, produces a similar effect on inhibin secretion as does testosterone, and (2) - the addition of estrogen itself has no effect on inhibin production by the same cells (Franchimont and others, 1981, Verhoeven and Franchimont, 1981). Steinberger (1981) has exposed Sertoli cells to higher concentrations of FSH, and for long periods of time and under these conditions, FSH also stimulates inhibin secretion. Moreover FSH and testosterone added together have a synergistic effect.

#### Hormonal Regulation in the female

Granulosa cells from antral bovine follicles in culture secrete progressively decreasing amounts of inhibin, whereas simultaneously progesterone secretion is increasing. Addition of FSH, LH, HCG or PMSG in the culture medium does not modify inhibin activity but stimulates progesterone production. In contrast, the presence of both aromatizable and non-aromatizable androgens, respectively testosterone and dihydrotestosterone, increase inhibin production without any change in progesterone secretion. Addition of estrogens does not modify inhibin secretion by granulosa cells while progesterone reduces it. Luteal cells in culture do not produce detectable levels of inhibin even in the presence of androgens (Franchimont and others, 1981; Henderson and Franchimont, 1981).

#### Comparison between Male and Female Regulations

The mechanisms involved in the regulation of inhibin secretion are identical in the male and female. Androgens directly stimulate the production of the hormone by Sertoli and granulosa cells. Progesterone which reduces inhibin production by granulosa cells remains to be tested in the male.

#### Heat Regulation

The production of inhibin by Sertoli cells *in vitro* is temperature dependent. Elevated temperature (37 - 38°C) leads to a reduction of inhibin secretion while cultures incubated at lower temperatures (31 - 32°C) continue to secrete inhibin (Demoulin and others, 1979, Steinberger, 1980). The effect of reduced temperature on the secretion of inhibin by granulosa cells *in vitro* is unknown. Based on these observations, two questions remain to be answered. Is the heat stability of male and female inhibin different? What about the inhibin production in male animals with normally abdominal testes such as elephants, etc...

### INHIBIN IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

#### Variations with Age

In the male rat, Steinberger (1979) has demonstrated that the ability of Sertoli cells to secrete inhibin is similar between 18 and 90 days of age. On the other hand, pituitary sensitivity to inhibin is significantly reduced in younger animals. Using a radioimmunoassay for inhibin, Vaze and others (1979) observed elevated concentrations in peripheral serum of 9 - 14 day old rats as compared to those of older animals. Moreover inhibin is inversely correlated with FSH levels. In the female, age related variations of inhibin are not yet available except in the human perimenopausal period. Ovarian extract of a 48 year old oligomenorrheic woman contained less inhibin than that of a younger woman with normal menstruations. Moreover, the FSH serum level in the older woman was elevated while LH and estradiol levels were similar to those of the younger patient (Fig. 2).

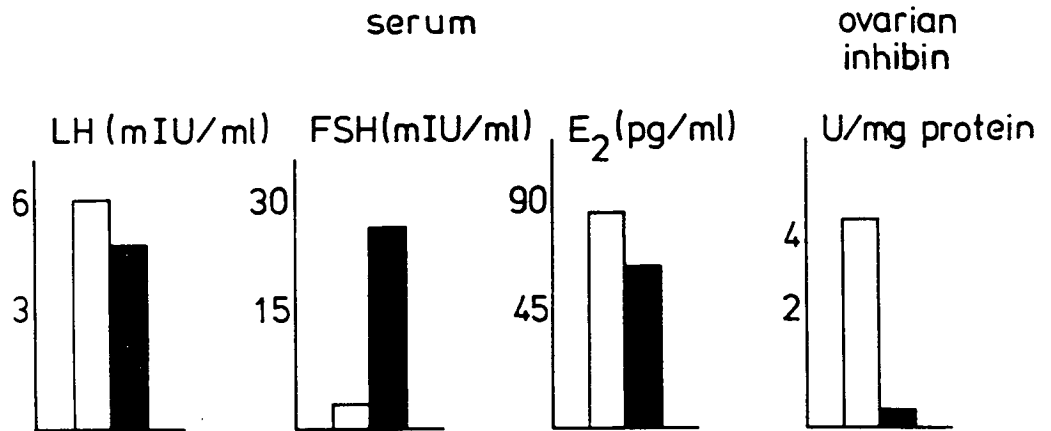


Fig. 2 Comparison between serum levels of LH, FSH, estradiol-17  $\beta$  and inhibin activity in ovarian extracts. Open columns : 42 year old woman with normal menstruations, closed columns : 48 year old woman who was oligomenorrhic.

This preliminary experiment argues for the postulated role of inhibin during the climateric period. Furthermore, lower inhibin activity is also observed in follicular fluids collected in premenopausal women (Franchimont and Lefaucheur, unpublished observation).

#### Cyclic Variations

Endocrine gland activity is modulated in the female by cyclic variations in ovarian secretions. In follicular fluids collected from various sized follicles, an inverse relationship between the sizes and inhibin activity is observed in pig (Lorenzen and others, 1978 ; Anderson and de Paolo, 1981), cow (Franchimont and others, 1981) and women (Channing and others, 1981 ; Franchimont and Lefaucheur, unpublished observation). Inhibin activity is also low in fluids collected from atretic follicles during the luteal phase (Chappel and others, 1980 ; Channing and others, 1981). Granulosa cell ability to secrete inhibin *in vitro* is however increasing with follicle maturation (Anderson and de Paolo, 1981). The authors suggest various hypotheses explaining the lower inhibin concentrations in pre-ovulatory follicles : destruction due to protease activities, rapid exit secondary to a better vascularisation and the presence of a prohormone. Detectable amounts of inhibin assessed by bioassay are also reported in rat ovarian venous blood ; inhibin and FSH levels being inversely correlated (review by Anderson and de Paolo, 1981). Moreover, administration of exogenous FSH reduces the inhibin activity in ovarian venous serum (Lee and others, 1981). Further investigations will explain the mechanisms of inhibin regulation *in vivo* i.e. direct stimulation by FSH or via steroid modifications.

Peritoneal fluids have been collected by laparoscopy in normally menstruating women. After charcoal treatment to remove steroids, the fluids stimulate LH and FSH secretion by pituitary cells in culture except for the post ovulatory fluids (Demoulin and others, 1981 b). A peritoneal fluid collected after ovulation has been chromatographed on sephadex G 75 (Fig. 3).

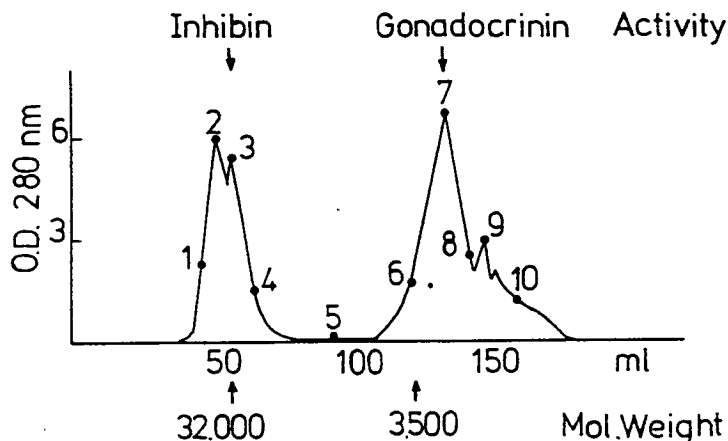


Fig. 3 Chromatography of charcoal treated postovulatory peritoneal fluid (human) on Sephadex G-75. Inhibin and gonadocrinin activity is assessed in ten fractions by monolayer pituitary cell culture assay.

Ten fractions are assayed for inhibin and gonadocrinin activity in the dispersed pituitary cell system. One fraction (n° 3) (M.W. + 32,000) has inhibin activity and another one (n° 7) (M.W. < 3,500) has gonadocrinin activity. The physiological meaning of the presence of inhibin in the abdominal cavity remains to be determined i.e. direct action on the ovaries, enzymatic destruction or reabsorption by general circulation to lower FSH secretion throughout the luteal phase.

#### Pathology

As early as 1972, Franchimont had observed a dramatic reduction of inhibin concentrations in the semen of azoospermic patients assessed by the inhibition of FSH secretion in castrated rats. A radioimmunoassay for human inhibin was developed later (Vaze and others, 1979). The results of this group were however conflicting (Vaze and others, 1980; Asch and others, 1980). Estimated by bioassay, inhibin concentrations in seminal plasma are strongly reduced or undetectable in azoospermia and significantly lowered after vasectomy (Scott and Burger, 1980). Similar results have been obtained in our laboratory using monolayer cell cultures as a bioassay. FSH concentrations in semen are not however correlated with those of inhibin (Table 1). The validity of bioassays for assessing inhibin concentration in native biological fluids remains limited. Indeed other substances such as gonadocrinin could interfere in this type of assay (Demoulin and others, 1981b).

The role of inhibin in female pathology is presently unknown. Inhibin activity is reduced in cystic fluids from human ovaries (Channing and others, 1981). Similarly, cystic fluids collected from bovine ovaries contain 2.5 times less inhibin activity than fluids from antral follicles. However, the total amount of inhibin is higher in ovarian cysts. (Franchimont, unpublished observation).



TABLE 1

Sperm Count (x 10 <sup>6</sup> / ml)	Inhibin Activity <sup>o</sup> (%)	Semen FSH (mU / ml)
> 40	40 + 5	3.06 + 2.14
10 - 20	16 + 2	2.75 + 1.87
5 - 10	15 + 7	1.91 + 0.57
1 - 5	6 + 4	3.33 + 2.14
1	9 + 4	1.89 + 0.63
< 0	2 + 5	2.17 + 0.63

<sup>o</sup> Suppression of FSH secretion *in vitro*

### CONCLUSION

At the present time, the existence of a protein hormone synthesized by the gonads and regulating FSH secretion is largely demonstrated in both sexes. Before complete purification and biochemical characterization of male and female inhibin, the affirmation of their molecular identity will remain hypothetical. Various arguments suggest a similarity :

1. male and female inhibin preparations reduce FSH secretion *in vivo* and *in vitro* in both sexes,
2. immunological cross-reactivity exists between male and female active materials,
3. inhibin secretion is stimulated by androgens in both Sertoli and granulosa cells *in vitro*.

Differences are observed in the *in vivo* regulation mechanisms. In the female, from puberty to menopause, inhibin secretion is modulated by menstrual cycle events. Similar cyclicity has never been described in the male.

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## INHIBIN PRODUCTION BY THE OVARY

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### INTRODUCTION

Inhibin may be defined as a peptidic factor of gonadal origin that specifically or selectively lowers the rate of secretion of FSH. This substance has been detected and partially purified from ovarian extracts (Hopkinson *et al.*, 1975, 1977; Chappel *et al.*, 1979), porcine (Marder *et al.*, 1977; Welschen *et al.*, 1977; Lorenzen *et al.*, 1978) and human (Chari *et al.*, 1979) follicular fluid as well as the culture medium of granulosa cells (Erickson and Hsueh, 1978).

In this paper, we intend to describe the assay of inhibin, to quantify the concentration of inhibin in follicular fluids and to approach the mechanism of inhibin secretion by granulosa and luteal cells.

### ASSAY OF INHIBIN

The assay of inhibin already described by Lee *et al.* (1979) is based on the inhibition of LHRH-induced FSH secretion by dispersed pituitary cells (Franchimont *et al.*, 1979a). Anterior pituitary cells from adult male Wistar rats are dispersed using the trypsin method of Hopkins and Farquhar (1973). The dispersed cells are suspended in 3 ml Dulbecco Modified Eagle's Medium (DMEM) (Dulbecco and Freeman, 1959), supplemented with 5% horse serum, 2.5% foetal calf serum, 1% glutamine and 1% non-essential amino acids and then distributed in culture

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dishes. Each culture dish (Falcon Plastics) contains  $1 \times 10^6$  cells and is incubated at  $37^\circ\text{C}$  in a water-saturated atmosphere of 95% air and 5%  $\text{CO}_2$  for 3 days. After this period, samples of standard and unknowns are added to five culture dishes for each concentration. The total volume of incubation was 1.6 ml. The incubation was continued for a further 3 days. After this period, the media were removed, and the culture plates were washed and then incubated for a further 6 h in DMEN with the samples at the same concentrations and with luteinizing hormone-releasing hormone (LHRH) at a final concentration of  $10^{-8}$  M. Each concentration of the samples is tested in 5-plicate in basal conditions and under the stimulatory effect of LHRH.

The reference preparation is derived from ovine testicular lymph (OTL) given an arbitrary potency 1 U/mg. The inhibin standard is added to dispersed rat anterior pituitary cell culture over a dose range of 250 to  $2,000 \mu\text{g ml}^{-1}$ .

Active preparations extracted from rete testis fluid have been extensively

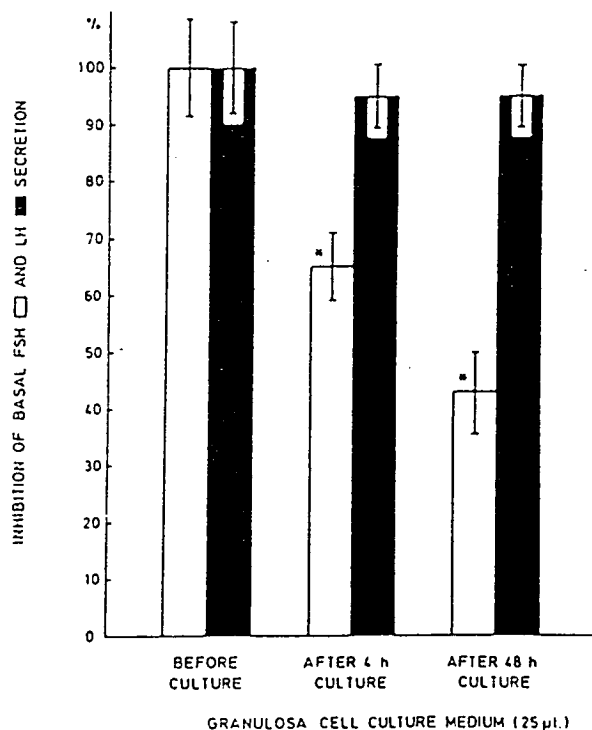


Fig. 1. Effect of  $25 \mu\text{l}$  granulosal cell culture medium on basal FSH and LH secretion by  $10^6$  dispersed anterior pituitary cells after 3 days of incubation. Unprimed culture medium is used as control and basal FSH and LH secretion in its presence represents 100%. Media of 4 h and 48 h cultures of granulosal cells significantly decrease basal FSH secretion without affecting LH secretion. Each bar represents the mean  $\pm$  1 standard deviation. \* $P < 0.001$ .

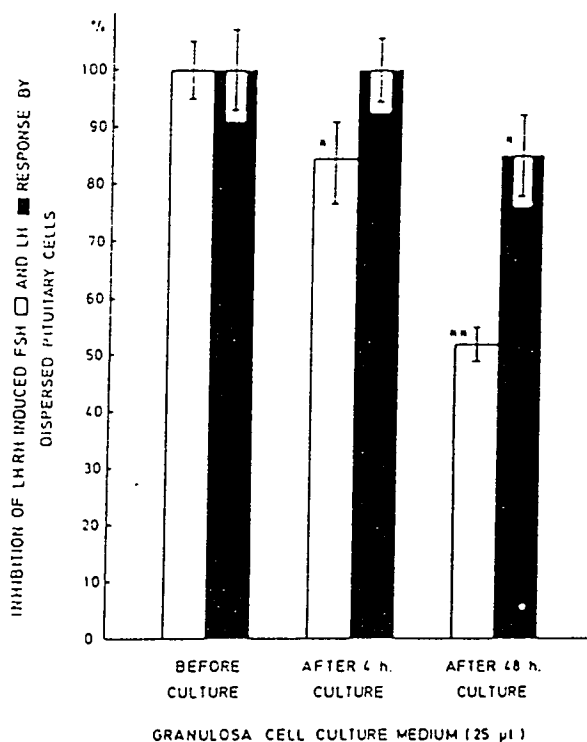


Fig. 2. Effect of 25  $\mu$ l granulosa cell culture medium on LHRH-induced FSH and LH release by  $10^6$  dispersed anterior pituitary cells. Unprimed culture medium is used as control and LHRH-induced FSH and LH release in its presence represents 100%. Medium of 4 h culture of granulosa cells significantly decreases FSH release without affecting LH. Medium of 48 h culture significantly depresses both FSH and LH release but the effect is much more marked for FSH than for LH. Each bar represents the mean  $\pm$  1 standard deviation. \* $P < 0.01$ ; \*\* $P < 0.001$ .

described previously (Franchimont *et al.*, 1978). Follicular fluids and culture medium are always pretreated with charcoal-dextran for the purpose of removing the sex steroids. Charcoal treatment involved the addition of activated charcoal (NORIT A 1%) pretreated with dextran (0.1%). The samples are mixed at 4° for 16 h followed by centrifugation at 3,000 g. The supernatants are filter-sterilized by passing through a 0.45  $\mu$ m cellulose millipore filter before use.

The concentrations of follicular fluid in the pituitary cell culture medium range from 1% to 5% (v/v) whereas the concentrations of granulosa and luteal cells culture medium are between 3% and 60% (v/ $\mu$ ). Control values are obtained using the same concentration of granulosa or luteal cell culture medium before any contact with the cells. Each amount of standard or unknowns and each volume of biological fluid are assayed five times at several concentrations. Specificity of the

method is ascertained in two ways (Franchimont *et al.*, 1979b). First, inhibin activity is defined by absence of effect on LH secretion in basal conditions after 3 days of incubation with inhibin preparations (Fig. 1). Furthermore, a preferential inhibitory effect on FSH secretion must be observed after stimulation of pituitary cells by LRRH (Fig. 2). Second, the secretion of pituitary hormones such as prolactin and TSH should not be affected by inhibin.

The precision and parallelism (Borth, 1976) for inhibin assay were evaluated. The FSH concentrations are expressed as percentage of control (no added inhibin). Fig. 3 represents the mean curve from 15 individual curves of inhibition of LHRH-induced FSH release by increasing amounts of OTL standard (OTL<sub>6</sub>)\*. The mean slope (b) is equal to 27.7 (19.4 - 34.2) and the significance of regression (Finney's g) is always higher than 0.01. RTF<sub>1A</sub>, a preparation of inhibin extracted from rete testis fluid with a M.W. higher than 10 000 (Franchimont *et al.*, 1978) and follicular fluid induce a dose dependent inhibition of LHRH-induced FSH

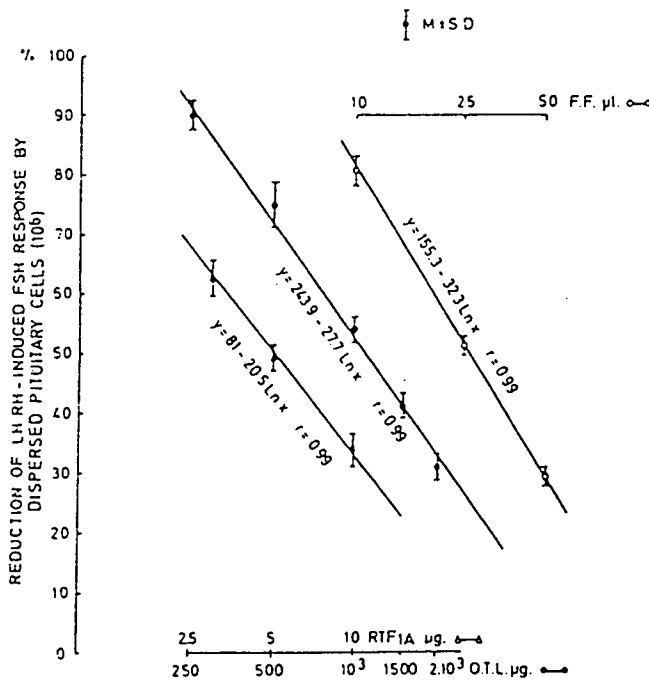


Fig. 3. Reduction of LHRH-induced FSH release by increasing amounts of several preparations of inhibin. Ordinate : reduction of FSH secretion expressed as percentage of the control value (100%)  $\pm$  1 SD. Abscissa : bovine follicular fluid (FF) fraction 1<sup>A</sup> of rete testis fluid (RTF<sub>1A</sub>) and the standard : OTL.

\*Kindly provided by Professor B. Hudson, Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Australia.



secretion. Variations in their slopes ( $G_2$ ) are not significant and, therefore, curves are considered as parallel. Index of precision ( $\lambda$ ) is ranged between 0.06 to 0.19. Inhibin preparations also decrease basal FSH concentrations in a dose dependent manner. But the inhibition curves are less steep ( $b = 19.3$ ) and less precise ( $\lambda$  between 0.41 - 0.15). For these reasons, LHRH-induced FSH release was chosen as the end point of inhibin assay.

#### INHIBIN IN OVARIAN VEIN AND FOLLICULAR FLUID

In female monkeys (Channing *et al.*, 1980) and rats (De Paolo *et al.*, 1979) inhibin activity is found in ovarian vein. In rats, the FSH-inhibiting activity in ovarian venous plasma varies inversely with peripheral plasma FSH concentrations. In monkeys, removal of large follicles from ovaries is followed by a decrease in FSH inhibiting activity of ovarian venous plasma and consequently there is a significant rise in serum FSH. Furthermore, in several animal species, inhibin activity has been found by *in vivo* assays in the follicular fluid pretreated with charcoal to remove sex steroids (De Jong and Sharpe, 1976; Marder *et al.*, 1977; Welschen *et al.*, 1977; Lorenzen *et al.*, 1978).

Data from the literature are contradictory concerning the levels of ovarian inhibin during follicular development. According to Lorenzen *et al.* (1978), the concentration of inhibin diminishes with the growth of the follicle in the pig. In contrast, Welschen *et al.* (1977) found inhibin in small bovine follicles (5-10 mm in diameter) and maximum concentrations were reached in medium and large (11-20 mm in diameter) follicles. By assaying inhibin *in vitro*, we have found that the concentration of inhibin is higher in small follicles from which a small volume of fluid is collected. In contrast, when the volume of follicular fluid increases, the concentration of inhibin decreases (Fig. 4).

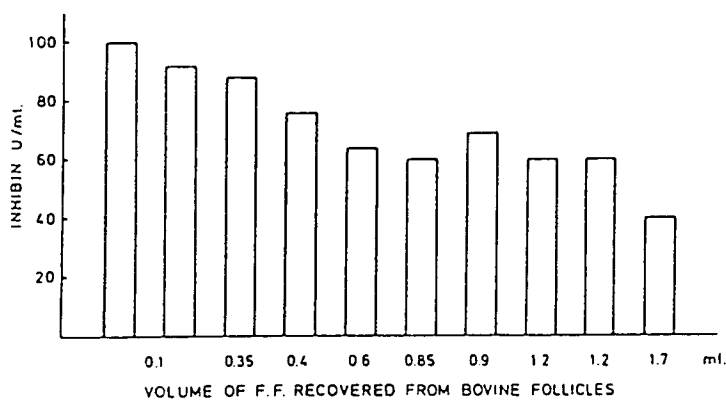


Fig. 4. Concentration of inhibin expressed in U/ml in follicular fluid according to the volume collected from individual antral follicles.

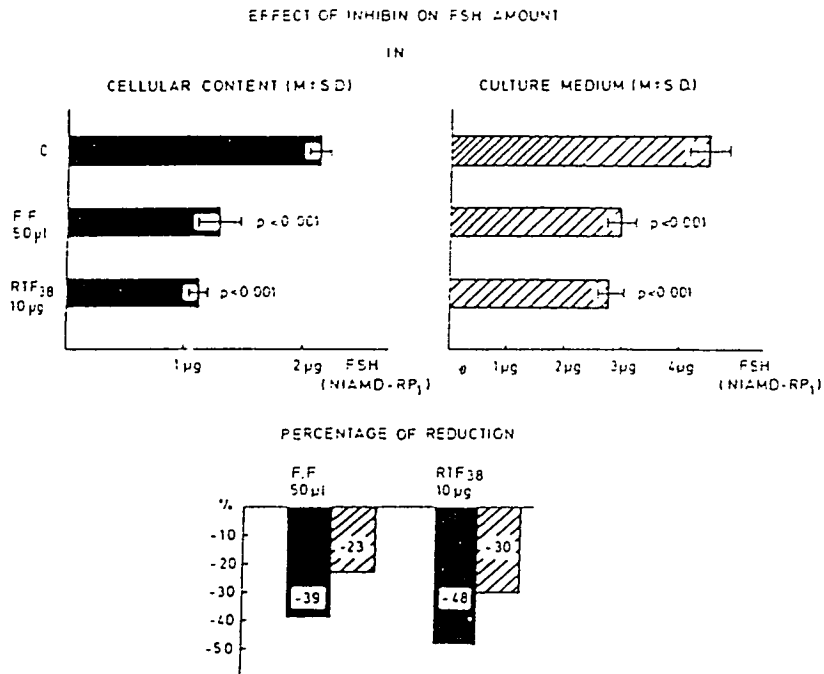


Fig. 5. Effect of 50  $\mu$ l of steroid-free bovine follicular fluid (FF) and 10  $\mu$ g of ram rete testis fluid (RTF<sub>38</sub>) on amounts of FSH cell content and in culture medium after 3 days of incubation. Bar C : control values observed in the absence of inhibin preparations. The upper part of the graph indicates absolute FSH amounts  $\pm$  SD and the lower part represents the reduction of FSH amounts in cellular content (black columns) and in culture medium (hatched columns) expressed as percentage, the control values representing 0%. Under these experimental conditions, no effect was observed on LH intracellular and culture medium contents.

*In vitro*, various preparations of inhibin (extract of RTF, follicular fluid from mare, etc.) lead to a concomitant reduction in the quantity of FSH in the culture medium and within the cells after 72 h of incubation in the absence of LHRH. This action is more marked on the cell content than on the quantities of FSH released into the culture medium (Fig. 5). Under our chosen experimental conditions, no effect was observed on the quantities of LH present in either the culture medium or the cells. These actions on the quantities of FSH in the two compartments show that inhibin preparations tested have an effect on FSH synthesis under basal conditions. In fact, if the actions were limited to an inhibition of FSH release, the level of FSH would be reduced in the culture medium whereas the quantities of FSH in the cells would remain the same or be even greater than in the control cells (Franchimont *et al.*, 1978).

## MECHANISMS OF INHIBIN SECRETION

To investigate the mechanism of inhibin secretion, monolayer granulosa and luteal cell culture were carried out. Indeed, Erickson and Hsueh (1978) had showed that the granulosa cells in culture secrete a substance that acts directly on pituitary cell culture and preferentially suppresses FSH secretion. The methods were extensively described by one of us (Henderson and Moon, 1979). Granulosa cells are obtained from bovine large antral follicles (7-15 mm). The harvested cells are pooled, washed three times with Minimum Essential Medium with Earle's Salts (EMEM) and supplemented with HEPES buffer (20 mM), glutamine (2 mM), antibiotics and non-essential amino acids. Each dish contained  $10^6$  cells which are cultured at 36 °C in a humidified incubator on 15 mm diameter round plastic coverslips in 1 ml culture medium consisting of 10% (v/v) foetal bovine serum and 90% EMEM without HEPES but supplemented with glutamine, antibiotics and non-essential amino acids.

Luteal cells are obtained from bovine corpora lutea. Small fragments of corpora lutea are incubated for 20 min at 37 °C with stirring in Hanks' Balanced Salt Solution supplemented with glutamine (2 mM), HEPES (20 mM) and antibiotics and contain 0.2% collagenase (Type II, Sigma). The medium is decanted and the released cells are collected by low speed centrifugation and stored at 4 °C. The remaining fragile tissue fragments are incubated again in the same medium containing 0.2% collagenase. All the released cells are pooled together, filtered through sterile gauze and washed four times to remove any trace of collagenase. Culture of  $10^6$  cells are set up exactly as described for the granulosa cells.

Pregnant mare serum gonadotropin (PMSG) has predominantly FSH like activity and induces ovarian follicular development in intact as well as hypophysectomized immature rats. In order to induce a follicular development 10 IU PMSG were injected subcutaneously to 21 day old female rats. Animals were killed 3 days later. Pseudopregnant corpora lutea were induced by sequential injection of 50 IU PMSG and 25 IU HCG s.c. at days 10 and 7 respectively before sacrifice. Rat dispersed granulosa and luteal cells were prepared according to the method of Croze and Franchimont (1981).

The progesterone content of culture medium was assayed directly by radioimmunoassay according to the method of Orczyk *et al.* (1974). Oestradiol was measured in diethylether extracted aliquot of culture media by radioimmunoassay using the antiserum and the methods described by Dorrington and Armstrong (1975).

#### Production of Inhibin and Progesterone by Bovine Granulosa and Luteal Cells

Luteal cells make 25 times more progesterone than granulosa cells over the first 24 h. During the next 24 h, progesterone production by granulosa cells rises, presumably as a consequence of luteinization while progesterone production by the luteal cells declines. Inhibin activity is undetectable in volumes of up to 50  $\mu$ l of culture medium of luteal cells during the first and second days of culture. In contrast, inhibin secretion by granulosa cells is elevated in the first day culture medium and declines in the second day culture medium (Fig. 6).

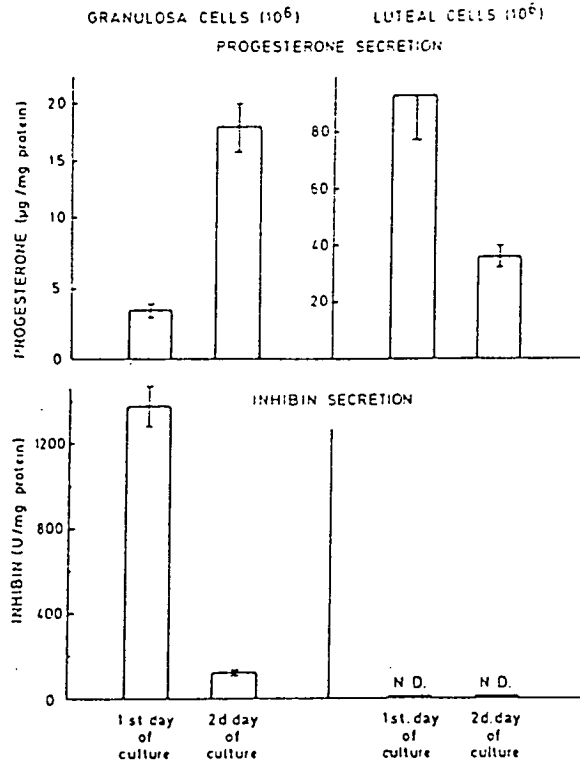


Fig. 6. Progesterone (upper pannel) and inhibin (lower pannel) secretion by granulosa (left parts) and luteal (right parts) cells during the first and the second day of culture. Results are expressed in  $\mu\text{g}$  for progesterone and in unit for inhibin per mg protein by measuring the total protein content of the cells remaining attached to the culture dish at the end of the culture.

The analysis of the results of several (four) experiments on bovine granulosa cell culture clearly demonstrates an inverse relationship between progesterone and inhibin secretion (Fig. 7).

#### Effect of $17\beta$ -Oestradiol and Testosterone on Progesterone and Inhibin Secretion by Bovine Granulosa Cells

Oestradiol has a marked inhibitory effect on progesterone production particularly during the second 24 h of culture when both concentrations (0.1 and 1  $\mu\text{g}/\text{ml}$ ) are inhibitory (Fig. 8). Only one microgram of  $17\beta$ -oestradiol is inhibitory over the first 24 h. Both concentrations of  $17\beta$ -oestradiol do not modify the inhibin secretion by the granulosa cells whatever the day of the culture.

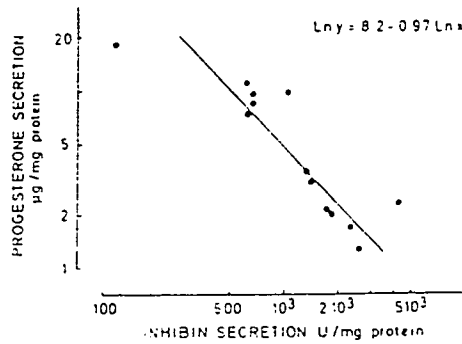


Fig. 7. Inverse relationship between progesterone and inhibin secretion by granulosa cells. Progesterone and inhibin secretions are respectively expressed in  $\mu\text{g}$  and unit per mg protein on log. scales. Coefficient of correlation ( $r$ ) is 0.76.

Testosterone at the dose of 100 ng and 1  $\mu\text{g}/\text{ml}$  of culture medium has no significant effect on progesterone production at either 24 h or 48 h of culture. In contrast, the addition of 1  $\mu\text{g}$  of testosterone to granulosa cell culture medium significantly increases inhibin secretion during the first and the second day of culture.

#### Stimulation of Aromatization and Inhibin Secretion in Immature Rats

Secretion of progesterone, oestradiol and inhibin by gonadotrophin-induced granulosa and luteal cells ( $10^4$  cells) was investigated for 4 days. The fourth day, PMSG 0.02 U/ml and testosterone  $5 \cdot 10^{-7}$  M were added to some cultures in order to stimulate the aromatization of testosterone to oestrogens. Progesterone secretion by luteal cells is more elevated than that by granulosa cells whereas inhibin secretion is higher for granulosa than for luteal cells. Oestradiol remained low over the whole culture period for both cellular types. The fourth day of culture, no inhibin was detectable in the culture medium of granulosa and luteal cells. Oestradiol was at the limit of detection and progesterone secretion was still sustained for luteal cells. Addition of PMSG and T to some cultures significantly increased the secretion of oestradiol and inhibin by granulosa and luteal cells without modifying the levels of progesterone (Table 1).

#### DISCUSSION

It is now possible to assay inhibin in biological fluids using an *in vitro* method consisting of the selective reduction of FSH secretion by dispersed anterior pituitary cells. Inhibition of LHRH-induced FSH secretion is, in our opinion, more precise and sensitive than the inhibition of basal FSH secretion. Criteria for the quality of a multiple parallel line bioassay are fulfilled. Thus, we con-

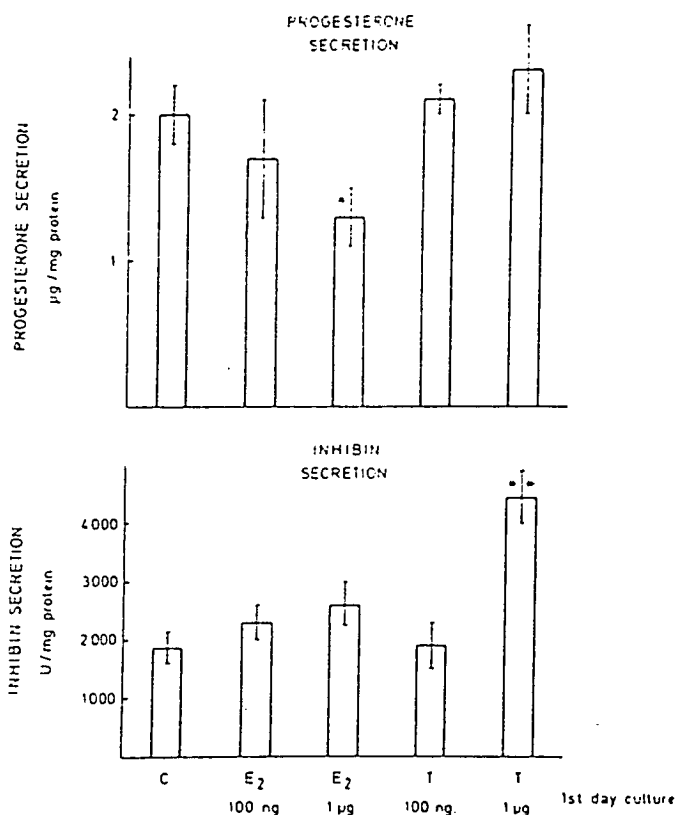


Fig. 8a. Effect of 0.1 and 1 μg of oestradiol (O<sub>2</sub>) and testosterone (T) on progesterone and inhibin secretion by granulosa cells during the first day of culture. \**P* < 0.05; \*\**P* < 0.01.

firm the usefulness of dispersed pituitary cell culture as a method for assaying inhibin (De Jong *et al.*, 1979; Eddie *et al.*, 1979; Hudson *et al.*, 1979; Lee *et al.*, 1979).

Biological identity of inhibin preparation extracted from ram rete testes fluid and of inhibin present in follicular fluid or in lyophilized ovine testicular lymph is assessed by the parallelism of curves of reduction of FSH release into the culture medium. As already demonstrated (Franchimont *et al.*, 1979), inhibin contained in mare follicular fluid decreases both the synthesis and the release of FSH without modifying the synthesis of LH. Only high volumes of follicular fluid are capable of inhibiting the LH release induced by LHRH added to the culture medium.

An inverse relationship exists between progesterone and inhibin production. Thus, when bovine granulosa cells undergo luteinization and differentiate into

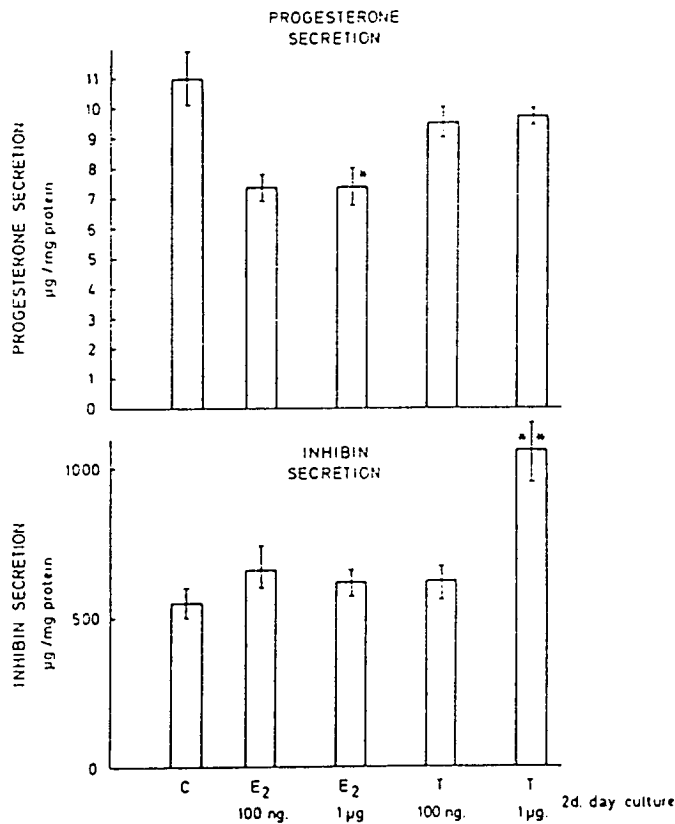


Fig. 8b. Effect of 0.1 and 1 µg of oestradiol (O<sub>2</sub>) and testosterone (T) on progesterone and inhibin secretion by granulosa cells during the second day of culture. \* $P < 0.05$ ; \*\* $P < 0.01$ .

luteal cells as indicated by histological changes and steadily increasing progesterone production, they progressively lose their capacity to make inhibin.

Luteal cells collected from bovine corpus luteum tissue have a very limited capacity, if any, to produce inhibin. Similarly, granulosa cells induced by injection of PMSG to immature female rats produce much more inhibin than PMSG-HCG-induced luteal cells in these animals.

Testosterone, but not oestradiol, stimulates inhibin production by granulosa cells. This positive effect of testosterone on inhibin secretion is concomitant with the stimulation of aromatization of androgens. This aromatization of exogenous androgens was clearly demonstrated by Henderson and Moon (1979) during the first 2 days of culture of large antral follicular cells. This positive effect on aromatization and on inhibin production does not require any exogenous FSH. Another demonstration of the association of androgen aromatization and inhibin pro-

*Table 1.* Secretion of progesterone (P<sub>4</sub>), 17 β-oestradiol (O<sub>2</sub>) and inhibin (Inh.) on the 4th day of granulosa and luteal cell culture (10<sup>4</sup>).

Treatment	Granulosa cells (10 <sup>4</sup> )			Luteal cells (10 <sup>4</sup> )		
	P <sub>4</sub> (ng)	O <sub>2</sub> (pg)	Inh. (mU)	P <sub>4</sub> (ng)	O <sub>2</sub> (pg)	Inh. (mU)
None (5) <sup>c</sup>	0.6 ± 0.09 <sup>a</sup>	30 ± 12	0	4.5 ± 0.2	33 ± 10	0
PMSG : 0.02 U/ml + T5·10 <sup>-7</sup> M (5) <sup>c</sup>	0.82 ± 0.10	1.100 ± 89 <sup>b</sup>	537 ± 45 <sup>b</sup>	5.5 ± 0.6	670 ± 68 <sup>b</sup>	262 ± 48 <sup>b</sup>

<sup>a</sup>M ± SE; <sup>b</sup>p < 0.01 compared with the untreated culture; <sup>c</sup>( ) number of culture.



duction is provided by cultures of gonadotrophin-induced granulosa and luteal cells in immature female rats. In control culture, no inhibin and very low amounts of oestradiol were detected after 4 days. In contrast, a significant increase of oestradiol and inhibin production was observed when PMSG and T were added to the culture.

It may be concluded that the principal source of inhibin is granulosa cells when they are capable of aromatizing testosterone into oestrogens and when they are non-luteinized. Luteinization of granulosa cells and subsequent luteal cell formation are associated with an increase of progesterone production and a loss in androgen aromatization and in inhibin production.

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INHIBIN: MECHANISMS OF PITUITARY ACTION AND REGULATION OF SECRETION\*

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Since 1972, numerous investigations have been directed towards the demonstration of the existence of inhibin, a gonadal peptide which specifically or preferentially decreases the secretion of follicle stimulating hormone (FSH).

The physiological role of this hormone, its actions on the pituitary, the hypothalamus and the gonads, its possible synergism with the sex steroids and the mechanisms which control its secretion have also been studied extensively.<sup>1,2</sup>

In this article, we shall provide some personal data on the (still incomplete) purification of inhibin, cyclic nucleotide modifications associated with inhibin-induced reduction of FSH secretion by pituitary cells in culture and the mechanisms regulating inhibin secretion by granulosa and Sertoli cells.

PURIFICATION OF INHIBIN

A number of investigators have attempted to purify this hormone.<sup>2</sup> At present, there are few, however, who believe that a completely pure preparation is available; its chemical structure and physico-chemical properties are still highly controversial.

High molecular weight inhibin. Inhibin extracted from bovine follicular fluid (BFF) and ram rete testis fluid (RTF) possesses some common characteristics. From the results obtained by Jansen et al<sup>3</sup> it may be concluded that inhibin in BFF is probably associated with a hydrophobic glycoprotein with a molecular weight of approximately 65,000 Daltons. We have confirmed these data and shown that inhibin-like activity extracted from RTF is also strongly retarded on matrex gel red A columns, binds to concanavalin A and is eluted with 1 M  $\alpha$  methyl-D-mannopyranoside. In Table 1, the recovery

TABLE 1

PURIFICATION OF HIGH MOLECULAR WEIGHT INHIBIN FRACTIONS FROM BOVINE FOLLICULAR FLUID (BFF) AND RAM RETE TESTIS FLUID (RTF) BY SEPHADEX G100, MATREX GEL RED A AND CONCANAVALIN A CHROMATOGRAPHY

Purification procedures	Bovine follicular fluid			Ram rete testis fluid		
	Protein content mg*	Biological specific activity U/mg**	Biological yield %	Protein content mg*	Biological specific activity U/mg**	Biological yield %
Starting material	1706	50	100	-	22	-
Sephadex G100 <sup>1</sup>	-	-	-	250	228	100
Matrex gel red A						
elution with Tris-HCl 50 mM +KCl 500 mM + urea 1 M	73.6	265	23	31.2***	776	42
elution with Tris-HCl 50 mM +KCl 1.25 M + urea 1M	6.7***	1075	9	5	276	2.4
Concanavalin A Sepharose 4 B						
not retained	0.74	1020	0.8	4.38	344	3
retained and eluted with α M-D-mannopyranoside 1M	1.27	2397	3.5	15.21	944	25

\* Determined by the method of Lowry *et al.*<sup>5</sup>

\*\* Biological specific activity is expressed in units per mg. The reference preparation was derived from ovine testicular lymph and given an arbitrary potency of 1 U/mg<sup>9</sup>

\*\*\* Submitted to Con. A chromatography

of proteins and biological activity and the biological specific activity of preparations obtained from both BFF and RTF are illustrated. Recovery of biological activity is poor but there is a 48 and 43 fold increase in specific biological activity for BFF and RTF respectively. In the case of RTF, concanavalin A chromatography does not greatly modify the specific activity of the most active fraction obtained after matrex gel red A chromatography.

Low molecular weight inhibin. Since 1978<sup>4</sup>, an inhibin-like activity has been found in a low molecular weight fraction (RTF<sub>3</sub>) extracted from rete testis and purified by chromatography on Sephadex G100 (Fig. 1). We have observed a similar fraction in BFF.

This fraction with a molecular weight less than 5,000 Daltons was filtered on a Sephadex G10 and inhibin-like activity identified in the void volume (RTF<sub>3-1</sub>) of the column. Further, this peak was submitted to a gel filtration on Sephadex G25 equilibrated and eluted with 10 mM ammonium bicarbonate buffer at pH 7.5. Two different biological activities were then dissociated. In the first protein elution peak (RTF<sub>3-1a</sub>) an inhibin-like activity was found as indicated by a reduction of basal FSH secretion and a decrease of LHRH-induced FSH and LH response by monolayer cultured pituitary cells (Table 2). The second peak (RTF<sub>3-1b</sub>) contained a substance(s) capable of stimulating basal LH secretion without modifying basal FSH secretion. This peak could contain substances related to gonadocrinins.<sup>6</sup> The low molecular weight inhibin fraction (RTF<sub>3</sub>) represents only 6.2% of the total inhibin-like activity of RTF.

#### EFFECTS OF INHIBIN PREPARATIONS ON CYCLIC NUCLEOTIDES IN VITRO

The aim of this work is to investigate the relationship between the effect of inhibin preparations on FSH and LH secretion and on cyclic nucleotide (cAMP and cGMP) production by isolated rat pituitary cells in culture. In a preliminary experiment<sup>7</sup> it was observed that inhibin preparations reduce cyclic AMP produced by pituitary cells in culture.

Methods. Monolayer pituitary cell cultures were prepared according to a method previously described.<sup>8</sup> Following various mechanical and enzymatic treatments, isolated cells are dispersed among Petri dishes ( $5 \times 10^5$  cells per dish) and allowed to recover for 48 to 72 hours. At that time, the medium is discarded and replaced by 1 ml of fresh culture medium containing inhibin preparations whose effects on the cell culture are being tested. This preincubation period takes place for 24, 48 or 72 hours and corresponds to the spontaneous secretion of the cells. At the end of the preincubation time, the

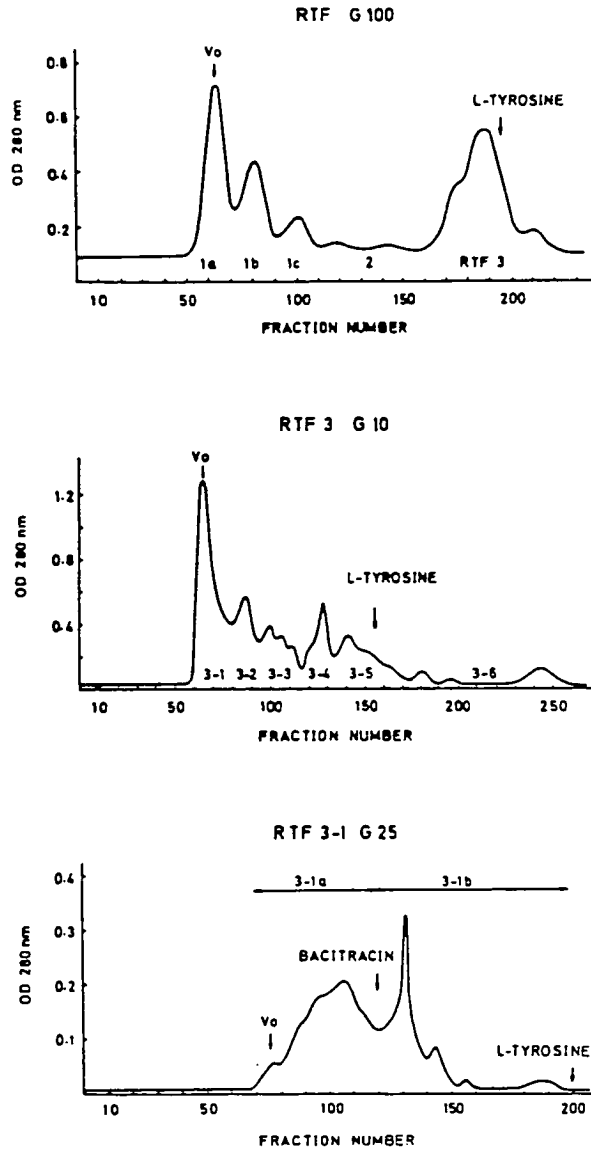


Fig. 1. Elution profiles of low molecular weight inhibin fraction RTF which has been first submitted to a G100 Sephadex filtration.  $RTF_3$ , the last peak of elution, has been filtered on Sephadex G10. The biological activity is localized in the first peak eluted in the void volume :  $RTF_{3-1}$ . This biologically active fraction is submitted to a Sephadex G25 column. Results are considered in Table 2.

TABLE 2

EFFECT OF SMALL MOLECULAR WEIGHT FRACTIONS FROM RAM RETE TESTIS FLUID (RTF) ON BASAL AND LHRH ( $10^{-8}$  M)-INDUCED SECRETION OF FSH AND LH BY MONOLAYER CULTURED PITUITARY CELLS

Material	Doses µg/ml	FSH ng/ml <sup>+</sup>		LH ng/ml <sup>++</sup>	
		Basal secretion	LHRH-induced release	Basal secretion	LHRH-induced release
Control	-	2909 ± 164	1730 ± 83	2339 ± 102	4685 ± 271
RTF <sub>3</sub>	20	3174 ± 430	1418 ± 112*	1991 ± 204	3746 ± 367*
Control	-	2304 ± 92	1430 ± 76	1978 ± 157	4871 ± 257
RTF <sub>3-1</sub>	10	2388 ± 92	1238 ± 59	2321 ± 100**	4210 ± 255**
	20	2371 ± 70	1073 ± 98**	2601 ± 211**	3397 ± 351**
RTF <sub>3-1</sub> <sup>a</sup>	10	2393 ± 69	1158 ± 12**	2088 ± 153	3810 ± 111**
	20	1947 ± 60*	970 ± 48**	2135 ± 280	3021 ± 398**
RTF <sub>3-1</sub> <sup>b</sup>	10	2402 ± 123	1331 ± 133	3066 ± 164**	4526 ± 176
	20	2402 ± 175	1405 ± 183	3524 ± 132**	4418 ± 326

<sup>+</sup> NIAMDD-FSH-RP<sub>1</sub>

<sup>++</sup> NIAMDD-LH-RP<sub>1</sub>

\* p < 0.005 compared to the control

\*\* p < 0.001 compared to the control



medium is collected for the various assays and 1 ml of fresh culture medium containing  $10^{-8}$  M LHRH and the same amounts of inhibin as in the preincubation period is added to the cells. Six hours later, the medium is collected. One ml distilled water is added to each culture dish which is frozen and then thawed. Cells fragments are scraped off with a rubber policeman; the cellular suspension is centrifuged and the supernatant is also used for the various assays. The stimulated secretion by the cells is measured during this incubation time.

Two inhibin preparations extracted from RTF were used with a biological activity of 220 U/mg and 975 U/mg. FSH and LH were assayed in culture medium and in cellular content in duplicate by double antibody methods utilizing NIAMDD rat pituitary gonadotrophin reagents supplied by the pituitary agency of NIH. The concentrations are expressed in term of NIAMDD-rat-FSH-RP<sub>1</sub> for FSH and NIAMDD-rat-LH-RP<sub>1</sub> for LH.

Prolactin (PRL), thyroid stimulating hormone (TSH) and growth hormone (GH) are also assayed in duplicate using NIAMDD kits. Cyclic AMP and GMP levels in media and in the supernatant from cellular fractions are determined in duplicate by double antibody methods using NEN kits (New England Nuclear - U.S.A.).

The amount assayed in culture medium is referred to as the release of hormones or cyclic nucleotides whereas the sum of the amounts found in culture medium and in cellular content is considered as the total production of pituitary cells in culture.

Results. Whatever the duration of preincubation, inhibin preparations do not modify the release and production of PRL, TSH and GH in basal and LH-RH stimulated conditions.

When 20  $\mu$ g of inhibin preparations (220 U/mg) are added to the pituitary cell culture, in basal conditions (i.e. without added LHRH), FSH and LH releases are significantly reduced after 24 and 48 hours of preincubation whereas only FSH release is decreased after 78 hours of preincubation (Fig. 2). Important modifications of cyclic nucleotides are observed in the presence of inhibin. Twenty-four hours after inhibin addition the level of cyclic AMP remains unchanged while the concentration of cyclic GMP is strongly increased. Forty-eight and 72 hours after adding inhibin to the cell culture, cyclic AMP concentration is significantly reduced while the concentration of cyclic GMP remains higher than that of control medium.

EFFECT OF INHIBIN ON FSH, LH, cAMP AND cGMP RELEASE BY PITUITARY  
CELLS IN BASAL CONDITIONS

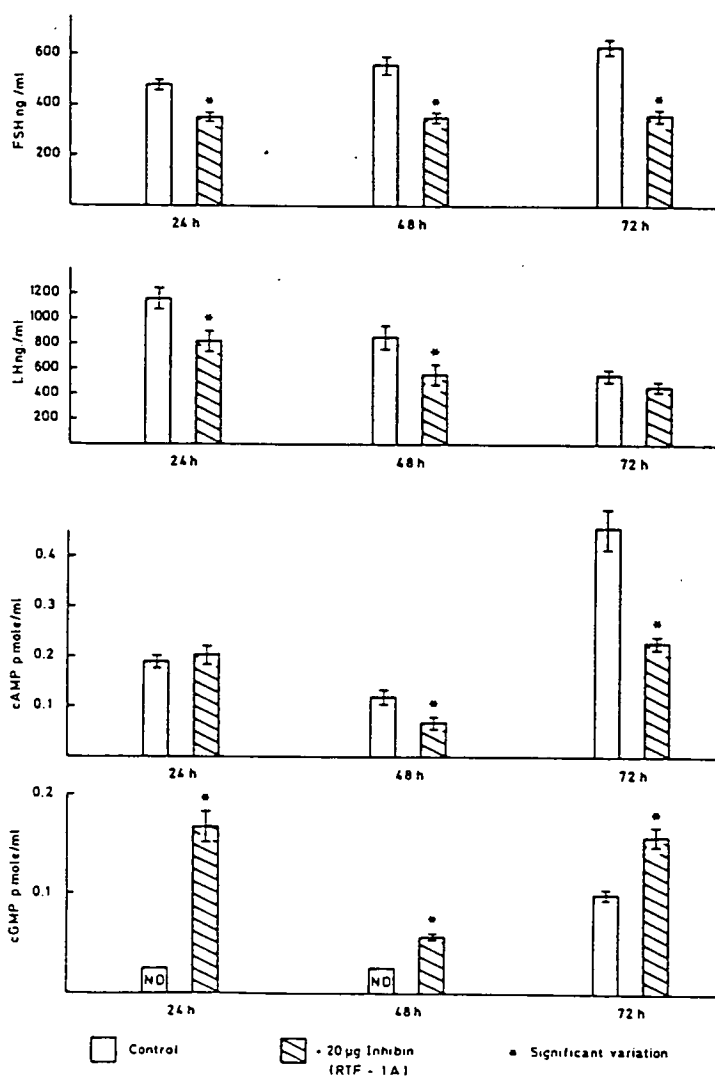


Fig. 2. Effects of 20 µg of inhibin preparation (1A = high molecular weight form obtained by Sephadex G100 filtration) after 24, 48 and 72 hours of preincubation on the release of FSH, LH, cyclic AMP and cyclic GMP into the culture medium.

LHRH-induced FSH and LH release is associated with an increase in cyclic AMP and GMP levels as shown by many authors.<sup>10-12</sup>

When pituitary cells are stimulated by LHRH, FSH and LH release (Fig. 3) and production are significantly reduced in the presence of 20 µg of inhibin preparation (220 U/mg) regardless of the length of the preincubation time. When LHRH is added to the culture medium 24 hours after the addition of

EFFECT OF INHIBIN ON FSH, LH, cAMP AND cGMP RELEASE BY  
LHRH-STIMULATED PITUITARY CELLS

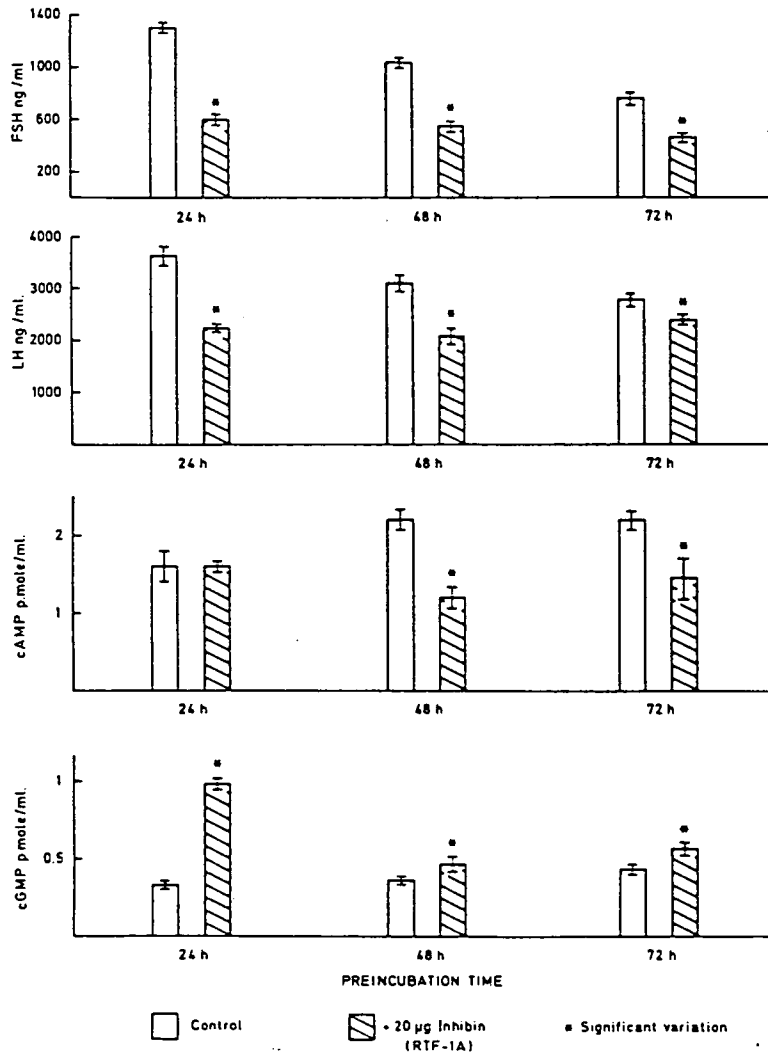


Fig. 3. Effects of 20 µg of 1A inhibin preparation after 6 hour incubation with  $10^{-8}$  M LHRH on the release of FSH, LH, cyclic AMP and cyclic GMP into the culture medium. The cells were preincubated with inhibin for 24, 48 or 72 hours.

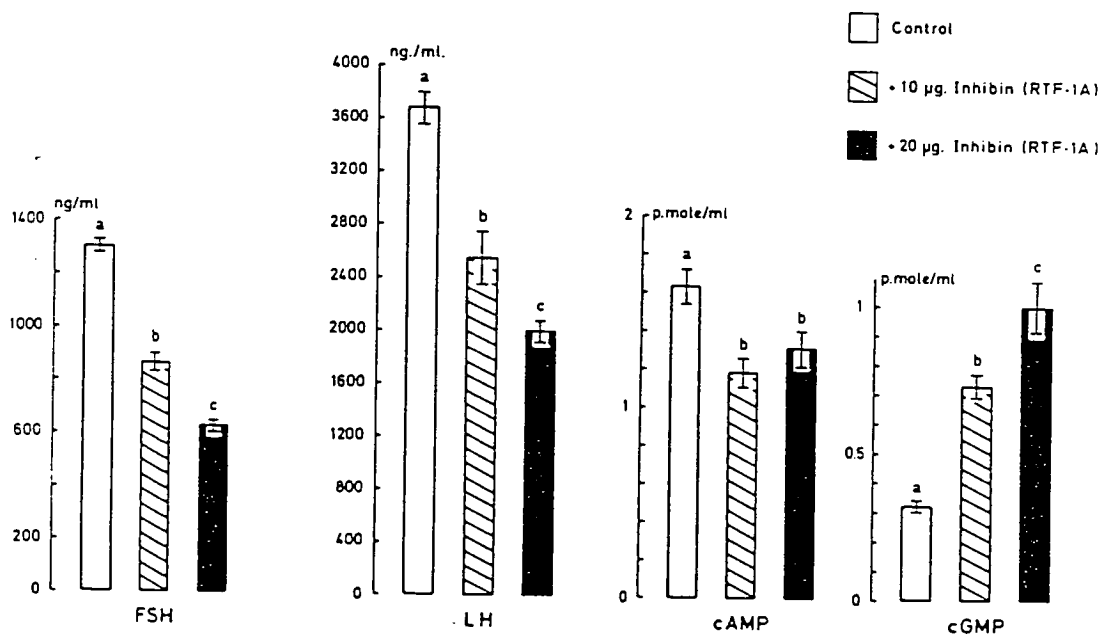
inhibin, the concentration of cyclic AMP is not modified while the concentration of cyclic GMP is strongly increased compared to that of control medium. On the other hand, when LHRH is added to the culture medium 48 or 72 hours after adding inhibin, the amount of cyclic AMP released is significantly reduced. The amounts of cyclic GMP released in the culture medium are always

higher than those of the control whatever the duration of preincubation of cells with inhibin before the addition of LHRH.

Increasing doses of inhibin incubated with cells for 48 hours reduce LHRH-induced FSH and LH release in a dose-dependent manner (Fig. 4). Although the secretion of cyclic AMP is lower in the presence of inhibin, there seems to be no dose-effect relationship since the inhibition of cyclic AMP remains unchanged when increasing amounts of inhibin are added to the culture medium. On the other hand, the stimulating effect of inhibin on cyclic GMP release appears to be directly related to the amounts of inhibin added to the culture medium.

DOSE EFFECT OF INHIBIN ON FSH, LH, cAMP AND cGMP RELEASE BY LHRH STIMULATED

PITUITARY CELLS



Internal significant variations are indicated by different letters: a, b, c

Fig. 4. Dose-dependent effects of 10 and 20 µg of 1A inhibin preparation after 48 hours of preincubation followed by 6 hour incubation with  $10^{-8}$ M LHRH on the release of FSH, LH and cyclic GMP in the culture medium. Effect of cyclic AMP is not related to the dose.

Similar results are observed with the purest preparation of inhibin from RTF (975 U/mg) used at the doses of 2 and 5 µg/ml. Various doses of inhibin preparations have been tested repeatedly on gonadotrophin and cyclic nucleotide releases in LHRH-stimulated conditions. Highly significant correlations exist between the reduction ( $\Delta$  = control group concentrations -

treated group concentrations) of FSH and LH release in culture medium and increase of cyclic GMP release ( $\Delta$  = treated group concentration - control group cGMP concentration). There is no significant relationship between cAMP variation on the one hand, and on the other hand, the reductions of FSH and LH release and cyclic GMP increase.

The effect of inhibin on FSH, LH, cAMP and cGMP is verified not only in terms of cell release but also in terms of total production by the pituitary cells. These results seem to indicate that, if inhibin modifies the secretion of FSH, LH, cyclic AMP and cyclic GMP, it also modifies their respective biosynthesis.

In order to complete this study, 3-isobutyl-1-methyl-xanthine, a phosphodiesterase inhibitor which is referred to as MIX, has been added to the culture medium. MIX alone at concentrations of 0.05, 0.1 and 0.2 mM does not significantly modify FSH and LH release and production in basal and LH-RH stimulated conditions. In contrast, cyclic AMP and GMP significantly increase. When inhibin is added to the culture medium, a similar inhibitory effect on FSH and LH is observed in the presence or the absence of MIX. This is the case when inhibin (10 and 20  $\mu$ g) preparation (220 U/mg) is added for 48 hours to the culture medium. At the end of this preincubation time, the medium has been collected and fresh medium containing inhibin  $10^{-8}$  M, LHRH and 0.2 mM MIX has been added back to the cells. In this case, the control medium contains LHRH and MIX. The results (Fig. 5) show that addition of MIX to stimulated cells does not modify the inhibitory effect of inhibin on FSH and LH released in the medium. Thus, the addition of inhibin leads to a dose-dependent reduction of both FSH and LH secretion. The addition of increasing amounts of inhibin to the culture medium does not decrease the cyclic AMP secretion of LHRH-stimulated cells. However, in the presence of MIX, inhibin still displays a stimulatory and dose-dependent effect on cyclic GMP release by the same LHRH-treated cells.

Discussion. These experiments show that in both basal and stimulated conditions inhibin induces an increase in cyclic GMP release and production which is dose-dependent and appears to occur simultaneously with the inhibition of FSH and LH release. In contrast, inhibin provokes a reduction of cyclic AMP release and production which is not dose-dependent and appears to be delayed in time with respect to FSH and LH modifications.

Therefore, these experiments suggest that cyclic GMP might act as the second messenger in the inhibition of FSH and LH secretion by inhibin. This

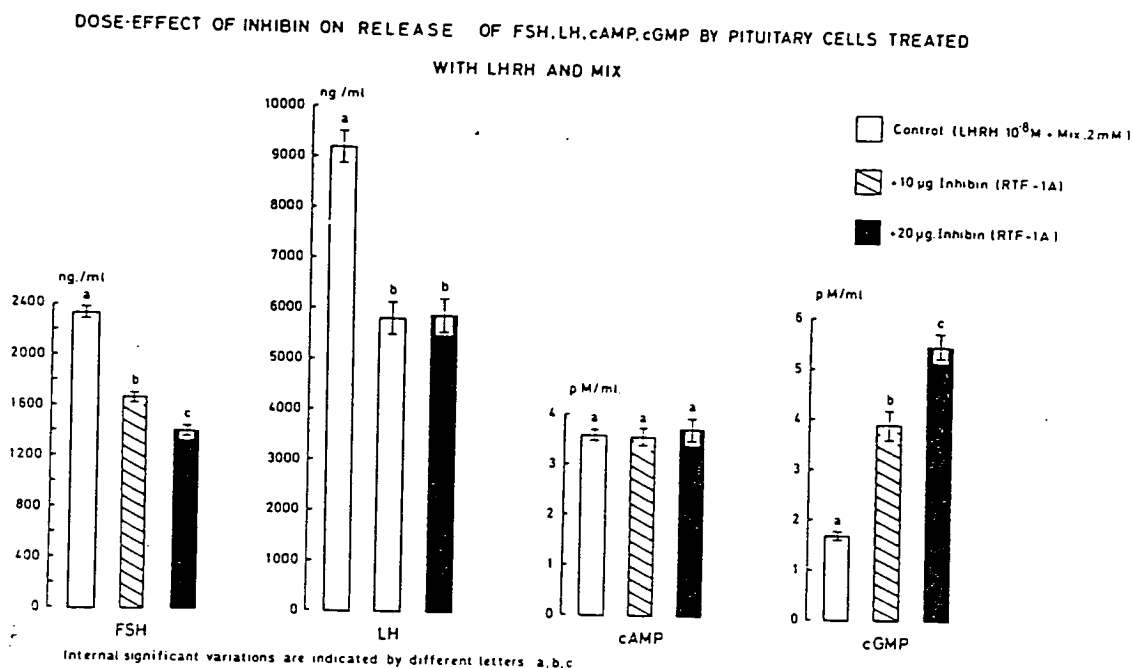


Fig. 5. Dose-dependent effects of 10 and 20 µg of 1A inhibin preparation on the release of FSH, LH and cyclic GMP in six hour incubations containing  $10^{-8}$  M LHRH and 0.2 M MIX. There was no modification of cyclic AMP release.

increase in cyclic GMP could, in turn, lead to a secondary reduction of cyclic AMP as it is demonstrated that cyclic GMP stimulated cyclic AMP hydrolysis.<sup>13</sup>

Cyclic GMP has already been demonstrated to be the second messenger for many inhibitory regulations. Thus, cyclic GMP increase is involved in the inhibition of melanin production by the hair follicle melanocytes *in vitro* under the influence of melatonin.<sup>14</sup> Similarly, Kano and Miyachi<sup>15</sup> have reported that the inhibition of testosterone production by melatonin in Leydig cells *in vitro* is accompanied by a rise in intracellular cyclic GMP but not in cyclic AMP. Furthermore, increase in cyclic GMP is associated with a reduction of thyroid hormone secretion in thyroid gland slices.<sup>16</sup>

#### REGULATION OF INHIBIN SECRETION

Follicular inhibin production. In several animal species, inhibin activity has been found in FF pretreated with charcoal to remove sex steroids.<sup>2</sup> It appears to be produced by granulosa cells. Erickson and Hsueh<sup>17</sup> showed that cultured granulosa cells secrete a substance that acts directly on cultured

pituitary cells and preferentially suppresses FSH secretion. In contrast, luteal cells are not capable of secreting inhibin in basal or experimental conditions.<sup>18</sup>

To investigate the mechanisms of inhibin secretion, monolayer cultures of granulosa cells were established according to the method of Henderson and Moon<sup>19</sup>, in the presence of 10% calf serum. Granulosa cells were obtained from large antral follicles (7-15 mm) from cows. Inhibin was assayed in the culture media on the basis of the inhibition of LHRH-induced FSH secretion by dispersed pituitary cells.<sup>1</sup> The reference preparation was derived from ovine testicular lymph (OTL P6) given an arbitrary potency of 1 U/mg.<sup>9</sup> Culture media were always pretreated with charcoal (1%) dextran (0.1%) at 4<sup>0</sup> C for 16 hours in order to remove sex steroids.

Granulosa cells produced inhibin and progesterone on the first day of culture. On subsequent days, progesterone secretion increased presumably as a result of luteinization, while inhibin production fell. The results of several experiments showed that there was an inverse relationship between progesterone secretion and inhibin production by granulosa cells in monolayer culture. Furthermore, addition of progesterone to the culture medium leads to a decrease of inhibin reduction.<sup>18-20</sup>

Neither purified porcine follicle stimulating hormone (FSH) nor luteinizing hormone (LH) (UCB, Brussels, Belgium, 100-1,000 ng/ml) nor bovine prolactin (NIAMDD-bPRL 6 : 50, 500, 2,500 ng/ml) modified inhibin secretion.

The addition of aromatizable (androstenedione and testosterone), nonaromatizable (dihydrotestosterone) and synthetic (methylestrenolone and mesterolone) androgens to the culture medium of granulosa cells led to a marked increase in inhibin production without any change in progesterone secretion (Fig. 6). Estradiol-17 $\beta$  secretion was increased on the first day of culture in the presence of testosterone and androstenedione, but was subsequently unchanged. Dihydrotestosterone, which cannot be aromatized, did not lead to any significant change in estradiol-17 $\beta$  secretion. Estrogens (estrone and estradiol-17 $\beta$ ) in doses of 1 to 5  $\mu$ g per ml of culture medium did not change inhibin secretion from granulosa cells.

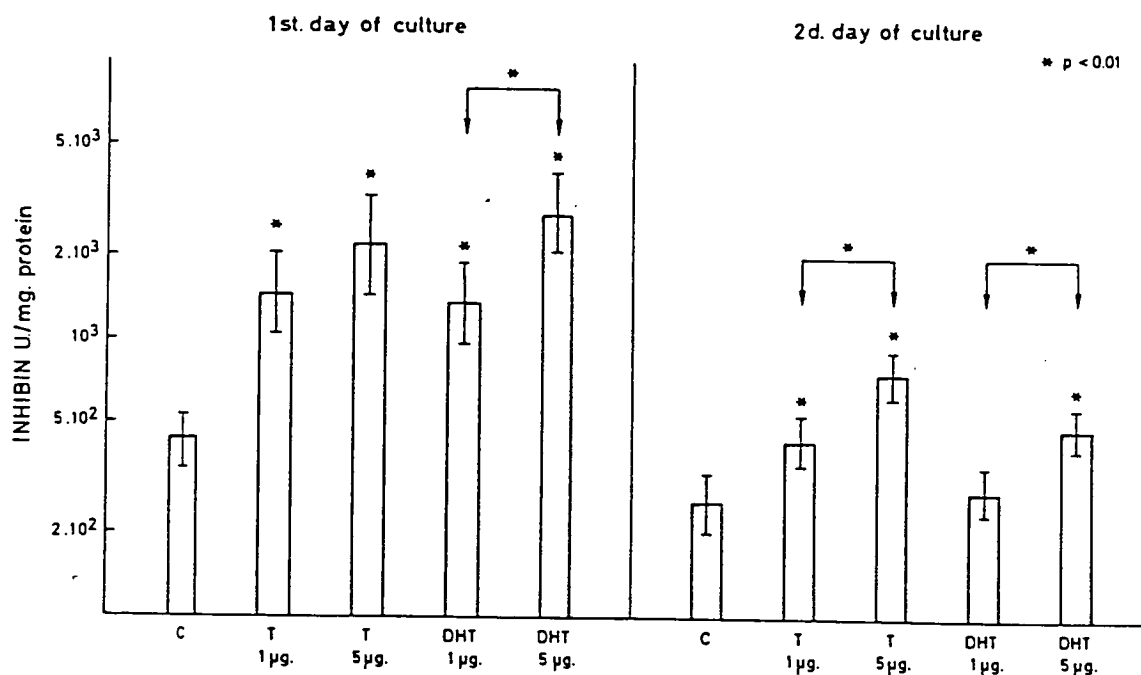


Fig. 6. Effects of 1 and 5 µg/ml testosterone (T) and dihydrotestosterone (DHT) on inhibin production by granulosa cells the first and the second day of culture.

Cyproterone acetate, an anti-androgen, at concentrations of 35 µM and 350 µM inhibited basal and testosterone (1 µg/ml = 3.5 µM)-stimulated inhibin production by granulosa cells during 24 hours of culture (Fig. 7). In addition both concentrations of cyproterone acetate inhibited progesterone production but not estradiol-17β production by granulosa cells.<sup>21</sup>

It may be concluded that inhibin production is decreased when the granulosa cell undergoes luteinization and increases progesterone secretion. The addition of exogenous progesterone significantly decreases inhibin secretion by cultured granulosa cells. Aromatizable, non-aromatizable and synthetic androgens stimulate inhibin secretion by these cells. They appear to act directly and not by being aromatized (as can occur with testosterone and androstenedione). In fact, estrogens have no effect on inhibin production, while dihydrotestosterone, which cannot undergo aromatization, is the most potent stimulus of inhibin secretion. Furthermore, the effect of cyproterone



acetate, an anti-androgen, provides another evidence that androgen may directly regulate inhibin production by granulosa cells and that this action may be receptor mediated process.

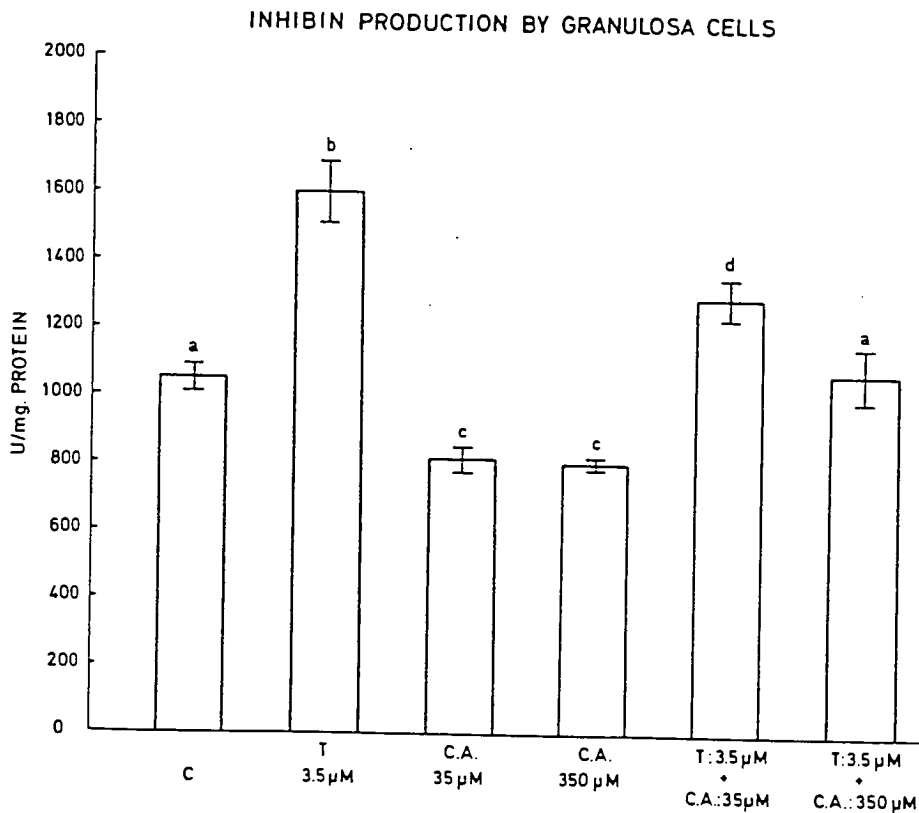


Fig. 7. Effect of testosterone (T) and cypropterone acetate alone or simultaneously on inhibin production by granulosa cells on the first day of culture.

Finally, under the conditions of our experiments, the gonadotrophins and prolactin do not appear to have any direct effect on inhibin secretion by granulosa cells.

Inhibin production by the testis. The testis is the source of inhibin, as shown by its presence in testicular extracts.

The in vitro experiments of Steinberger and Steinberger<sup>22</sup> have shown unequivocally that the Sertoli cell is directly involved in the synthesis and secretion of inhibin, called for this reason Sertoli cell factor. Other evidence for the role of the Sertoli cells in the secretion of inhibin and absence of effect of germinal cells on inhibin production has been provided by Demoulin et al<sup>23</sup> using mouse testes maintained in organ culture. The presence of inhibin in culture medium of Sertoli cells was confirmed by others.<sup>24,25</sup>

On the other hand, isolated germ cells or peritubular cells show no secretion of inhibin-like substance.<sup>26</sup>

Little data are available concerning the mechanisms regulating inhibin secretion in males. Steinberger<sup>26</sup> demonstrated that Sertoli cells obtained from rats which were hypophysectomized for 10 days show little or no inhibin secretion. Daily treatment of hypophysectomized animals for 10 days with either FSH, LH or testosterone restores inhibin secretion to intact control levels. Treatment with FSH and LH or FSH plus testosterone has a synergistic effect on inhibin secretion compared to treatment with each hormone alone.

We have investigated inhibin secretion by isolated Sertoli cells.<sup>20-27</sup> A Sertoli cell enriched fraction was prepared according to the method of Verhoeven et al.<sup>28</sup> This method consists of successive treatments with collagenase and pancreatin as initially proposed by Welsch and Wiebe.<sup>29</sup>

Inhibin production and estradiol-17 $\beta$  secretion have been studied at days 7 and 9 as aromatization of testosterone to estradiol-17 $\beta$  is maximum at that time<sup>28</sup> and as morphological investigation by phase contrast microscopy reveals homogeneous monolayers of Sertoli cells. No germ cells were observed. The absence of Leydig cells is indicated by the low or undetectable basal concentrations of testosterone and lack of stimulation of its secretion by HCG.

Inhibin was assayed by the inhibition of LHRH-induced FSH release and was expressed in units of the ovine testicular lymph reference preparation (OTL P6).

Without any treatment, the production of estradiol-17 $\beta$  and of inhibin was low or undetectable on days 7 and 9 of Sertoli cell cultures. The addition of HCG in doses of 5, 25 and 45 mIU/ml induced no change in the production of either substance on these days. Pregnant mare serum gonadotrophin (PMSG), with predominantly FSH-like activity (in doses of 0.02 to 10 IU/ml), and purified ovine FSH (0.1 to 1  $\mu$ g/ml for 4 hours) also failed to produce any change in inhibin secretion over the subsequent 24 hours.<sup>20-31</sup>

Testosterone alone (0.5  $\mu$ M) led to a small but significant increase in estradiol-17 $\beta$  production. This aromatization was much more marked when PMSG (0.02 U/ml) was added to the culture medium with testosterone. Testosterone alone produced a dose-dependent increase in inhibin production (Fig. 8). The effect was not due to estradiol-17 $\beta$  production by the Sertoli cells, however, as estradiol-17 $\beta$  (0.5, 1 and 5  $\mu$ g/ml) had no effect on inhibin production.<sup>31</sup> Furthermore, this stimulating effect of androgens was not neutralized by an inhibitor of aromatase activity. In contrast, dihydrotestosterone, which

cannot be aromatized and synthetic androgens stimulated Sertoli cell inhibin secretion (Fig. 8; Table 3). The anti-androgen cyproterone acetate, neutralized the effect of testosterone on inhibin secretion when added in a tenfold excess.<sup>30</sup>

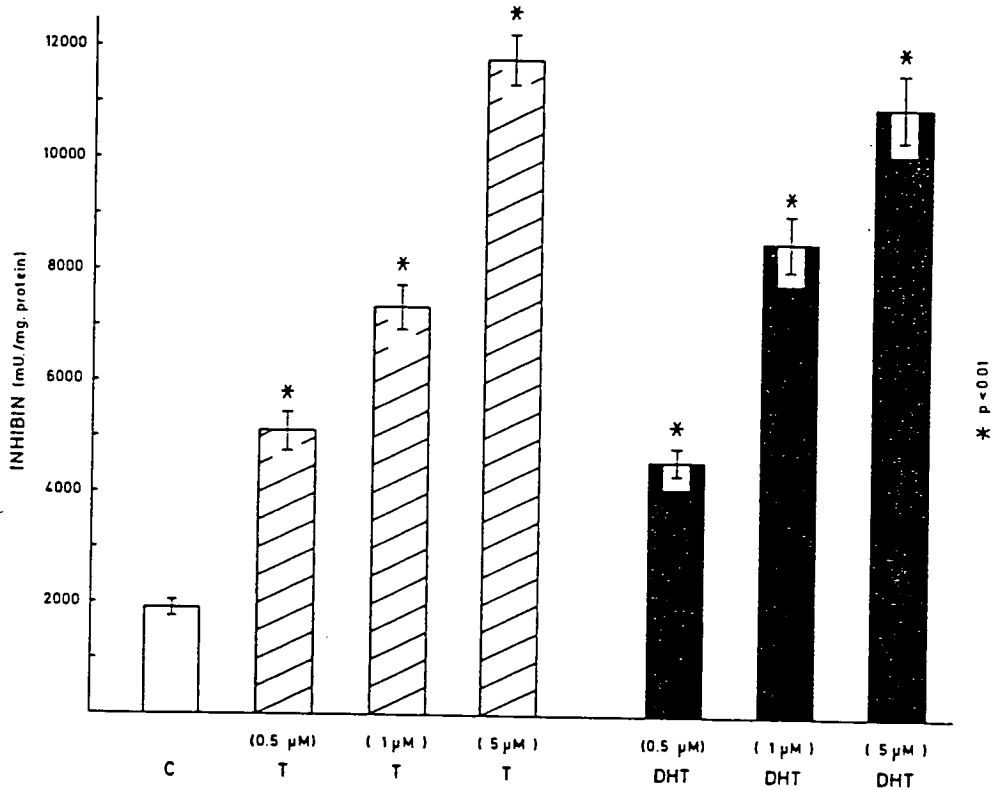


Fig. 8. Effect of 0.5, 1 and 5  $\mu\text{M}$  testosterone (T) and dihydrotestosterone (DHT) on inhibin production by Sertoli cells on the 7th day of culture.

Finally, when spermatocytes and spermatogonia are co-cultured with Sertoli cells, there is no modification of inhibin secretion.

In our current state of knowledge, no direct argument can be put forward for a germ cell signal which would inform the Sertoli cell regarding the state of spermatogenesis and which would stimulate inhibin secretion. On the other hand, it is certain that the androgens, testosterone and dihydrotestosterone are at the same time essential for spermatogenesis and for inhibin production.

TABLE 3

EFFECTS OF ANDROGEN AND CYPROPTERONE ACETATE ON INHIBIN PRODUCTION BY SERTOLI CELLS IN VITRO

Treatment	Inhibin concentration mU/mg protein M $\pm$ S.D.
Control	1605 $\pm$ 59.4 <sup>a</sup>
Testosterone 0.5 $\mu$ M	2770 $\pm$ 52.6 <sup>b</sup>
Cypropterone Ac. 0.5 $\mu$ M	1382 $\pm$ 185.6 <sup>a</sup>
Cypropterone Ac. 5 $\mu$ M	1692 $\pm$ 50.8 <sup>a</sup>
T : 0.5 $\mu$ M + Cyprop. Ac. 0.5 $\mu$ M	2382 $\pm$ 133.0 <sup>b</sup>
T : 0.5 $\mu$ M + Cyprop. Ac. 5 $\mu$ M	1894 $\pm$ 151.1 <sup>a</sup>

Different letters indicate significantly different results

#### CONCLUSIONS

The existence of inhibin may be accepted on the basis of numerous experiments. Its chemical nature is not yet elucidated but it is clear that a high degree of polymorphism exists. At the pituitary level, inhibin preparations induce a reduction of FSH and, to a lesser degree, of LH secretion which is associated with a dose-dependent increase of cyclic GMP. The variations of cyclic GMP are simultaneous to those of FSH and LH secretion. Cyclic GMP is a possible candidate as second messenger mediating the inhibitory effect of inhibin. Cyclic AMP variations are not related to the dose of inhibin and to the amplitude of gonadotrophin inhibition. Its modifications are delayed compared to those of gonadotrophins and cyclic GMP and should be secondary to the stimulatory effect of cyclic GMP on cyclic AMP hydrolysis.

Our in vitro experiments show that inhibin secretion by granulosa and Sertoli cells is stimulated by aromatizable, non-aromatizable and synthetic androgens. This stimulatory effect seems to be a direct one and mediated through androgen receptor activation. The estrogens do not influence inhibin secretion by both cell types. Progesterone decreases inhibin secretion by granulosa cells, yet its effects on Sertoli cells are still unknown.

The direct role of gonadotrophins is equally unclear. Prolactin does not appear to modify inhibin secretion by granulosa cells.

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## Inhibin production by bovine ovarian tissues *in vitro* and its regulation by androgens

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**Summary.** No detectable amounts of inhibin were produced by cultured ovarian stroma or luteal tissue. Follicular tissue produced inhibin *in vitro* and removal of the granulosa cells from the follicle wall caused inhibin production to fall by 80%. Granulosa cells alone had the greatest ability of any ovarian cell type to produce inhibin *in vitro*, and are probably the major site of follicular inhibin production.

Cyproterone acetate at concentrations of 35 and 350  $\mu\text{M}$  inhibited basal and testosterone (3.5  $\mu\text{M}$ )-stimulated inhibin production by cultured intact follicle wall and granulosa cells. In addition, each concentration of cyproterone acetate inhibited progesterone but not oestradiol-17 $\beta$  production by the follicle wall and granulosa cell cultures. The synthetic, non-aromatizable androgens, methylestrenolone and mesterolone, at concentrations of 5 and 25  $\mu\text{M}$ , mimicked the effect of testosterone and stimulated granulosa cell inhibin production, methylestrenolone being the more potent. These findings provide further evidence that androgens regulate follicular inhibin and progesterone production and that these may be receptor-mediated processes, and suggest that inhibin production may be a general property of androgenic compounds.

Preliminary examination of the physicochemical characteristics of inhibin indicated that the inhibin activity of bovine granulosa cell culture medium was (a) retained by an Amicon XM100A filter with a nominal molecular weight cut-off point of 100 000; and (b) destroyed by heating to 80°C for 30 min.

### Introduction

There is increasing evidence to indicate that inhibin produced by the ovary is physiologically important in the regulation of pituitary FSH secretion, and consequently follicular development (Franchimont *et al.*, 1979, 1981; Henderson & Franchimont, 1981). Follicular granulosa cells secrete inhibin *in vitro* (Erickson & Hsueh, 1978; Anderson & de Paolo, 1981; Henderson & Franchimont, 1981) and are therefore considered to be the site of ovarian inhibin production. However, little information is available on the ability of other ovarian cell types to produce inhibin. The purpose of this study was to compare the relative efficiency of intact bovine follicular tissue, isolated thecal tissue and granulosa cells, and ovarian stromal and luteal tissue to produce inhibin *in vitro*. In addition, in view of the finding that androgens may regulate ovarian inhibin production (Henderson & Franchimont, 1981), the effects on inhibin production of the anti-androgen, cyproterone acetate, and the synthetic androgens, mesterolone and methylestrenolone, were studied. A preliminary examination of the physicochemical characteristics of ovarian inhibin produced *in vitro* was also undertaken.

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### Materials and Methods

**Reagents.** Cyproterone acetate (6 $\alpha$ -chloro-17 $\alpha$ -hydroxy-1,2 $\alpha$ -methylene-4,6-pregnadiene-3,20-dione-17-acetate) and mesterolone (17 $\beta$ -hydroxy-1 $\alpha$ -methyl-5 $\alpha$ -androstan-3-one) were obtained from Schering A.G., Berlin. Methylestrenolone (17 $\beta$ -hydroxy-17-methylestr-4-en-3-one) was obtained from Organon, Oss, Holland. All other steroids were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset, U.K.

**Cell and tissue culture.** Ovaries were obtained from healthy adult cows within 1 h of their slaughter at a local abattoir. Antral follicles present on the surface of the ovary and containing  $\geq 0.3$  ml follicular fluid ( $\geq 10$  mm diameter) were dissected out and trimmed free of adhering tissue. Follicular fluid was aspirated by syringe and needle, and the collapsed follicle was slit open and bisected. One half was used for cultures of intact follicle wall. Granulosa cells were removed from the other half of the follicle wall by scraping with a platinum wire loop. The cells were collected into Eagle's Minimum Essential Medium (modified) with Earle's salts (Eagle, 1959) supplemented with Hepes buffer (20 mM), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50  $\mu$ g/ml), amphotericin B (2.5  $\mu$ g/ml) and non-essential amino acids (Eagle, 1959). (All reagents obtained from Flow Laboratories, Irvine, U.K.) After washing twice with the above medium, the cells were counted in a haemocytometer, and their viability was determined by uptake of nigrosin dye. Representative portions of the intact follicle wall, and the follicle wall remaining after the granulosa cells had been removed were taken for histological examination. The follicle wall minus granulosa cells was termed thecal tissue and cultured as such, although histological examination showed that considerable amounts of stromal tissue were also present. Using scalpel blades, minces were prepared of the intact follicle wall and thecal tissues, and from samples of ovarian stroma and corpus luteum. Aliquants of these minces corresponding to 1–2 mg protein, and a minimum of  $1 \times 10^5$  'live' granulosa cells were cultured separately in multi-welled tissue-culture Petri dishes (Sterilin, Middlesex, U.K.) at 37°C in 1 ml of the above medium containing 10% newborn calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). The gas phase was air. Steroids were added to the cultures in ethanol (testosterone, mesterolone and methylestrenolone) or methanol (cyproterone acetate). Control cultures received vehicle alone. The concentration of organic solvent in the culture medium never exceeded 1%. Aliquants of the tissue minces and granulosa cells were also taken to determine the endogenous concentrations of inhibin and steroids. These samples were homogenized in 1 ml culture medium, which was then freeze-dried and assayed for inhibin, or in 1 ml ethanol which was then assayed for steroids. After culture for 24 h, the medium was removed and aliquants taken and freeze-dried for subsequent inhibin determination while the remainder was stored frozen until assayed for steroids. The granulosa cells and tissue fractions were washed thoroughly with medium devoid of serum. The granulosa cells were stored frozen until assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Patterson (1979). The tissue fractions were taken up in 1 ml of 1 M-sodium hydroxide and assayed for protein by the method of Lowry *et al.* (1951). Inhibin and steroid production by the ovarian tissues was calculated by subtracting the values obtained for the endogenous tissue concentrations of inhibin and steroid from those present in the culture medium after the 24-h culture period.

**Inhibin assay.** Inhibin activity was determined using a bioassay based on the inhibition of LH-RH-stimulated FSH secretion by cultured rat anterior pituitary cells as described and validated by Henderson & Franchimont (1981). The freeze-dried aliquants of cell culture medium taken for inhibin assay were reconstituted with distilled water. Steroids were removed by mixing for 16 h at 4°C with activated charcoal (Norit A, 1%) pretreated with dextran (0.1%) followed by centrifugation at 3000 g (Henderson & Franchimont, 1981). The supernatants were filter-sterilized by passing through a 0.45  $\mu$ m cellulose millipore filter before use. Aliquants were then tested for their ability to inhibit LH-RH ( $10^{-8}$  M)-stimulated FSH secretion by cultured rat anterior pituitary cells during a 6-h incubation period. Inhibin activity of the cell culture medium was expressed

relative to the potency of a standard reference preparation of inhibin derived from ovine testicular lymph which has been designated an arbitrary potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979). Regression analysis and parallel line assay statistics (Borth, 1976) were used to relate the inhibin activity of the culture medium to that of the inhibin standard.

**Radioimmunoassays.** The gonadotrophin concentrations in media from the pituitary cell cultures were measured in duplicate by double-antibody methods using NIAMDD rat pituitary gonadotrophin reagents supplied by the pituitary agency of NIH. The concentrations were expressed in terms of NIAMDD rat-FSH-RP1 and rat-LH-RP1. The sensitivities of the assays were 5 ng FSH/ml and 10 ng LH/ml. The inter- and intra-assay coefficients of variation were 3 and 8% for the FSH assay and 5 and 12% for the LH assay respectively.

Steroid concentrations in the cell culture medium and ethanolic extracts of the ovarian tissues were determined using the specific radioimmunoassays described previously (McNatty, Gibb, Dobson, Thurley & Findlay, 1981). The limit of sensitivity of the assays (per tube) was 25 pg for progesterone, 5 pg for oestradiol-17 $\beta$  and 10 pg for testosterone. The intra- and inter-assay coefficients of variation were each <10% and <16% respectively.

**Fractionation of bovine granulosa cell culture medium.** Granulosa cell culture medium was fractionated by filtering consecutively through XM100A, XM50 and PM10 Amicon filter membranes (Amicon Ltd, Massachusetts, U.S.A.). These filters have nominal molecular weight cutoff points of >100 000, >50 000 and >10 000 respectively. Aliquants of each filtrate and retentate were assayed for protein (Lowry *et al.*, 1951) and inhibin activity.

**Statistics.** Unless otherwise stated, the statistical significance of observed differences between different treatment groups was determined by analysis of variance in conjunction with Fisher's least significant difference test. When Bartlett's test indicated heterogeneity of variance, data were transformed to logarithms to equalize the variances before statistical analysis. The level of significance was set at  $P < 0.05$ .

## Results

### *Inhibin production by various ovarian cell types*

Table 1 shows that, under both basal and testosterone stimulated conditions, only follicular tissue produced inhibin *in vitro*, and that granulosa cells were the most active cell type. Removal of granulosa cells from the intact follicle wall to leave a thecal shell caused inhibin production to fall by 80%. Oestradiol-17 $\beta$  production by thecal tissue was <2% of that produced by the intact follicle wall. No detectable amounts of inhibin or oestradiol-17 $\beta$  were present in the homogenates of ovarian stroma and luteal tissue, or produced by these tissues during culture for 24 h. Although

Table 1. Inhibin and oestradiol-17 $\beta$  production by bovine ovarian tissues cultured for 24 h

Tissue cultured	Testosterone (1 $\mu$ g/ml) present	Oestradiol-17 $\beta$ (ng/mg protein)	Inhibin (U/mg protein)
Follicle wall	—	6.4 $\pm$ 1.4 <sup>a</sup>	26 $\pm$ 1 <sup>a</sup>
(theca + granulosa)	+	8.1 $\pm$ 1.6 <sup>a</sup>	54 $\pm$ 2 <sup>b</sup>
Theca	—	0.03 $\pm$ 0.01 <sup>b</sup>	4.9 $\pm$ 0.3 <sup>c</sup>
	+	0.13 $\pm$ 0.05 <sup>c</sup>	12 $\pm$ 2 <sup>d</sup>
Granulosa	—	ND	833 $\pm$ 37 <sup>e</sup>
	+	5300 $\pm$ 520 <sup>d</sup>	1846 $\pm$ 103 <sup>f</sup>
Stroma	— or +	ND	ND
Corpus luteum	— or +	ND	ND

Values are mean  $\pm$  s.e.m. for  $n = 5$ . Mean values with different superscripts in the same column are significantly different ( $P < 0.01$ ). ND, not detectable.

producing small or undetectable amounts of inhibin, the thecal, stromal and luteal tissues seemed functional *in vitro*. Appreciable amounts of testosterone ( $0.8 \pm 0.1$  ng/mg protein) were produced by the thecal tissue, while the luteal and stromal tissues produced  $212 \pm 38$  and  $0.3 \pm 0.04$  ng progesterone/mg protein respectively during the 24-h culture period.

*Effect of cyproterone acetate on follicular inhibin and steroid production*

Table 2 shows that cyproterone acetate inhibited testosterone-stimulated inhibin production ( $P < 0.001$ ) and progesterone production ( $P < 0.001$ ) by follicle wall cultured for 24 h. Cyproterone acetate had no effect on oestradiol-17 $\beta$  production ( $P > 0.05$ ). Similar results were obtained using granulosa cells in culture (Table 2). Cyproterone acetate inhibited basal ( $P < 0.01$ ) and testosterone-stimulated inhibin production ( $P < 0.002$ ), with the effect being dose-dependent in those cultures receiving testosterone ( $P < 0.05$ ). The inhibition of granulosa cell progesterone production was dose-dependent in the presence and absence of exogenous testosterone ( $P < 0.001$ ). Cyproterone acetate had no effect on the production of oestradiol-17 $\beta$  ( $P > 0.05$ ) which was only detectable in those granulosa cell cultures receiving exogenous testosterone.

Table 2. Effect of testosterone (T) and cyproterone acetate (CA) on inhibin production and steroidogenesis by bovine follicle wall or granulosa cells alone cultured for 24 h

Additions to culture medium	Inhibin production (U/mg protein)	Steroid production (ng/mg protein)	
		Progesterone	Oestradiol-17 $\beta$
<b>Follicle wall</b>			
None	$9.3 \pm 0.6^a$	$15.4 \pm 1.4^a$	$3.0 \pm 0.2^a$
3.5 $\mu$ M-T	$19.4 \pm 1.7^b$	$13.0 \pm 1.5^a$	$4.2 \pm 0.6^b$
35 $\mu$ M-CA	$9.4 \pm 0.6^a$	$5.1 \pm 0.6^b$	$3.0 \pm 0.3^a$
350 $\mu$ M-CA	$8.8 \pm 0.6^a$	$3.5 \pm 0.7^b$	$2.6 \pm 0.1^a$
3.5 $\mu$ M-T + 35 $\mu$ M-CA	$12.3 \pm 0.3^c$	$5.5 \pm 0.6^b$	$4.6 \pm 0.6^b$
3.5 $\mu$ M-T + 350 $\mu$ M-CA	$10.9 \pm 0.8^{ac}$	$3.3 \pm 0.4^b$	$5.1 \pm 0.2^b$
<b>Granulosa cells</b>			
None	$1050 \pm 44^a$	$1105 \pm 75^a$	ND
3.5 $\mu$ M-T	$1600 \pm 87^b$	$1055 \pm 81^a$	$1376 \pm 182^a$
35 $\mu$ M-CA	$815 \pm 39^c$	$110 \pm 14^b$	ND
350 $\mu$ M-CA	$800 \pm 16^c$	$40 \pm 11^c$	ND
3.5 $\mu$ M-T + 35 $\mu$ M-CA	$1292 \pm 62^d$	$93 \pm 11^b$	$1519 \pm 96^a$
3.5 $\mu$ M-T + 350 $\mu$ M-CA	$1073 \pm 76^a$	$37 \pm 7^c$	$1107 \pm 89^a$

Values are mean  $\pm$  s.e.m.,  $n = 5$ . Mean values with different superscripts in the same columns for each tissue type are significantly different,  $P < 0.05$ . ND, not detectable.

*Effect of testosterone and synthetic androgens on inhibin and progesterone production by granulosa cells*

Table 3 shows that the synthetic androgens methylestrenolone and mesterolone mimicked the effect of testosterone in stimulating inhibin production by bovine granulosa cells cultured for 24 h ( $P < 0.001$ ). The stimulatory effects of testosterone and mesterolone were dose-dependent ( $P < 0.001$  and  $P < 0.05$  respectively) but that of methylestrenolone was not. Methylestrenolone was more active than mesterolone in stimulating inhibin production ( $P < 0.001$ ) and both concentrations of methylestrenolone tested were as active as 5  $\mu$ M-testosterone. Methylestrenolone at a concentration of 25  $\mu$ M inhibited progesterone production ( $P < 0.001$ ). Only those cultures receiving testosterone produced detectable amounts of oestradiol-17 $\beta$ . The amounts of oestradiol-17 $\beta$  produced were (mean  $\pm$  s.e.m.)  $337 \pm 25$  and  $338 \pm 18$  ng/mg protein for the cultures receiving 5 and 25  $\mu$ M-testosterone respectively.

Table 3. Effect of testosterone, methyltestosterone and mesterolone on inhibin and progesterone production by bovine granulosa cells cultured for 24 h

Additions to culture medium	Inhibin production (U/mg protein)	Progesterone production (ng/mg protein)
None	196 ± 9 <sup>a</sup>	243 ± 47 <sup>ab</sup>
Testosterone		
5 µM	607 ± 11 <sup>b</sup>	237 ± 12 <sup>ab</sup>
25 µM	1004 ± 20 <sup>c</sup>	255 ± 19 <sup>a</sup>
Methyltestosterone		
5 µM	629 ± 12 <sup>b</sup>	183 ± 14 <sup>b</sup>
25 µM	643 ± 17 <sup>b</sup>	60 ± 4 <sup>c</sup>
Mesterolone		
5 µM	422 ± 19 <sup>d</sup>	261 ± 19 <sup>a</sup>
25 µM	492 ± 32 <sup>c</sup>	232 ± 18 <sup>ab</sup>

Values are mean ± s.e.m. for  $n = 5$ . Mean values with different superscripts in the same column are significantly different,  $P < 0.05$ .

The above effects of testosterone, cyproterone acetate and the synthetic androgens on inhibin production were not artefacts caused by residual amounts of the test substances, still present after dextran-charcoal treatment of the culture media, modifying FSH production by the cultured pituitary cells in the inhibin bioassay. Dextran-charcoal-treated culture media containing the test substances, but not exposed to granulosa cells or other ovarian tissue, had no effect on pituitary cell FSH production. In addition, measurement of LH-RH in the pituitary cell culture medium throughout the 6-h incubation period indicated that there was no difference in the kinetics of LH-RH disappearance in the presence of granulosa cell culture medium whether primed or not with androgen. It is unlikely, therefore, that the androgens were acting to stimulate production of an ovarian peptidase which breaks down LH-RH resulting in reduced FSH production and an apparent increase in inhibin activity.

#### *Physicochemical characteristics of ovarian inhibin*

Fractionation of bovine granulosa cell culture medium (containing  $177 \pm 11$  U/mg protein,  $n = 5$ ) on the basis of molecular size showed that inhibin activity was only present in the retentate after filtration through an Amicon XM100A membrane which has a nominal molecular weight cut-off point of 100 000 ( $257 \pm 13$  U/mg protein  $n = 5$ ;  $P < 0.002$ , Student's  $t$  test). No inhibin activity was present in the  $M_r < 100$  000 filtrate, or in the retentates and filtrates following successive filtration of the  $M_r < 100$  000 filtrate through Amicon XM50 and PM10 membranes with nominal molecular weight cut-off points of 50 000 and 10 000 respectively. The inhibin activity of granulosa cell culture medium was destroyed by heating to 80°C for 30 min.

#### Discussion

The present study demonstrates that only ovarian follicular tissue has the ability to produce inhibin *in vitro*. Ovarian stroma and luteal tissue neither contained nor produced detectable amounts of inhibin. The finding with cultured luteal tissue is similar to that with dispersed luteal cells in culture (Henderson & Franchimont, 1981). Follicular granulosa cells are probably the major site of ovarian inhibin production. Granulosa cells were the most active cell type with respect to inhibin production *in vitro*, and removal of granulosa cells from the intact follicle wall to leave a thecal shell

caused inhibin production to fall to <20% of its original value (Table 1). The thecal tissue appeared devoid of granulosa cells as assessed by histological examination of representative sections, and by the fact that thecal oestradiol-17 $\beta$  production, under basal and testosterone stimulated conditions, was <2% of that produced by the cultures of intact follicle wall (Table 1). Previous studies have indicated that granulosa cells are the principal site of bovine follicular aromatization (Lacroix, Eechaute & Leusen, 1974). However, in view of the great ability of granulosa cells to secrete inhibin *in vitro*, the possibility that the thecal inhibin production might be due to residual contaminating granulosa cells cannot be excluded.

Androgens, through their ability to stimulate inhibin secretion, as demonstrated in Table 1, may be involved in the regulation of ovarian inhibin production (Henderson & Franchimont, 1981). This is supported by the present finding that the anti-androgen, cyproterone acetate, inhibited inhibin production by cultured intact bovine follicular tissue (Table 2). This effect is probably the result of an action on the follicular granulosa cells which are the major source of follicular inhibin. In addition, cyproterone acetate inhibited inhibin production by cultured bovine granulosa cells (Table 2). Cyproterone acetate exerts its anti-androgenic effect through inhibiting the association of androgen with cytosolic receptors and preventing the nuclear retention of androgen-receptor complexes (Belham & Neal, 1971; Peets, Henson & Neri, 1974). A cytosolic receptor which after binding testosterone, is translocated to the nucleus is present in rat granulosa cells (Schreiber & Ross, 1976). The present finding, that cyproterone acetate inhibits testosterone-stimulated inhibin production, suggests that a similar cytosolic testosterone receptor capable of nuclear translocation may be present in bovine granulosa cells, and that receptors of this type may mediate androgenic stimulation of granulosa cell inhibin production. Cyproterone acetate also inhibited basal inhibin production by granulosa cells (Table 2), thereby implicating androgen involvement in this process. Bovine granulosa cells isolated from antral follicles contain appreciable amounts of endogenous testosterone, in the order of 0.2-10 ng/mg protein (Henderson & Moon, 1979). The culture medium also contained small amounts of testosterone (<20 pg/ml) derived from the calf-serum. Together these small amounts of testosterone may be responsible, at least in part, for basal inhibin production by granulosa cells, and it is through blocking the action of this androgen that cyproterone acetate exerts its inhibitory effect on basal inhibin production. Bovine granulosa cells have only a very limited ability, if any, to synthesize androgens (Lacroix *et al.*, 1974; Henderson & Swanston, 1978). It is therefore tempting to speculate that follicular inhibin production may involve a co-operative interaction between theca and granulosa cells similar to that proposed for follicular oestradiol-17 $\beta$  production (Armstrong & Dorrington, 1977) with thecal cell-derived androgen being utilized to stimulate inhibin production by granulosa cells.

In addition to its effect on inhibin production, cyproterone acetate inhibited progesterone production by cultured follicular tissue and granulosa cells (Table 2). Oestradiol-17 $\beta$  production was unaffected. Androgens have previously been implicated in the regulation of granulosa cell progesterone production. *In vitro*, androgens stimulate progesterone production by cultured granulosa cells from immature, hypophysectomized oestrogen-treated rats (Lucky, Schreiber, Hillier, Schulman & Ross, 1977; Nimrod, Rosenfield & Otto, 1980), an effect which can be inhibited by cyproterone acetate and other anti-androgens (Hillier, Knazek & Ross, 1977). *In vivo*, intra-ovarian implants of the anti-androgen, flutamide, reduce the ability of porcine granulosa cells to secrete progesterone *in vitro* (Schomberg, Williams, Tyrey & Ulberg, 1978). While androgens failed to stimulate progesterone production by bovine follicular tissue and granulosa cells in this and other studies (Henderson & Moon, 1979; Henderson & Franchimont, 1981), a role for androgens in regulating bovine follicular progesterone production is suggested by the inhibitory action of cyproterone acetate. It is unlikely that the reduction in inhibin production by granulosa cells after cyproterone acetate treatment occurs as a consequence of the fall in progesterone production or *vice versa*. Previous studies indicate that progesterone inhibits granulosa cell inhibin production (Henderson & Franchimont, 1981) while-purified preparations of inhibin inhibit granulosa cell progesterone production (Franchimont *et al.*, 1981).

The ability to stimulate granulosa cell inhibin production is not restricted to the natural androgens, but is mimicked by the synthetic androgens, methylestrenolone and mesterolone (Table 3), and the ability to stimulate inhibin production may be a general property of androgenic compounds. In addition, these studies with non-aromatizable synthetic androgens confirm the previous finding that the stimulatory effect of androgens on inhibin is direct and not dependent upon their aromatization to oestrogen (Henderson & Franchimont, 1981). Androgen stimulation of inhibin production is probably, therefore, a receptor-mediated event.

The assigned molecular weight of inhibin is usually between 10 000 and 30 000 although inhibin activity has been reported in molecular weight fractions of > 100 000 and < 5000 (Franchimont *et al.*, 1979; de Jong, Jansen & van der Molen, 1981). This inconsistency is probably due to (1) the different methods of measuring inhibin activity and determining molecular weight, (2) the fact that few of the studies have been performed with purified inhibin, and (3) because inhibin appears to be biochemically heterogeneous (Franchimont *et al.*, 1979). The inhibin activity of bovine granulosa cell culture medium after ultrafiltration was only found in the  $M_r$  > 100 000 fraction. Using a similar ultrafiltration method, Cahoreau, Blanc, Dacheux, Pisselet & Courot (1979) found the inhibin activity of ram rete testis fluid to be in the  $M_r$  > 100 000 fraction. It is possible, however, that the concentrating effect of ultrafiltration promotes aggregation of lower molecular weight forms. The loss of inhibin activity of bovine granulosa cell culture medium by heating to 80°C for 30 min is consistent with the finding that inhibin present in Sertoli cell culture medium (Steinberger & Steinberger, 1976) and rete testis fluid (Franchimont *et al.*, 1979) is also thermolabile.

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## Effect of follicular atresia on inhibin production by bovine granulosa cells *in vitro* and inhibin concentrations in the follicular fluid

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**Summary.** The ability of bovine granulosa cells to produce inhibin and to synthesize oestradiol-17 $\beta$  increased with increasing follicle size in healthy but not atretic follicles. Granulosa cells from small ( $\leq 5$  mm diam.) healthy follicles were indistinguishable from cells of atretic follicles in terms of their ability to produce inhibin and to aromatize androgen. However, granulosa cells from healthy and atretic follicles, irrespective of size, differed markedly in their morphological appearance after culture for 24 h. Testosterone (1  $\mu\text{g/ml}$ ) stimulated inhibin production by granulosa cells from healthy and atretic follicles while FSH (100 ng/ml) stimulated inhibin production by granulosa cells from healthy follicles only. The relative ability of granulosa cells from different sizes of healthy and atretic follicles to produce inhibin *in vitro* was reflected in inhibin concentrations in follicular fluid. There was a significant positive correlation between inhibin concentration in follicular fluid and the number of granulosa cells per follicle. There was also a significant positive correlation between follicular diameter and inhibin concentration in follicular fluid, but only in healthy follicles. These findings show that both follicular size and atresia influence follicular inhibin production.

### Introduction

Inhibin is produced by the ovary of several primate and non-primate species and may be physiologically important in the regulation of pituitary FSH secretion (Franchimont *et al.*, 1981; Channing *et al.*, 1982; Demoulin *et al.*, 1982). Our previous studies of bovine ovarian tissues have demonstrated that inhibin is present in follicular fluid, that it is produced predominantly by granulosa cells and that its production by granulosa cells can be stimulated by androgens (Henderson & Franchimont, 1981, 1983). At present, little information is available on how follicular inhibin production is influenced by the size and health of follicles. This was investigated in the present study by comparing inhibin production by granulosa cells *in vitro* and inhibin concentrations in follicular fluid between follicles of differing size and health obtained from oestrous cyclic cows.

### Materials and Methods

#### *Ovarian tissues and collection of follicular fluid and granulosa cells*

Ovaries were obtained from 18 adult, non-pregnant, parous Angus cows from Day -3 to Day +1 of the oestrous cycle (Day 0 is day of oestrus). Under a dissecting microscope ( $\times 10$ - $\times 40$  magnification) all antral follicles  $\geq 2.0$  mm diameter were individually dissected out of each pair of ovaries into sterile Medium 199 with Earle's salts (Eagle, 1959) supplemented with HEPES buffer (20 mM), L-glutamine (2 mM), gentamicin (50  $\mu\text{g}/\text{ml}$ ) (Gibco, Grand Island, New York, U.S.A.), sodium heparin (50 i.u./ml) (Weddel Pharmaceuticals Ltd, London, U.K.) and 0.1% bovine serum albumin (Fraction V; Sigma Chemical Co., St Louis, MO, U.S.A.) (Medium A). After recording the follicular diameter and examining the thecal vasculature, the follicle was incised to release the contents. The released follicular fluid was examined for the presence or absence of debris and then aspirated through a fine-bore capillary tube. An aliquant of fluid was removed and stored at  $-20^\circ\text{C}$  until freeze-dried, or until pooled with fluid from similar follicles and freeze-dried, and subsequently assayed for inhibin activity. The internal follicle wall was washed gently and repeatedly with  $\sim 2$  ml Medium A and the released clumps of granulosa cells were dispersed by pipetting several times through a finely drawn Pasteur pipette. The oocyte was isolated and assessed subjectively as being healthy or degenerate as previously described (McNatty *et al.*, 1983) and then discarded. The follicle wall was washed several more times, and the total number of granulosa cells in the pooled washings counted by using a haemocytometer and their viability was determined by uptake of nigrosin dye. The colour of the theca interna was noted.

#### *Follicle classification*

Each follicle was classified as healthy or atretic on the basis of its morphological appearance. Follicles considered to be healthy were those with: visible thecal capillaries when viewed at  $\times 10$  magnification under a dissecting microscope; no debris in the follicular fluid; an oocyte of healthy appearance,  $\geq 26\%$  of the maximum number of recoverable granulosa cells for a follicle of a given size (McNatty *et al.*, 1982, 1984) and a pink to red theca interna. Follicles were considered to be atretic if one or more of these 5 criteria was not applicable.

#### *Granulosa cell cultures*

To obtain sufficient granulosa cells to establish triplicate cultures for each treatment, it was often necessary to pool cells from follicles of a similar size and classification from each pair of ovaries. In the 18 pairs of ovaries studied, granulosa cell cultures were established from the following numbers of healthy and atretic follicles: 75 healthy follicles of which 22 were studied individually and the cells from the remaining follicles pooled into 13 different groups, and 602 atretic follicles of which 2 were studied individually and the cells from the remainder pooled into 45 different groups. Before culture, the collected granulosa cells were centrifuged at 200 *g* for 10 min, resuspended in Medium A devoid of sodium heparin and bovine serum albumin, but containing 10% newborn calf serum (Gibco, New York, U.S.A.) (Medium B), and recounted. Aliquants of approximately 400 000 'live' granulosa cells in 0.5 ml Medium B were pipetted into individual wells of multi-welled tissue-culture Petri dishes (Sterilin, Middlesex, U.K.), with each well containing an 18 mm<sup>2</sup> glass cover-slip. A further 0.5 ml Medium B alone or containing added testosterone (2  $\mu\text{g}/\text{ml}$ ) (Sigma), FSH (200 ng/ml, NIH-FSH-S15) or LH (200 ng/ml, NIH-LH-B9) was added and the cells incubated for 24 h at  $37^\circ\text{C}$  in a humidified incubator (Contherm Scientific, Petone, N.Z.) gassed with 5%  $\text{CO}_2$ :95% air. To determine endogenous inhibin content, aliquants of the granulosa cells were also homogenized in 1 ml Medium B and stored frozen until freeze-dried and subsequently assayed for inhibin activity. After 24 h of culture, the granulosa cells were examined under an inverted phase-contrast microscope and counted. The medium was next removed and

aliquants freeze-dried for subsequent assay of inhibin, and the remaining medium stored frozen until assayed for oestradiol-17 $\beta$ . The granulosa cells were washed with Medium B devoid of serum, fixed with Smear Fix (Raymond A. Lamb, London, U.K.) and stained with haematoxylin and eosin.

#### *Inhibin assay*

Inhibin activity was determined using a bioassay based on the inhibition of LHRH-stimulated FSH secretion by rat cultured anterior pituitary cells as described and validated previously (Henderson & Franchimont, 1981), but with the following minor modifications. (1) The concentration of activated charcoal (Norit A) and dextran used to remove endogenous steroids from the culture medium and follicular fluid was reduced from 1% and 0.1% to 0.1% and 0.01% respectively. We have found that the higher concentrations of charcoal and dextran cause a slight but consistent reduction in inhibin activity of up to 20%. (2) Follicular fluid was only diluted 1/10 to 1/20 before treatment with charcoal and dextran. Studies with semi-purified inhibin from rete testis fluid indicate that at low concentrations inhibin may be adsorbed during charcoal-dextran treatment, but at high concentrations inhibin is unaffected by the charcoal-dextran treatment. (3) The inhibin standard preparation was derived from ovine rete testis fluid. One unit of activity of this standard corresponds to 1 unit of activity of the previous standard used by us which was derived from ovine testicular lymph and had been assigned an arbitrary potency of 1 U/mg (Eddie, Baker, Higginson & Hudson, 1979).

The freeze-dried aliquants of cell culture medium and follicular fluid taken for inhibin assay were reconstituted with distilled water. Steroids were removed by mixing for 16 h at 4°C with activated charcoal (Norit A, 0.1%) pretreated with dextran (0.01%) followed by centrifugation at 3000 g. The supernatants were filter-sterilized by passing through a 0.45  $\mu$ m cellulose millipore filter before use. Aliquants were then added to rat anterior pituitary cells, previously cultured for 3 days, and their ability to inhibit LHRH ( $10^{-8}$  M)-stimulated FSH production during a 6-h incubation period was tested 3 days later. Aliquants of the inhibin standard and sample preparations were each tested in quadruplicate. As described previously, there was close parallelism between the dose-response curve obtained with the inhibin standard preparation and the curves generated from different dilutions of culture medium and follicular fluid (Henderson & Franchimont, 1981). Regression analysis and parallel-line assay statistics (Borth, 1976) were used to relate the inhibin activity of the culture medium and follicular fluid to that of the inhibin standard. The limit of sensitivity of the assay (per tube) was 0.1 U inhibin. Inhibin production by granulosa cells was calculated by subtracting the amount of endogenous inhibin in the granulosa cells from that present in the culture medium after 24 h. Dextran-charcoal-treated culture medium with and without testosterone (1  $\mu$ g/ml), FSH (100 ng/ml) or LH (100 ng/ml) but not exposed to granulosa cells had no effect on pituitary cell FSH production.

#### *Radioimmunoassays*

The gonadotrophin concentrations in media from the pituitary cell cultures were measured in duplicate by double-antibody methods using NIAMDD rat pituitary gonadotrophin reagents supplied by the pituitary agency of NIH. The concentrations were expressed in terms of NIAMDD rat-FSH-RP1 and rat-LH-RP1. The sensitivities of the assays were 5 ng FSH/ml and 10 ng LH/ml. The intra- and inter-assay coefficients of variation were 3 and 8% for the FSH assay and 5 and 12% for the LH assay respectively.

Oestradiol-17 $\beta$  was measured in unextracted granulosa cell culture medium using a specific radioimmunoassay described previously (McNatty, Gibb, Dobson, Thurley & Findlay, 1981). Steroids showing >1% cross-reactivity with the oestradiol-17 $\beta$  antiserum (WA-27) were oestrone (7.3%), oestriol (1.4%) and oestradiol-17 $\alpha$  (1.4%); testosterone was <0.02%. The sensitivity of the assay was 5 pg/tube. The intra- and inter-assay coefficients of variations were <11%.

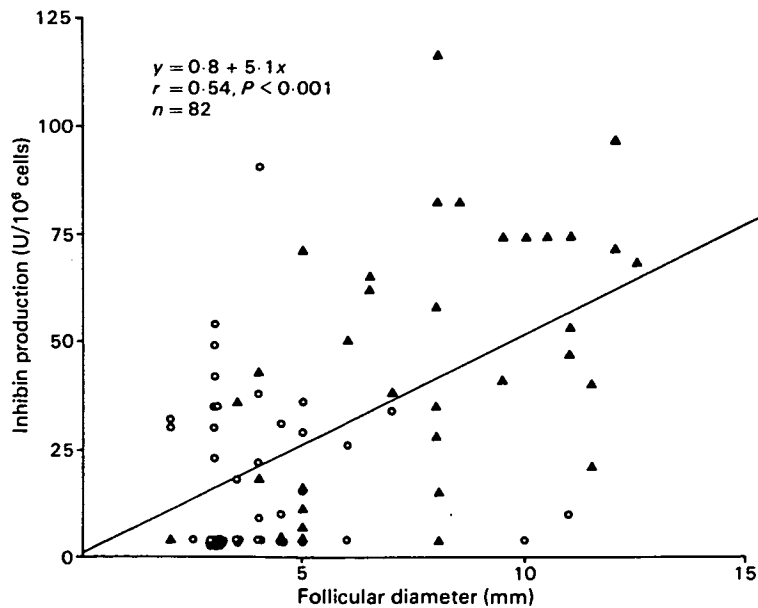
### Statistics

Unless otherwise stated, the statistical significance of observed differences between different treatment groups was determined by analysis of variance in conjunction with Newman-Keuls multiple range test. When Bartlett's test indicated heterogeneity of variance, data were transformed to logarithms to equalize the variances before statistical analysis. The level of significance was set at  $P < 0.05$ .

### Results

#### *Granulosa cell inhibin production in relation to follicular diameter, degree of atresia and aromatase activity*

Text-figure 1 shows that there was a significant correlation between inhibin production by granulosa cells and the diameter of the follicles from which the granulosa cells were obtained ( $r = 0.54$ ,  $P < 0.001$ ,  $n = 82$ ). When regression analysis was performed on the data from healthy and atretic follicles separately, a significant correlation was observed for healthy follicles ( $r = 0.52$ ,  $P < 0.002$ ,  $n = 35$ ) but not for atretic follicles ( $r = -0.05$ ,  $P > 0.05$ ,  $n = 47$ ). The results in Table 1 show that in healthy follicles, but not in atretic follicles, inhibin production by granulosa cells and their ability to synthesize oestradiol-17 $\beta$  (aromatase activity) was influenced by follicular diameter. Mean inhibin production and aromatase activity of granulosa cells from healthy follicles  $\leq 5$  mm diameter was significantly less ( $P < 0.01$ ) than that of granulosa cells from healthy follicles  $> 5$  mm diameter, but not significantly different ( $P > 0.05$ ) from that of cells from atretic follicles. Contingency table analysis indicated that a significant relationship existed between the ability of granulosa cells to produce inhibin and their ability to synthesize oestradiol-17 $\beta$  ( $P < 0.001$ ). In cultures with an aromatase activity (see legend to Table 1 for definition) for  $\geq 10$  ng oestradiol-17 $\beta$ /10<sup>6</sup> cells, inhibin production in 16 of 24 cultures was  $\geq 50$  U/10<sup>6</sup> cells (maximum 116 U/10<sup>6</sup> cells). When the aromatase activity was  $< 10$  ng oestradiol-17 $\beta$ /10<sup>6</sup> cells, inhibin production was  $\geq 50$  U/10<sup>6</sup> cells in only 1 of 29 cultures (maximum 54 U/10<sup>6</sup> cells).



**Text-fig. 1.** Relationship between follicular diameter and inhibin production by granulosa cells cultured for 24 h. ▲, Healthy follicles; ○, atretic follicles.

**Table 1.** Effect of follicular atresia and follicular diameter on inhibin production and aromatase activity of bovine granulosa cells cultured for 24 h

	Follicular diameter (mm)	Inhibin production (U/10 <sup>6</sup> cells)	Aromatase activity (ng oestradiol-17 $\beta$ /10 <sup>6</sup> cells)*
Healthy	$\leq 5$	21 $\pm$ 7 <sup>a</sup> (10)	14 $\pm$ 6 <sup>a</sup> (8)
	> 5	55 $\pm$ 6 <sup>b</sup> (25)	104 $\pm$ 19 <sup>b</sup> (25)
Atretic	$\leq 5$	18 $\pm$ 3 <sup>a</sup> (42)	6 $\pm$ 2 <sup>a</sup> (29)
	> 5	16 $\pm$ 6 <sup>a</sup> (5)	13 $\pm$ 6 <sup>a</sup> (5)

Values are mean  $\pm$  s.e.m. for the no. of determinations indicated in parentheses. Mean values with different superscripts in the same column are significantly different (<sup>a</sup>*b**P* < 0.01).

\* Cells cultured with testosterone (1  $\mu$ g/ml) for 24 h. (Inhibin production was determined in cultures not exposed to testosterone, and no detectable amounts of oestradiol-17 $\beta$  were produced by these cultures.)

#### *Effect of testosterone, FSH and LH on inhibin production by granulosa cells from healthy and atretic follicles*

Inhibin production by granulosa cells from healthy follicles > 5 mm diameter was significantly stimulated by testosterone (1  $\mu$ g/ml) from a mean  $\pm$  s.e.m. value of 60  $\pm$  6 to 185  $\pm$  25 U inhibin/10<sup>6</sup> cells (*n* = 23, *P* < 0.001) during culture for 24 h. Inhibin production by granulosa cells from small healthy follicles ( $\leq 5$  mm diam.) and atretic follicles (both of which produced similar amounts of inhibin under basal conditions, Table 1) was stimulated by testosterone (1  $\mu$ g/ml) during culture for 24 h from an overall mean  $\pm$  s.e.m. value of 16  $\pm$  4 to 58  $\pm$  11 U inhibin/10<sup>6</sup> cells (*n* = 29, *P* < 0.001) an amount not significantly different from that produced by granulosa cells from healthy follicles > 5 mm diameter (60  $\pm$  6 U inhibin/10<sup>6</sup> cells; *P* > 0.05).

FSH (NIH-FSH-S15, 100 ng/ml) significantly stimulated inhibin production by granulosa cells from healthy follicles (4–11 mm diam.) from a mean  $\pm$  s.e.m. value of 73  $\pm$  9 to 103  $\pm$  16 U inhibin/10<sup>6</sup> cells (*n* = 8, *P* < 0.05, Wilcoxon signed rank test) in 24 h of culture. FSH had no significant effect on inhibin production by granulosa cells from atretic follicles. LH (NIH-LH-B9, 100 ng/ml) had no significant effect on inhibin production by granulosa cells from healthy or atretic follicles.

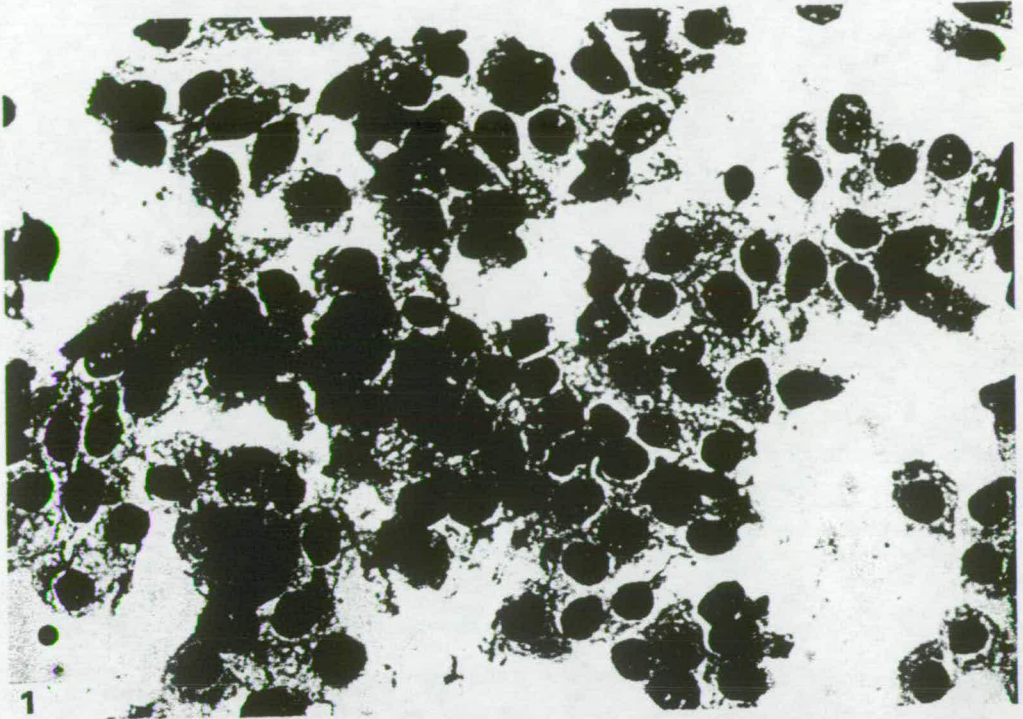
#### *Morphology after culture for 24 h of granulosa cells from healthy and atretic follicles*

After culture for 24 h there were marked differences in the morphological appearance of granulosa cells from healthy and atretic follicles which were independent of follicle size. Irrespective of treatment, granulosa cells from healthy follicles were characterized by an homogeneous population of cuboidal, epithelial-like cells with a small cytoplasmic-nuclear ratio (Pl. 1, Fig. 1). In contrast, irrespective of treatment, granulosa cells from atretic follicles were heterogeneous, generally fibroblastic in appearance, had vacuole-filled cytoplasm and a high cytoplasmic-nuclear ratio (Pl. 1, Fig. 2).

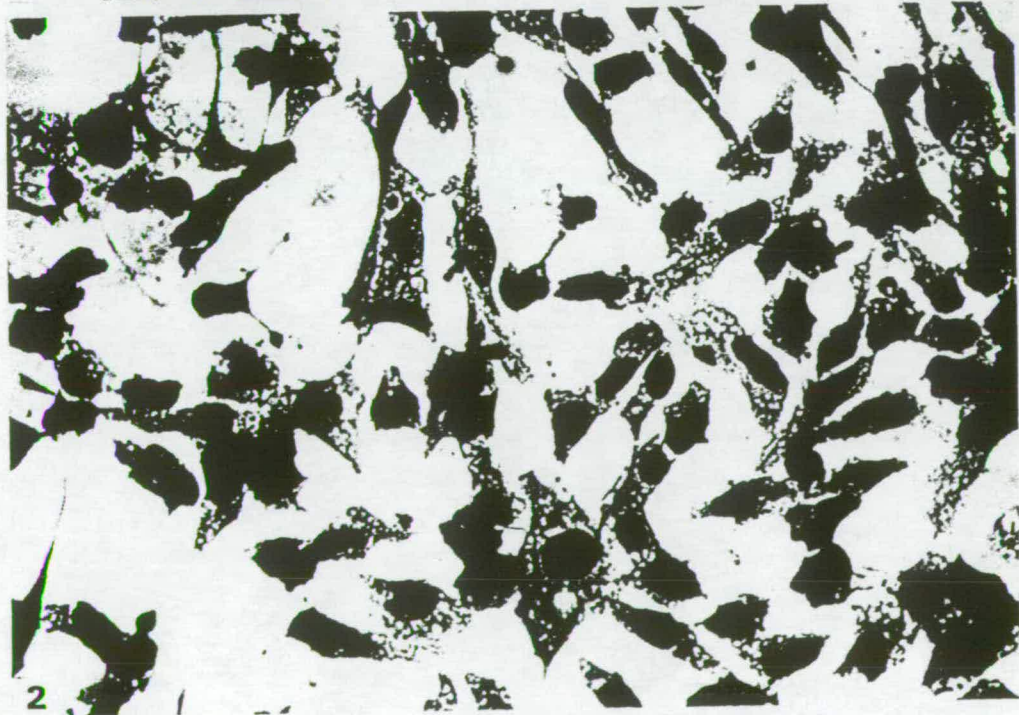
#### *Inhibin concentrations in bovine follicular fluid in relation to follicular atresia, diameter and follicular granulosa cell number*

Table 2 shows that in healthy, but not atretic, follicles mean concentrations of inhibin in follicular fluid increased significantly as follicular diameter increased. The mean concentration of inhibin in follicular fluid of small healthy follicles ( $\leq 5$  mm diam.) was not significantly different

PLATE 1



1



2

Morphological appearance of granulosa cells cultured for 24 h and stained with haematoxylin and eosin from healthy (Fig. 1) and atretic (Fig. 2) follicles.  $\times 560$ .

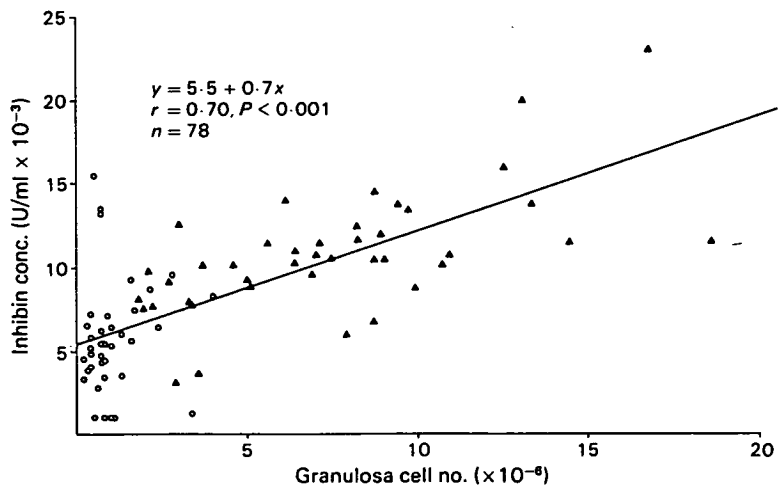
(Facing p. 5)

from that of atretic follicles. Regression analysis showed that a significant correlation existed between inhibin concentrations ( $\text{U/ml} \times 10^{-3}$ ) in follicular fluid ( $y$ ) and follicular diameter (mm) ( $x$ ) in healthy follicles ( $y = 4.4 + 0.7x$ ,  $r = 0.56$ ,  $P < 0.001$ ,  $n = 41$ ) but not in atretic follicles ( $y = 5.7 - 0.01x$ ,  $r = 0.01$ ,  $P > 0.05$ ,  $n = 37$ ). In healthy but not atretic follicles the mean number of granulosa cells per follicle increased significantly as follicle size increased (Table 2). The mean number of granulosa cells per follicle was significantly lower in atretic than in healthy follicles. There was a significant correlation between the number of granulosa cells per follicle and inhibin concentrations in follicular fluid (Text-fig. 2). When the data for healthy and atretic follicles were analysed separately, follicular granulosa cell number ( $x$ ) and fluid inhibin concentrations ( $y$ ) were significantly correlated in healthy ( $y = 6.6 + 0.6x$ ,  $r = 0.63$ ,  $P < 0.001$ ,  $n = 41$ ) but not in atretic follicles ( $y = 5.1 + 0.7x$ ,  $r = 0.20$ ,  $P > 0.05$ ,  $n = 37$ ).

**Table 2.** Effect of follicular atresia and follicular diameter on inhibin concentrations in follicular fluid and follicular granulosa cell number

	Follicular diam. (mm)	$n$	Inhibin conc. ( $\text{U/ml} \times 10^{-3}$ )	Granulosa cell number ( $\times 10^{-6}$ )
Healthy	$\leq 5$	6	$6.7 \pm 1.1^a$	$3.2 \pm 0.5^d$
	$> 5$ to $\leq 10$	22	$10.7 \pm 0.5^b$	$6.7 \pm 0.5^e$
	$> 10$	13	$13.1 \pm 1.2^c$	$11.2 \pm 1.2^f$
Atretic	$\leq 5$	13	$6.4 \pm 0.9^a$	$1.1 \pm 0.2^g$
	$> 5$ to $\leq 10$	17	$5.0 \pm 0.6^a$	$1.0 \pm 0.2^g$
	$> 10$	7	$6.5 \pm 1.5^a$	$1.4 \pm 0.5^g$

Values are mean  $\pm$  s.e.m. for  $n$  determinations. Mean values with different superscripts in the same column are significantly different ( $P < 0.01$  except for  $^{bc}P < 0.05$ ).



**Text-fig. 2.** Relationship between the number of granulosa cells per follicle and inhibin concentration in bovine follicular fluid. ▲, Healthy follicles; ○, atretic follicles.

### Discussion

The present study demonstrates that follicular production of inhibin is influenced by both the health and size of follicles, as assessed by the relative ability of granulosa cells to produce inhibin *in vitro* and the concentrations of inhibin in follicular fluid. Granulosa cells from atretic and small

( $\leq 5$  mm diam.) healthy follicles produced similar amounts of inhibin *in vitro*. As follicular diameter increased, there was an increase in the ability of granulosa cells from healthy, but not atretic, follicles to produce inhibin *in vitro*. Granulosa cell aromatase activity was also influenced by follicle size and health, in a similar way to that of cellular inhibin production, and these two cellular functions were significantly related. Although granulosa cells from small healthy follicles could not be distinguished from those of atretic follicles, in terms of cellular ability to produce inhibin, or aromatize androgen, granulosa cells from healthy and atretic follicles, irrespective of size, differed markedly in their morphological appearance after culture for 24 h (Plate 1). The reason for these morphological differences is unknown. While FSH has previously been shown to stimulate inhibin production by cultured Sertoli cells (Steinberger, 1981; Le Gac & de Krestler, 1982), this study is the first to show that FSH can stimulate inhibin production by granulosa cells, but only by cells taken from healthy follicles. Our previous failure to show a significant effect of FSH on granulosa cell inhibin production (Henderson & Franchimont, 1981) was probably due to using pools of cells taken from follicles not classified with respect to atresia.

The increasing ability of granulosa cells from healthy follicles to produce inhibin as follicular diameter increased may be a consequence of prior gonadotrophin action on the follicles. LH stimulates production of thecal androgens (Henderson, Kieboom, McNatty, Lun & Heath, 1984; McNatty *et al.*, 1984) which may in turn increase the ability of granulosa cells to produce inhibin (Henderson & Franchimont, 1981, 1983). FSH may directly increase production of inhibin by granulosa cells from healthy follicles. In contrast, LH does not stimulate androgen production by the theca interna from large atretic follicles (Henderson *et al.*, 1984; McNatty *et al.*, 1984) and FSH does not stimulate inhibin production by granulosa cells from atretic follicles. Failure to respond to gonadotrophins may thus account for the reduced ability of granulosa cells from atretic follicles to produce inhibin, relative to cells from large healthy follicles. A role for androgens in particular is supported by the finding that testosterone stimulates inhibin production by granulosa cells from small healthy ( $\leq 5$  mm diam.) and atretic follicles to the basal amounts produced by cells from healthy follicles  $> 5$  mm diameter. Thecal-derived androgens and FSH may also positively influence granulosa cell aromatase activity (Hillier, van Hall, van den Boogaard, de Zwart & Keyzer, 1982). Thus follicular responsiveness and prior exposure to gonadotrophins may also account for differences in granulosa cell aromatase activity between cells from healthy and atretic follicles.

The concentrations of inhibin in follicular fluid reported in this study are higher than those reported previously (Henderson & Franchimont, 1981), which were probably underestimated because higher concentrations of charcoal and dextran were used to remove endogenous steroids from more diluted follicular fluid. Tsonis *et al.* (1983) have also reported that differences in the concentration of charcoal used to treat ovine follicular fluid and the dilution of the inhibin sample before charcoal treatment influenced the amount of inhibin activity measured. The values for inhibin concentrations in ovine follicular fluid are quite comparable to those in bovine follicular fluid (present study). The relative ability of granulosa cells from healthy and atretic follicles of different sizes to produce inhibin *in vitro* was reflected in the relative concentrations of inhibin present in follicular fluid (Table 2). Atretic follicles contained fewer granulosa cells than did healthy follicles. This may have also contributed to the lower concentrations of inhibin present in follicular fluid of atretic follicles  $> 5$  mm diameter compared to healthy follicles  $> 5$  mm diameter. The correlation between inhibin concentrations in follicular fluid and the number of granulosa cells per follicle (Text-fig. 2) provides strong, supportive evidence to the view that granulosa cells are the predominant source of ovarian inhibin (Henderson & Franchimont, 1983). Previous reports of the relationship between inhibin concentrations in follicular fluid and follicular diameter have been confusing. With increased follicular diameter, inhibin concentrations have been reported to decrease in follicular fluid of the pig (Lorenzen, Channing & Schwartz, 1978) and cow (Henderson & Franchimont, 1981) and to increase in the sheep (Tsonis *et al.*, 1983) and cow (Welschen, Hermans, Dullaart & de Jong, 1977). However, the health of the follicle was not considered in these



studies. In the present study, inhibin concentrations in follicular fluid were related to follicular diameter in healthy, but not atretic follicles (Table 2). A significant positive correlation between follicular diameter and fluid inhibin concentrations was found for healthy follicles, but no significant correlation was found for atretic follicles. Differences in the results of previous studies may therefore be attributable to the relative proportions of healthy and atretic follicles that were studied.

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REGULATION OF INHIBIN SECRETION BY GRANULOSA CELLS IN VITRO \*

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Evidence has accumulated over the past ten years to demonstrate that inhibin produced by the gonads is physiologically important in regulating pituitary FSH secretion. In males and females, steroid free follicular fluid and ram rete testis fluid selectively reduce FSH levels in vivo and/or selectively or preferentially inhibit FSH production by anterior pituitary cells in vitro (see 1 for review). The experiments of Erickson and Hsueh (2) indicated that granulosa cells are the source of this follicular inhibin while in vivo experiments have shown that inhibin is secreted into the ovarian vein (3-4); and that the ovarian secretion of inhibin could be dependent on a specific action of FSH (5) giving further support to the hypothesis that inhibin is involved in the feedback regulation of FSH.

Although the sites of inhibin biosynthesis have been well defined, there is little information on the regulation of ovarian inhibin production. The aim of the present work was to review in vitro models for studying in vitro production of ovarian inhibin and to investigate the factors which may modify inhibin production by the ovarian cells.

INHIBIN ASSAY

The assay of inhibin activity was based on the selective or preferential inhibition of basal or GnRH-induced FSH secretion by cultured anterior pituitary cells as described previously (6). Culture medium incubated in the absence of ovarian cells was used as control medium for adding to the pituitary cell culture medium. The standard reference preparation, kindly supplied by Professor B. Hudson (Howard Florey Institute of Experimental Physiology and Medicine, Melbourne, Australia) was derived from ovine testicular lymph (OTL) and has been given an arbitrary potency of 1 U/mg. This inhibin standard has been

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added to the pituitary cell cultures at doses of 250 to 2000  $\mu\text{g/ml}$ . The spent media of the ovarian cells were treated with charcoal (1%) - dextran T 70 (0.1%) (Pharmacia Fine Chemicals) prior to testing for inhibin activity. To monitor for specificity, we have relied upon parallelism between dose-response lines of test substances (i.e. spent media) and standards. Inhibin activity in culture media was expressed relative to the activity of the OTL standard.

#### Influence of serum on inhibin production

The effect of foetal bovine serum or newborn calf serum has been studied using cultures of rat ovarian cells as described by Croze and Franchimont (7,8). Briefly female rats aged 20 days were injected with 10 I.U. pregnant mare serum gonadotrophin (Gestyl; N.V. Organon Oss, Holland). Seventy three hours later, the animals were sacrificed, the ovaries collected, cleaned of their adipose tissue and pressed through stainless-steel grids (60 mesh; Falcon, Oxnard, CA, USA). The cells were recovered by agitating the grids in culture medium (Ham's F10 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50  $\mu\text{g/ml}$  streptomycin and 10% FBS).  $2 \times 10^6$  cells per dish (35 x 10 mm; Flow Laboratories, Irvine, U.K.) were generally incubated in 2 ml culture medium and kept at 37°C in a humidified atmosphere consisting of 95% air - 5%  $\text{CO}_2$ . The substances whose effects on inhibin production by the rat ovarian cells were tested were added to the culture medium at the time the cells were seeded in the Petri dishes. Twenty-four hours later the culture medium was discarded. The cells were washed and fresh culture medium was added back to the cells. Twenty-four hours after the culture medium had been changed, it was collected and used for steroid determination and inhibin activity assay.

#### Influence of serum on inhibin and progesterone secretion

Figure 1 illustrates the results of experiments in which daily inhibin and progesterone productions were measured in the absence or in the presence of 10% foetal bovine serum (FBS). It appears that the inhibin activity in the culture medium from rat ovarian cells incubated without serum is only detectable during day 2 when determined in basal conditions. In the presence of 10% FBS, the inhibin production is always greater than the inhibin activity found in culture media from cells incubated with serum-free medium. Moreover, it can be seen that the inhibin activity measured in the presence of 10% FBS increases from day 2 to day 4. On the contrary, the secretion of progesterone diminishes with the duration of the culture period regardless of the presence of FBS. Nevertheless, progesterone secretion is higher in the presence of FBS than in the absence of FBS.

Progesterone concentration decreases with the duration of the culture period suggesting an inverse relationship between inhibin production and progesterone concentration in the culture medium from rat

ovarian cells cultured with 10 % FBS. From this study (7), it clearly appears that serum is an essential constituent of the culture medium in order for the cells to sustain their inhibin production.

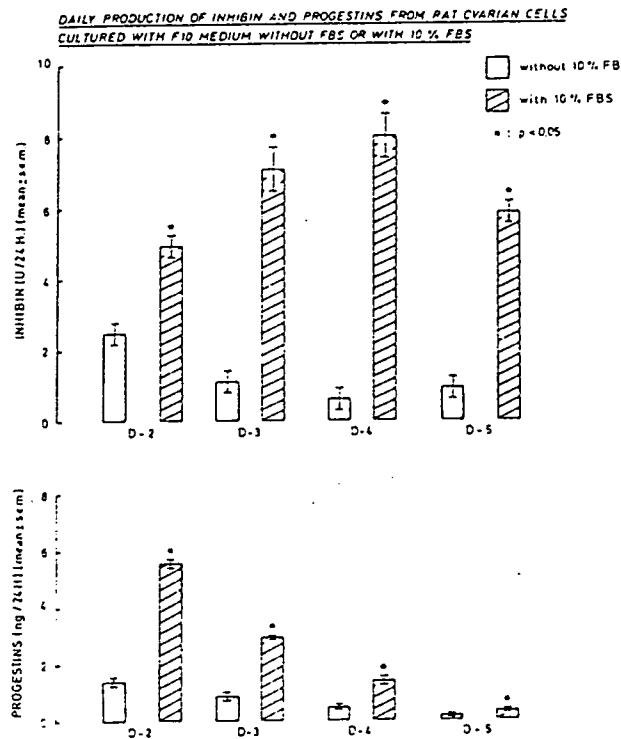


Figure I : Daily production (mean + s.e.m.) of inhibin and progesterone from rat ovarian cells cultured with F-10 medium without FBS or with 10 % FBS.

Comparison of the effect of foetal bovine serum (FBS) and newborn calf serum (NBCS) on the production of inhibin and progestagens

The concentrations of progestagens, androgens, oestrogens, FSH, and LH were measured in the FBS and in the NBCS (7). These 2 sera differ from each other by their steroid concentrations and their gonadotrophin contents. The concentrations of progestagens are similar in both types of sera while the concentrations of androgens and oestrogens are significantly greater in FBS. The concentration of FSH is significantly greater in the NBCS while there is no difference in LH concentration. The daily mean production of inhibin by ovarian cells incubated with culture medium supplemented with FBS or NBCS is not statistically different during day 2. But from day 3 to day 5, inhibin production is significantly greater for cells exposed to FBS, as determined in basal conditions. On the contrary, progestagen secretion is greater for ovarian cells cultured with NBCS than with FBS during this experimental period. Therefore, a lower amount of inhibin is measured in the culture media from ovarian cells which secrete greater amount of progestagens in the presence of NBCS and FBS is better able to maintain inhibin production than NBCS. Thus this positive effect of serum on inhibin production depends on the source of serum since serum from bovine fetuses was better able to maintain the production of inhibin than was that from newborn calves. This difference was probably not due to harmful effect because ovarian cells cultured with newborn calf serum produced more progestagens than did cells incubated with FCS. The fact that the ovarian cells incubated with the sera having the higher concentration of FSH (newborn calves) produced lower amounts of inhibin does not seem to be in agreement with a simple direct physiological interaction between the two hormones and contrasts with the stimulatory effect of FSH on the secretion of inhibin by Sertoli cells (9,10).

Effect of adsorption of foetal bovine serum with charcoal-dextran on inhibin and steroid production

Different batches of FBS unadsorbed or adsorbed with 1 % charcoal: 0.1 % dextran were tested for their effect on the secretion of inhibin and progestagens. Adsorption of FBS does not modify progestagen concentration while it reduces greatly the concentrations of both androgens and oestrogens to undetectable levels. A study on the effect of charcoal:dextran treatment on gonadotrophin concentration showed that adsorption of sera does not significantly modify the amount of serum FSH while it significantly reduces the concentration of LH.

It appears (figure 2) that the daily mean production of inhibin by ovarian cells exposed to unadsorbed serum is maintained although the production during day 5 is significantly lower than the production

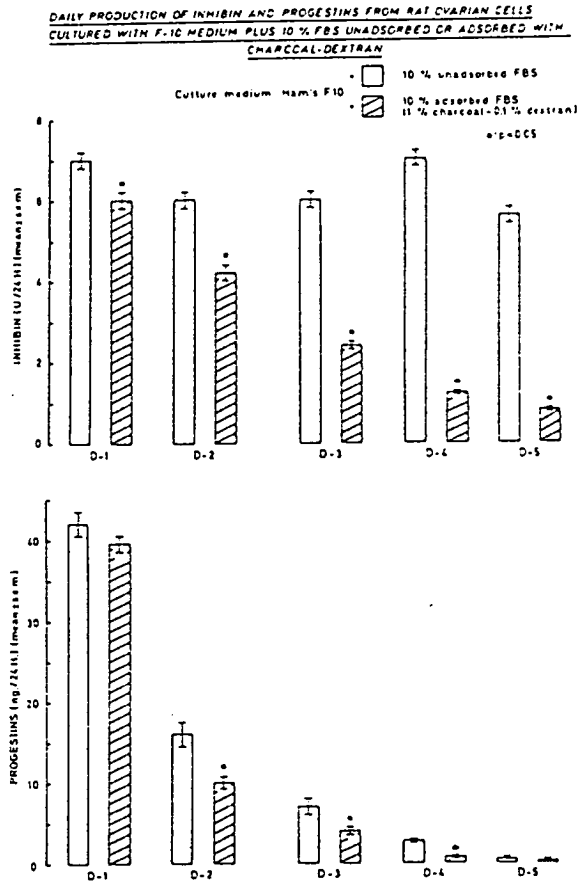


Figure 2

during day 1 and day 4. In contrast, cells incubated with adsorbed serum are unable to sustain their inhibin production. At day 1, the production of inhibin in the presence of adsorbed serum is already lower than the production observed in the presence of unadsorbed serum and it gradually decreases with the day of incubation.

The production of progestagens by ovarian cells exposed to unadsorbed serum and charcoal:dextran-adsorbed serum progressively decreases with the time of incubation. During the first 24 hours of

culture and day 5, the production of progestagens is not significantly different for the cells incubated with either unadsorbed or adsorbed serum. However, from day 2 to day 4, the production by cells exposed to unadsorbed serum is always greater than the production by cells incubated with adsorbed serum.

The ability of FCS to support inhibin production from rat ovarian cells seems to be linked to some extent to substances adsorbed by charcoal:dextran. However, it is unlikely that progestagens have a beneficial effect on inhibin production since treatment with charcoal:dextran failed to adsorb progestagens. Furthermore, a lower amount of inhibin was measured in the culture media from ovarian cells which secreted greater amounts of progestagens in the presence of newborn calf serum and it has also been reported that a high exogenous progesterone concentration decreases the production of inhibin by bovine granulosa cells (6). The possibility that the endogenous androgens and oestrogens present in the intact serum may be partly responsible for the maintenance of inhibin production cannot be excluded since both steroids were adsorbed by the charcoal:dextran treatment and ovarian cells incubated with FBS that contained more androgens than did the newborn calf sera secreted more inhibin. Finally, it has been demonstrated that bovine granulosa cells exposed to exogenous androgens secrete more inhibin (6, 15). Epidermal and fibroblast growth factor, insulin and somatomedins which favour mitosis, protein synthesis of granulosa cells in vitro (11, 12, 13, 14) could be other charcoal:dextran-adsorbable serum constituents involved in inhibin production.

#### INHIBIN PRODUCTION BY OVARIAN CELLS

Detailed studies (table I) in which inhibin, oestradiol-17 $\beta$  and progesterone production by various bovine ovarian tissues cultured for 24 hours was determined, demonstrate that only ovarian follicular tissue has the ability to produce inhibin in vitro (15).

Removal of granulosa cells from the intact follicle wall to leave a thecal shell causes inhibin production to fall by 80%. Oestradiol 17 $\beta$  production by thecal tissue is < 2% of that produced by the intact follicle wall. No detectable amount of inhibin or oestradiol 17 $\beta$  are present in the homogenates of ovarian stroma and luteal tissue or produced by these tissues during culture for 24 hours whereas large amount of progesterone are detected demonstrating that these cells are functional in vitro. Therefore, granulosa cells are the only ovarian cell type which produce large amounts of inhibin.

The analysis of several granulosa cell culture experiments revealed an inverse relationship between progesterone and inhibin production ( $\ln y = 5.28 - 0.79 \ln x$ ; coeff. of linear correlation ( $r$ ) = 0.83 with  $P < 0.001$ ) (6). Thus, follicular granulosa cells are



Table I. Inhibin and steroid production by bovine ovarian tissues cultured for 24 hours

Tissue cultured	Inhibin (U/mg protein)	Oestradiol 17 $\beta$ (ng/mg protein)	Progesterone (ng/mg protein)
Follicle wall (theca + granulosa)	26 $\pm$ 1 <sup>a</sup>	6.4 $\pm$ 1.4 <sup>a</sup>	1540 $\pm$ 140 <sup>a</sup>
Theca	4.9 $\pm$ 0.3 <sup>b</sup>	0.03 $\pm$ 0.01 <sup>b</sup>	
Granulosa	833 $\pm$ 37 <sup>c</sup>	ND	1105 $\pm$ 75 <sup>b</sup>
Stroma	ND	ND	1623 $\pm$ 175 <sup>a</sup>
Corpus luteum	ND	ND	9800 $\pm$ 257 <sup>c</sup>

Values are mean  $\pm$  s.e.m. for n = 5. Mean values with different superscripts in the same column are significantly different ( P < 0.01 ). ND , not detectable.

probably the major site of ovarian inhibin production but this production decreases as the granulosa cells undergo luteinization.

#### Effect of exogenous steroids on inhibin production and steroidogenesis by granulosa cells

The results presented elsewhere (6,15,16) demonstrate that androstenedione at 5  $\mu\text{g/ml}$  and testosterone at 1 and 5  $\mu\text{g/ml}$  significantly stimulated granulosa cell inhibin production. In contrast, progesterone at 1 and 5  $\mu\text{g/ml}$  significantly inhibited inhibin production. Oestrogens had no significant effect on inhibin production (6).

In a separate experiment, the effects of the same doses of an aromatizable and non-aromatizable androgen (testosterone and 5  $\alpha$ -dihydrotestosterone (DHT) respectively) on inhibin production by granulosa cells were determined. Both androgens induced a significant and dose-related increase in inhibin production.

The synthetic androgens, methylestrenolone and mesterolone were shown to mimic the effects of testosterone in stimulating inhibin production by granulosa cells whereas cyproterone acetate, a potent competitor for the androgen receptor, neutralizes the effect of testosterone when added in a 10 fold excess. The inhibition of androgen stimulated inhibin production is not due to the progestagenic actions of the antiandrogen since cyproterone acetate as such does not modify inhibin production. Therefore, one might propose that inhibition of androgen stimulation of inhibin production results from inhibiting the association of androgen with cytosolic receptors and from preventing the nuclear retention of androgen-receptor complexes (17,18).

#### Effect of follicular atresia on inhibin production by bovine granulosa cells in vitro

The studies reported above have demonstrated that inhibin is present in follicular fluid, that it is produced predominantly by granulosa cells, and that its production by granulosa cells can be stimulated by androgens. However, little information is available on how follicular inhibin production is influenced by the age and health of follicles. Granulosa cells were collected from healthy and atretic follicles classified according to morphological criteria (19,20).

As shown in figure 3, no significant difference was observed in inhibin production in vitro between granulosa cells from small ( $\leq 5\text{mm}$  diameter) healthy or atretic follicles. Moreover, inhibin production by granulosa cells from large atretic follicles did not differ from that of cells from small follicles. However, granulosa cells from large

healthy follicles produced significantly more inhibin than cells from small follicles (21).

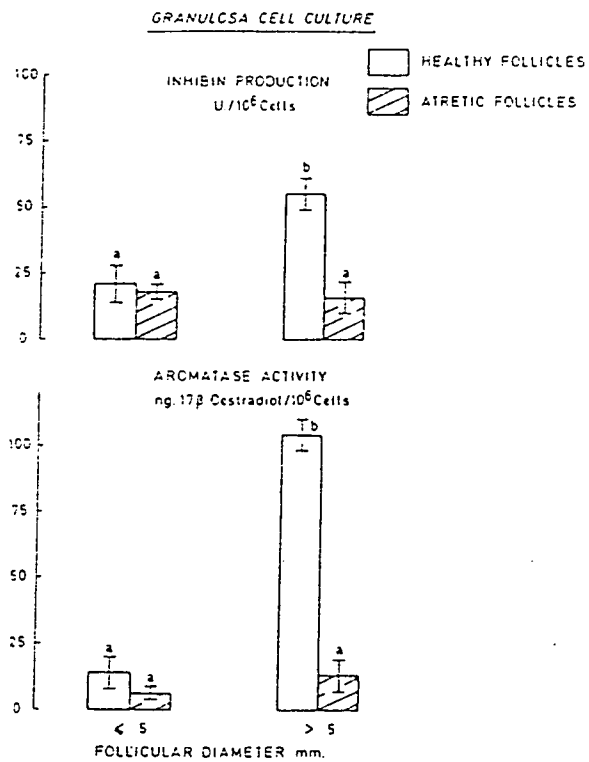


Figure 3 : In vitro inhibin production/10<sup>6</sup> cells according to follicular diameter and health or atresia of the follicles from which the granulosa cells were obtained.

The capacity of granulosa cells from small and large healthy or atretic follicles to enzymatically convert androgens to oestradiol  $17\beta$  (aromatase activity) followed a similar pattern to that of inhibin. Indeed, there was a significant relationship between the capacity of granulosa cells to produce inhibin and synthesize oestradiol  $17\beta$  (table II). Thus, as healthy follicles increase in age, the granulosa cells acquire an increased capacity to both produce inhibin and synthesize oestradiol  $17\beta$ .

Table II

		AROMATASE ACTIVITY		Culture Number
		<10 ng $17\beta$ E <sub>2</sub> /10 <sup>6</sup> cells	≥10 ng $17\beta$ E <sub>2</sub> /10 <sup>6</sup> cells	
INHIBIN PRODUCTION	< 50 U/10 <sup>6</sup> cells	28	1	29
	> 50 U/10 <sup>6</sup> cells	8	16	24
				TOTAL : 53

Contingency table analysis :  $P < 0.001$

Effects of gonadotrophins and prolactin on granulosa cell inhibin production

While steroids have been shown to influence inhibin production by granulosa cells (see above) the effects of gonadotrophins have been less well studied. Figure 4 shows that ovine FSH has a slight but significant stimulatory effect on inhibin production by granulosa cells taken from healthy follicles (4-11 mm diameter) (21). In contrast, FSH had no effect on inhibin production by granulosa cells from atretic follicles of the same age range. Neither LH or HCG nor prolactin was found to influence directly granulosa cell inhibin production (16). However, LH may influence granulosa cell inhibin production indirectly, through its capacity to stimulate thecal androgen production, the androgen in turn directly stimulating granulosa cell inhibin production.

EFFECT OF FSH ON INHIBIN PRODUCTION: COMPARISON OF GRANULOSA CELLS FROM HEALTHY AND ATRETIC FOLLICLES

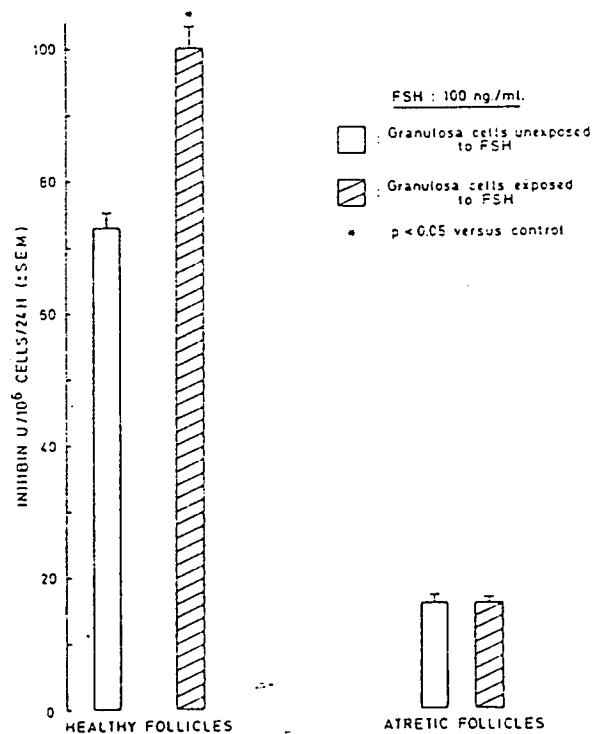


Figure 4

### PROPOSAL

Thus, one might propose that the following scheme of events may occur during preovulatory follicular development. Following luteolysis, the pituitary is released from the negative feedback effects of the steroids and plasma concentrations of FSH and LH increase. This promotes the growth and maturation of the follicle (s) which will ultimately ovulate and then respond with an increased production of oestradiol  $17\beta$ . Follicular inhibin production will also be stimulated in these maturing healthy follicles as discussed above. While plasma LH and oestradiol concentrations continue to rise throughout the follicular phase, plasma FSH concentrations fall during the mid to late follicular phase due to the suppressive actions on the pituitary of the increased amounts of oestradiol and inhibin being secreted by the follicle. The falling plasma concentrations of FSH prevent any additional follicles from being stimulated to mature and thereby limits the number of follicles which can attain ovulatory maturity.

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**Paper no.:** 35

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**Title:** Ovarian inhibin: a hormone with potential to increase ovulation rate in sheep.

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## Ovarian inhibin: a hormone with potential to increase ovulation rate in sheep

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### ABSTRACT

There is increasing evidence that a non-steroidal compound, inhibin, produced by the ovary is involved in the regulation of follicle stimulating hormone (FSH) production by the pituitary. Our studies have shown that ovarian inhibin is present in follicular fluid, that it is synthesised exclusively by follicular granulosa cells, that androgens regulate its synthesis and that it is predominantly a product of healthy follicles, i.e., follicles with the potential to attain ovulatory maturity. It is suggested that androgens produced by luteinising hormone action on follicular thecal tissue stimulate granulosa cell inhibin production which then suppresses pituitary FSH secretion thereby limiting the number of follicles that can be stimulated to develop to ovulatory maturity. Ovulation rates of Romney ewes actively immunised with a semi-purified preparation of inhibin derived from bovine follicular fluid were significantly higher than those of control ewes ( $2.06 \pm 0.16$  v  $1.31 \pm 0.06$  ovulations/ewe).

**Keywords** Inhibin; granulosa cells; theca; androgens; sheep; cow; ovulation rate

Adequate exposure to the pituitary gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH) is necessary for a follicle to develop and mature sufficiently so that it can ultimately ovulate and release an egg capable of fertilisation. Together LH and FSH act on the follicle to stimulate oestradiol-17 $\beta$  production, and oestradiol-17 $\beta$  with FSH promotes the development of the follicle by stimulating processes including antrum development, cell proliferation, receptor and enzyme induction and oocyte maturation. In the absence of FSH, follicles never attain ovulatory maturity but degenerate (i.e., undergo atresia). Conversely, increased exposure to FSH reduces the incidence of follicular atresia thereby allowing more follicles to attain ovulatory maturity. FSH and gonadotrophins with FSH-like activity, e.g., PMSG can be used to increase ovulation rate, albeit unpredictably, in sheep and cattle. Thus, pharmacological manipulation of pituitary FSH secretion provides a potential means of regulating follicular atresia to produce an ovulation rate compatible with a desired prolificacy.

### Regulation of Ovarian Inhibin Production

While feedback effects of ovarian steroids is the generally accepted means by which pituitary FSH secretion is regulated, there is increasing evidence that the ovary also produces a non-steroidal compound, named inhibin which regulates FSH secretion (Franchimont *et al.*, 1981; Channing *et al.*, 1982). Inhibin is defined

as a non-steroidal compound of gonadal origin which specifically or selectively inhibits pituitary secretion of FSH. Its activity is normally measured by an appropriate bioassay, e.g., suppression of FSH production by cultured rat anterior pituitary cells (Henderson and Franchimont, 1981). Inhibin has not yet been fully characterised. However, data from several laboratories indicate that inhibin is likely to be an acidic protein (isoelectric point  $\sim$  pH = 5) with a molecular weight  $> 10\ 000$  and it may contain a carbohydrate moiety (Grady *et al.*, 1982). Inhibin activity has been found in the follicular fluid of all primate (monkey and human) and non-primate (cow, sheep, pig, mare) species thus far examined. Studies with bovine ovarian tissues indicate that follicular granulosa cells are the source of ovarian inhibin. Inhibin is secreted *in vitro* by cultured granulosa cells but not by cultured thecal tissue, ovarian stroma nor corpus luteum tissue (Henderson and Franchimont, 1983). In addition, there is a significant positive correlation between inhibin concentrations in bovine follicular fluid and the number of granulosa cells per follicle (Henderson *et al.*, 1984). Inhibin has also been shown to be produced by sheep (Henderson, Franchimont and McNatty, unpublished), pig and monkey (Channing *et al.*, 1982) granulosa cells.

Neither LH, FSH nor prolactin have any effect on the capacity of granulosa cells to produce inhibin *in vitro*, suggesting that granulosa cell inhibin production is not directly regulated by gonadotrophin action on that cell type (Henderson and Franchimont, 1981).

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Androgens do, however, influence granulosa cell inhibin production; androstenedione, testosterone,  $5\alpha$ -dihydrotestosterone and the synthetic androgens mesterolone and methylestrenolone each stimulating inhibin production by granulosa cells in a dose dependent manner (Henderson and Franchimont, 1981; 1983). In contrast to androgens, oestradiol and oestrone have no effect on granulosa cell inhibin production. Theca interna cells, which lie adjacent to granulosa cells in the follicle are the major source of follicular androgens and LH stimulates thecal androgen production (McNatty *et al.*, 1984). Thus, one can envisage an interaction between granulosa cells and theca interna whereby LH acting on the follicle stimulates thecal androgen production and this androgen, in turn, stimulates granulosa cell inhibin production.

The majority of follicles in an ovary never ovulate, but undergo atresia. A study was performed in our laboratory to determine if inhibin was predominantly a product of the relatively few healthy follicles with the potential to attain ovulatory maturity or a product of atretic follicles. It was found that granulosa cells from healthy bovine follicles produced significantly more inhibin than cells from atretic follicles ( $49 \pm 7$  v  $16 \pm 4$  U inhibin/ $10^6$  cells,  $n=21$ ). In addition, the mean concentration of inhibin in bovine follicular fluid from non-atretic follicles was also significantly higher than that in fluid of atretic follicles ( $11 \pm 1$  v  $6 \pm 1$  U/ml follicular fluid,  $n=21$ ) (Henderson *et al.*, 1984).

#### Inhibin and the Regulation of Pituitary FSH Production.

Taking these findings together and using the sheep as a model, one can propose that during the follicular phase the following scheme of events might occur, as outlined schematically in Fig. 1. Following the initiation of luteal regression, plasma progesterone concentrations start to fall. This releases the pituitary from the negative feedback effects of progesterone and there is a rise in the peak frequency and amplitude of LH, causing an increase in the plasma concentration of LH. This together with elevated plasma FSH concentrations promotes the growth and maturation of the follicle(s) which will ultimately ovulate, which respond with an increased production of oestradiol. Follicular inhibin production would also be stimulated by androgens, produced by LH action on the theca interna, stimulating granulosa cell inhibin production. While plasma LH and oestradiol concentrations continue to rise throughout the follicular phase, plasma FSH concentrations fall during the mid-follicular phase (Baird *et al.*, 1981) due to the suppressive actions of oestradiol and inhibin on the pituitary. The falling plasma concentrations of FSH prevent any additional follicles being stimulated to mature and thereby limit the number of follicles that can attain ovulatory maturity. Inhibin concentrations in both peripheral and ovarian

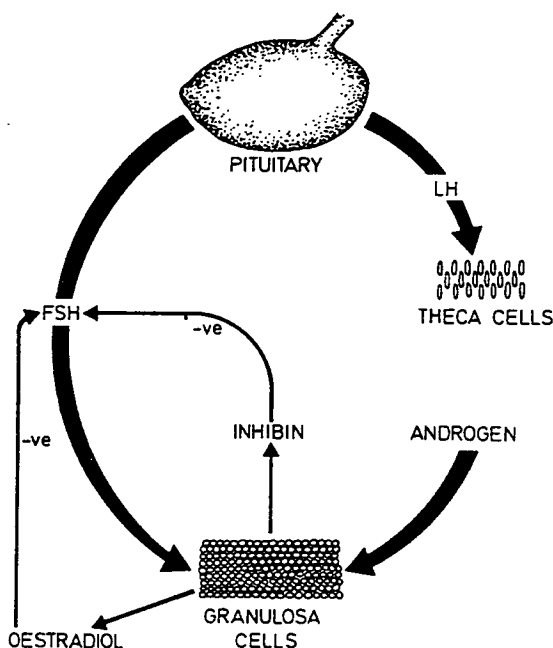


FIG. 1 Relationship between pituitary gonadotrophin secretion and ovarian inhibin production.

vein blood are too low to be measured by current bioassays for inhibin. Thus, one cannot determine if there is indeed an inverse relationship between blood concentrations of FSH and inhibin during the follicular phase. However, if inhibin is physiologically important in the regulation of FSH secretion in sheep, then active immunisation against inhibin might neutralise any circulating inhibin and thereby reduce the suppression of FSH occurring during the follicular phase, so allowing additional follicles to attain ovulatory maturity. To test this hypothesis, inhibin was partially purified from bovine follicular fluid by chromatographic methodology (Franchimont *et al.*, 1983). Adult parous Romney ewes on day 10 of an oestrous cycle were actively immunised with this preparation emulsified in Freund's complete adjuvant (1 ml emulsion containing 1 mg protein was injected subcutaneously into the gracular or axillary region). Control ewes were immunised with Freund's complete adjuvant alone. Each ewe was immunised twice at 30-day intervals. The number of ovulations occurring in each of 4 successive oestrous cycles immediately following the first immunisation was determined by laparoscope examination of the ovaries of each ewe, 9 to 12 days after each display of oestrous behaviour, and counting the number of corpora lutea present. The number of sheep on which the effects of active immunisation against inhibin could be studied was restricted to 4 because of the limited amount of inhibin preparation available. Nevertheless, despite this limitation, a significant effect

**TABLE 1** Ovulation rates in 4 successive oestrous cycles of 4 control and 4 inhibin immunised Romney ewes.

	Sheep No.	No. of ovulations oestrous cycle				Mean No. of ovulations/ewe	Mean ovulation rate $\pm$ s.e.m.
		1	2	3	4		
Control	934	1	1	2	1	1.25	1.31 $\pm$ 0.06
	962	1	1	1	2	1.25	
	738	2	1	1	1	1.25	
	662	1	2	1	2	1.50	
Inhibin immunised	782	2	2	2	2	2.00	2.06 $\pm$ 0.16
	701	3	2	3	2	2.50	
	716	1	2	2	2	1.75	
	651	2	2	2	2	2.00	

on ovulation rate was observed (Table 1), the inhibin immunised ewes having a higher mean ovulation rate compared to the controls ( $2.06 \pm 0.16$  v  $1.31 \pm 0.06$  ovulations/ewe, mean  $\pm$  s.e.m. for  $n=4$ ,  $P < 0.05$ ; Wilcoxon rank sum test). This finding not only provides further support for the notion that inhibin is physiologically important in the regulation of ovarian function in the ewe but suggests that immunisation with inhibin to increase ovulation rate might have some potential as a means of increasing fecundity.

It is of interest to note that immunisation with androgens increases ovulation rate and fecundity (cf. fecundity drugs Multi-Lamb and Fecundin). Androgens stimulate follicular inhibin production (Henderson and Franchimont, 1981; 1983) and immunisation against inhibin increases ovulation rate. Thus it is conceivable that the effects of the fecundity drugs are mediated by causing changes in follicular inhibin production. Immunisation against androgen may reduce circulating androgen concentrations thereby reducing follicular inhibin production which in turn reduces the suppression of pituitary FSH secretion and so allows more follicles to be stimulated to develop to ovulatory maturity.

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**Section 4: Papers related to studies of the Booroola**

**Paper no.:** 36

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**Title:** Ovarian activity in Booroola x Romney ewes which  
have a major gene influencing their ovulation rate.

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120

## Ovarian activity in Booroola × Romney ewes which have a major gene influencing their ovulation rate

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**Summary.** A marked difference in both the function and composition of individual ovarian follicles was noted in Booroola × Romney ewes (6–7 years of age) which had previously been segregated on at least one ovulation rate record of 3–4 (F+ ewes, N = 21) or < 3 (++) ewes, N = 21).

Follicles in F+ ewes produced oestradiol and reached maturity at a smaller diameter than in ++ ewes. In F+ ewes (N = 3), the presumptive preovulatory follicles were  $4.4 \pm 0.5$  (s.e.m.) mm in diameter and contained  $2.1 \pm 0.3 \times 10^6$  (s.e.m.) granulosa cells, whereas in ++ ewes (N = 3), such follicles were  $7.3 \pm 0.3$  mm in diameter and contained  $6.5 \pm 0.8 \times 10^6$  cells. During a prostaglandin (PG)-induced follicular phase, the secretion rate of oestradiol from ovaries containing 3 presumptive preovulatory follicles in F+ ewes was similar to that from ovaries with only one such follicle in ++ ewes.

We suggest that the putative 'gene effect' in F+ ewes is manifested during early follicular development and that it may be mediated via an enhanced sensitivity of granulosa cells to pituitary hormones. As a consequence, the development of 3 preovulatory follicles in F+ ewes may be necessary to provide a cell mass capable of producing the same quantity of oestradiol as that from one preovulatory follicle in ++ ewes.

### Introduction

There is evidence to suggest that high fecundity Booroola ewes contain a major gene which influences their ovulation rate (Davis, Montgomery, Allison, Kelly & Bray, 1982; Piper & Bindon, 1982). Homozygous (FF), heterozygous (F+) and non-carriers of the putative gene have tentatively been segregated on the basis of at least one ovulation rate recording of  $\geq 5$ , 3 or 4, and 1 or 2 respectively (Davis *et al.*, 1982).

The endocrine basis for the high ovulation rate in Booroola ewes has recently been investigated by several groups (see Bindon & Piper, 1982, for review). It has been proposed on the basis of differences in pituitary FSH content between Booroola Merino and non-Booroola Merino controls that increased FSH secretion may contribute to the increased ovulation rate in Booroola ewes (Robertson, Ellis, Foulds, Findlay & Bindon, 1984). Support for this notion has been provided by the studies of Cummins, O'Shea, Bindon, Lee & Findlay (1983), who showed that the inhibin content of ovaries of Booroola ewes was about one-third of that in non-Booroola Merinos. However, it is also possible that the different ovulation rates of FF, F+ and ++ Booroolas are due to differences in the sensitivity of ovarian follicles to gonadotrophins rather than to absolute differences in the circulating concentrations of hormone although the two mechanisms may not be

mutually exclusive. For example, Piper, Bindon, Curtis, Cheers & Nethery (1982) and Kelly, Owens, Crosbie, McNatty & Hudson (1983) have shown that F+ Booroola ewes contain ovarian follicles that are more sensitive to PMSG than those from ++ Booroola ewes.

Further insight into the mechanisms regulating the ovulation rate in FF, F+ and ++ Booroola ewes might result from examination of follicular and luteal activity and steroid biosynthesis. The aim of this study was to investigate some of these aspects in F+ and ++ Booroola × Romney ewes.

### Materials and Methods

*Animals and procedures.* Twenty-one Booroola × Romney ewes (6–7 years of age) with at least one ovulation rate record of  $\geq 3$  but  $< 5$  were classified as F+. A further 21 Booroola × Romney ewes of similar age with 4–5 previous annual recordings of ovulation and lambing rates  $< 3$  were classified as ++.

All the animals were injected with a prostaglandin (PG) derivative (cloprostenol, 125  $\mu\text{g}$  s.c.; ICI-Tasman Vaccine Laboratories, New Zealand) on Day 10 of the oestrous cycle (oestrus = Day 0) to induce luteolysis. At intervals after PG administration (0, 3, 6, 12, 24, 36 and 48 h) ovarian (10–20 ml from both left and right ovarian veins) and peripheral (50 ml) venous blood was collected from 3 ++ and 3 F+ ewes anaesthetized with thiopentone sodium (Intraval; May and Baker, Wellington, New Zealand). At 0 h blood samples were collected immediately after PG injection. The purpose of treating the ewes with PG was to study follicular growth and steroidogenesis at precisely determined stages of corpus luteum (CL) regression (McNatty *et al.*, 1982). During ovarian venous blood collection the rate of blood flow was also measured (McNatty, Dobson, Gibb, Kieboom & Thurley, 1981b). Immediately after the blood had been collected, the ovaries of each animal were removed and all follicles  $\geq 1$  mm in diameter and CL were isolated as previously described (McNatty *et al.*, 1982, 1984a).

*Blood samples.* All blood samples were centrifuged at 4000  $g$  at 4–6°C for 20 min within 30 min of collection, and the plasma samples stored at –20°C until assayed.

*Follicle classification.* To assess the state of atresia of the various follicles, the following factors were considered; the presence or absence of thecal blood capillaries when the intact (but cleanly dissected) follicles were observed at  $\times 10$  magnification; the presence or absence of debris in follicular fluid; the presence and status of the oocyte (healthy, degenerate or absent); the total number of granulosa cells expressed as a percentage of the maximum number of cells observed in a follicle of that size; and the colour (or appearance) of the theca interna (red, pink or white) (McNatty *et al.*, 1984a, b). In F+ ewes, the maximum number of granulosa cells recorded in 1, 2, 3, 4 and 5 mm diameter follicles was  $1 \times 10^6$ ,  $2 \times 10^6$ ,  $2.6 \times 10^6$ ,  $3.3 \times 10^6$  and  $3.3 \times 10^6$  respectively. In ++ ewes, the maximum number in 1, 2, 3, 4, 5, 6, 7 and 8 mm follicles was  $1.1 \times 10^6$ ,  $2.3 \times 10^6$ ,  $3.0 \times 10^6$ ,  $4.2 \times 10^6$ ,  $5.0 \times 10^6$ ,  $7.0 \times 10^6$ ,  $9.3 \times 10^6$  and  $6.5 \times 10^6$  respectively. Oocytes were said to be degenerating if they were free of a cumulus cell matrix or if they showed signs of cytolysis, necrosis or loss of spherical shape.

When the above variables for each genotype were subjected to hierarchical cluster analysis (Genstat, 1981), they separated into four identical clusters termed Grades 1, 2a, 2b and 3 with each having a similarity coefficient of 0.85. Grade 1 contained follicles with a vascularized theca interna which was red, pink or white, no debris in the follicular fluid,  $> 25\%$  of the maximum number of granulosa cells for a given follicle size and a healthy-looking oocyte. Grade 2a contained follicles with a vascularized red, pink or pale theca with debris in the follicular fluid,  $< 75\%$  of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. Grade 2b contained follicles with an avascular white theca interna, no debris in the follicular fluid,  $< 75\%$  of the maximum number of granulosa cells for a given follicle size, and a

healthy or degenerate oocyte or no oocyte. Grade 3 contained follicles with a white avascular theca interna, debris in the follicular fluid, <50% of the maximum number of granulosa cells for a given follicle size, and a healthy or degenerate looking oocyte or no oocyte. For the purpose of this communication, the data from the Grade 1 follicles are considered to be from non-atretic follicles (McNatty *et al.*, 1984b), whereas the pooled data from the Grade 2a, 2b and 3 follicles are referred to as those from atretic follicles (McNatty *et al.*, 1984b).

*The granulosa cell aromatase assay.* The assay was identical to that described by McNatty *et al.* (1984a). Briefly the washed cells ( $60\text{--}600 \times 10^3$ ) in 0.5 ml Medium 199 containing sodium bicarbonate (0.85 g/l), Earle's salts, L-glutamine (0.10 g/l), Hepes buffer (20 mM) and 1% BSA (w/v) (Medium A) were incubated with 0.5 ml of a solution of 2000 ng testosterone/ml Medium A, for 3 h at 37°C in a shaking water bath. Aromatase activity was assessed after measuring the oestradiol content of the supernatant after the cells plus medium were centrifuged and separated at the end of the incubation. Under these conditions the rate of oestradiol formation was linear for the first 3 h for granulosa cells from all atretic follicles and most non-atretic follicles. But in some (~30%) non-atretic follicles  $\geq 3$  mm diameter, the rate of metabolism was not linear after 2 h so that the results in some instances underestimate extant aromatase activity by up to 20%.

Whenever possible aromatase assays were performed on individual follicles. However, on most occasions, cells from atretic follicles of similar size were pooled.

*Theca interna perfusions.* To determine the ability of theca interna to secrete androstenedione, samples of theca interna were perfused *in vitro* as described for bovine tissue (McNatty *et al.*, 1984c). Briefly, 10–25 mg theca interna was placed into glass columns ( $4 \times 0.7$  cm; Econo-column, Bio-Rad Laboratories, Ca., U.S.A.) containing 50 mg Sephadex G-25 and prewashed for 1 h with a perfusion medium consisting of sterile Krebs–Ringer–bicarbonate buffer containing 1% BSA (w/v) which was gassed continuously with 50% O<sub>2</sub>, 45% N<sub>2</sub> and 5% CO<sub>2</sub>. An aliquant of the theca was homogenized in 1 ml ethanol to determine its endogenous steroid content. Also, a small amount of theca was fixed for histological examination to examine the purity of the tissue before perfusion. The theca on the column was perfused at 37°C for 3 h at a flow rate of 1.4 ml/min with 12-min fractions being collected. LH (NIH-LH-S23; 200 ng/ml) was introduced into the perfusion medium for 20 min after the tissue had been perfused for 1 h. At the end of the 3-h period, the tissue was fixed for histological examination and the medium from each tube stored at –20°C until assayed for androstenedione by a specific radioimmunoassay (McNatty *et al.*, 1984a). The androstenedione output from theca was expressed as the cumulative output (ng) per 10 mg theca interna over the 3-h period during and after LH stimulation.

Although it is known that the androstenedione output from ovine theca interna perfused at 1.4 ml/min is much greater than that from theca in static cultures (i.e. 10–20 times) or after perfusion at 0.1, 0.2 or 0.8 ml/min (2–10 times), the flow rate for optimal steroidogenesis has not been determined because of insufficient quantities of theca interna. The optimum flow rate of 1.4 ml/min for bovine theca interna (McNatty *et al.*, 1984c) was therefore adopted for the present study.

*Hormone assays.* All steroids were measured using previously published RIA procedures (McNatty, Gibb, Dobson, Thurley & Findlay, 1981a; McNatty *et al.*, 1982). Androstenedione, testosterone and oestradiol-17 $\beta$  were extracted from peripheral blood (10 ml) or ovarian venous blood (1 ml) with diethyl ether ( $2 \times 5$  vols) and then separated by the Lipidex 5000 or Sephadex LH-20 column chromatographic methods outlined elsewhere (McNatty *et al.*, 1981a). The ovarian steroid secretion rates were calculated from a knowledge of haematocrit, blood flow and steroid concentration (McNatty *et al.*, 1981b). Progesterone in plasma was measured in 1 ml peripheral plasma (McNatty *et al.*, 1982). In follicular fluid, progesterone, androstenedione and oestradiol were measured directly in diluted (10–100-fold with 0.1 M-phosphate-buffered saline, PBS, pH 7.2) aliquants of follicular fluid. Testosterone was measured in follicular fluid after it had been diluted (10–200-fold) with PBS and extracted twice with 5 volumes of diethyl ether. Androstenedione in



Krebs-Ringer solution (from the thecal columns) and oestradiol in the aromatase assay solutions were measured directly without extraction.

Details of the working solutions and specifications of the progesterone (WA-26), androstenedione (WA-965), testosterone (WA-36) and oestradiol-17 $\beta$  (WA-27) antisera are provided elsewhere (McNatty *et al.*, 1984a).

The detection limit of steroids in follicular fluid was 1 ng/ml. The detection limits of progesterone, androstenedione, testosterone and oestradiol in plasma were 200, 2, 1 and 1 pg/ml respectively. The intra- and inter-assay coefficients of variation for all the above steroid assays were <8% and <12% respectively.

*Statistical procedures.* The data on numbers of follicles or granulosa cells, hormones in follicular fluid or plasma, aromatase activity in granulosa cells and androstenedione output from thecal tissues were first normalized by log-transformation and the values averaged, when appropriate, for each animal. Thereafter the data were subjected to analysis of variance and/or unpaired Student's *t* test; the latter was used when comparing the overall means between genotypes. The effects of PG treatment on the above parameters were subjected to contingency table analysis.

## Results

### Ovarian follicular activity in ++ and F+ ewes

There was no significant effect of time after PG treatment (i.e. 0-6 h, 12-24 h, 36-48 h) on the mean proportion of non-atretic follicles ( $\geq 1$  mm diameter) in ovaries of F+ or ++ ewes ( $P > 0.25$ ; contingency table analysis).

There was no significant effect of genotype on the total number of follicles  $\geq 1$  mm diameter (i.e. irrespective of time after PG treatment). The mean  $\pm$  s.e.m. number of follicles ( $\geq 1$  mm diameter) per ewe in F+ (21 animals) and ++ (21) ewes was  $40 \pm 3$  and  $44 \pm 3$  respectively. Moreover, the mean number of non-atretic follicles ( $\geq 1$  mm diam.) per ewe for each genotype was also similar (21 F+ ewes,  $15.7 \pm 1.0$  follicles; 21 ++ ewes,  $16.3 \pm 0.9$  follicles). The numbers of non-atretic follicles for each genotype with respect to follicular diameter are summarized in Table 1. There were significantly more ( $P < 0.01$ ) large and significantly fewer ( $P < 0.05$ ) intermediate follicles in ++ ewes than in F+ ewes.

**Table 1.** Number of non-atretic follicles and concentrations of progesterone, testosterone and oestradiol in follicular fluid of non-atretic follicles with respect to genotype (F+ or ++) and follicular diameter

Follicular diam. (mm)	Genotype	No. of non-atretic follicles/ewe†	Steroid concentrations in follicular fluid* (ng/ml)		
			Progesterone	Testosterone	Oestradiol
$\leq 2.5$	F+	$12.3 \pm 1.2$	27 (17-44)	53 (35-78)	16 (12-23) <sup>b</sup>
	++	$13.0 \pm 1.1$	23 (15-36)	66 (49-88)	5 (4-5) <sup>b</sup>
3-4.5	F+	$3.1 \pm 0.3^a$	5 (3-7)	18 (12-26) <sup>a</sup>	43 (26-72) <sup>b</sup>
	++	$2.2 \pm 0.4^a$	8 (4-14)	31 (23-43) <sup>a</sup>	7 (5-12) <sup>b</sup>
$\geq 5$	F+	$0.3 \pm 0.1^b$	4 (2-7)	15 (10-23)	56 (25-265)
	++	$1.1 \pm 0.1^b$	4 (2-7)	15 (9-23)	70 (47-106)

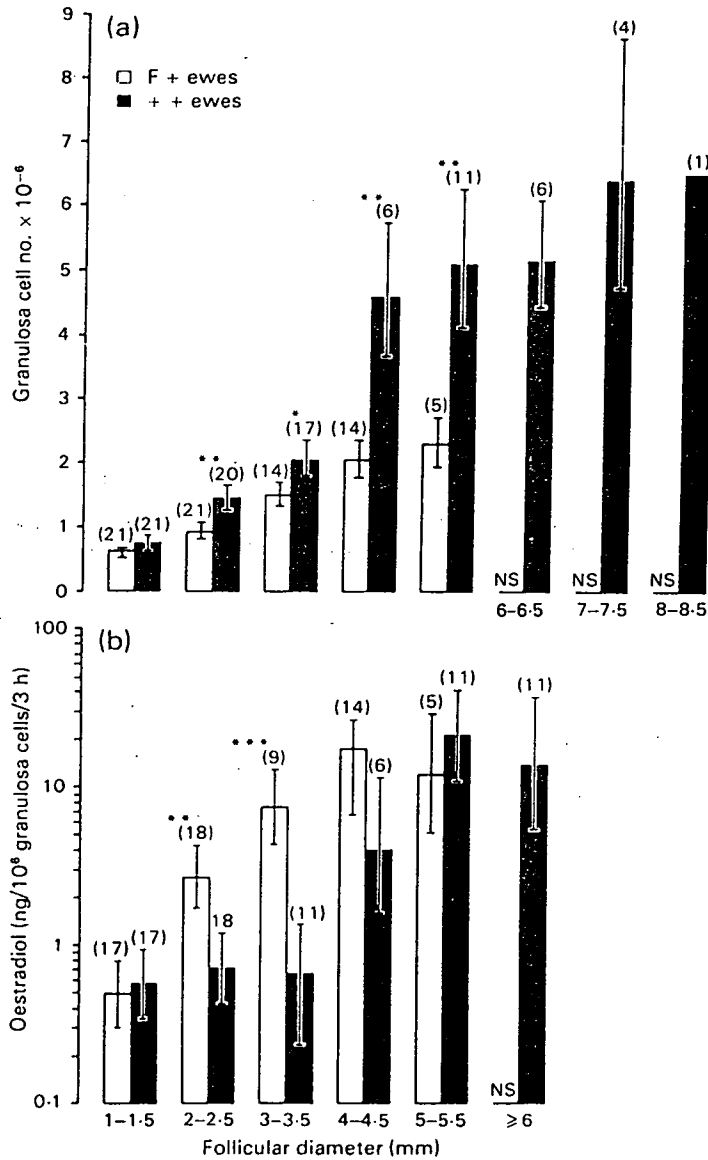
† Values are means  $\pm$  s.e.m.

\* Values are geometric means (and 95% confidence limits). Steroid data from 18-96 follicles were averaged for each ewe with respect to follicle diameter with the overall means from each genotype compared by Student's *t* test on log-transformed data ( $N = 21$  for both F+ and ++ ewes). The data on follicle numbers were also obtained from the same 21 F+ and 21 ++ ewes.

For each follicular diameter, the results in each column sharing a common superscript are significantly different from one another: <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  (Student's *t* test on log-transformed data).

Effect of genotype on the number of granulosa cells

For ewes of each genotype, the numbers of granulosa cells in non-atretic follicles with respect to follicular diameter are summarized in Text-fig. 1(a). At all diameters from 2 to 5 mm there were significantly more cells in follicles of ++ ewes than in F+ ewes. There were no non-atretic follicles ≥ 6 mm in diameter in F+ ewes.



Text-fig. 1. The number of granulosa cells (a) and levels of aromatase activity in granulosa cells (b) from different-sized non-atretic follicles of Booroola × Romney ewes previously segregated as heterozygous (F+) carriers or non-carriers (++) of a major gene influencing ovulation rate. NS = no samples. Results are geometric means with vertical bars representing 95% confidence limits. Numbers in parentheses refer to number of ewes from which granulosa cells were studied. A total of 21 F+ and 21 ++ ewes were investigated. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

*Effect of genotype on follicular fluid concentrations of steroid*

For ewes of each genotype, there was no significant relationship between the proportions of non-atretic follicles with high and low concentrations of progesterone (high,  $\geq 20$  ng/ml; low  $< 20$  ng/ml), or testosterone (high,  $\geq 25$  ng/ml; low  $< 25$  ng/ml) or oestradiol (high,  $\geq 50$  ng/ml; low,  $< 50$  ng/ml) in follicular fluid and time (i.e. 0–6 h, 12–24 h, 36–48 h) after PG treatment ( $P > 0.05$  for all steroids; contingency table analysis).

*Progesterone and testosterone*

There were no significant differences between non-atretic and atretic follicles in the follicular concentration of progesterone or testosterone in the ewes of either genotype. Moreover, for non-atretic or atretic follicles there was no significant effect of genotype on the progesterone concentrations in small, medium or large follicles (see Table 1 for data on non-atretic follicles). For testosterone, however, the concentrations in medium-sized non-atretic follicles of F+ ewes were significantly lower ( $P < 0.05$ ) than in non-atretic follicles of ++ ewes (Table 1).

Irrespective of genotype the concentrations of progesterone and testosterone were both 2–4-fold higher in small follicles than in medium and large follicles. Moreover, the concentrations of testosterone were 2–3.8-fold higher than those of progesterone over all size ranges.

*Oestradiol-17 $\beta$* 

The concentrations of oestradiol in non-atretic follicles of ++ and F+ ewes with respect to follicle size are shown in Table 1. In small and medium-sized follicles, the oestradiol concentrations in follicles of F+ ewes were significantly higher (both  $P < 0.01$ ) than in follicles of ++ ewes. In small follicles, the testosterone concentrations exceeded those of oestradiol for both genotypes, but in intermediate sized follicles, the oestradiol concentrations in F+ but not ++ follicles exceeded those of testosterone. In ++ follicles, the oestradiol concentrations only exceeded those of testosterone in large follicles.

Irrespective of follicular diameter, the concentration (geometric means and 95% confidence limits) of oestradiol in atretic follicles from F+ ewes ( $N = 21$ ) and ++ ewes ( $N = 21$ ) were 5 (5–7) and 5 (4–6) ng/ml respectively.

*Effect of genotype on aromatase activity in granulosa cells*

For F+ ewes, there was no significant relationship in non-atretic follicles between the frequency of follicles with granulosa cells having low, medium or high levels of aromatase activity *in vitro* (i.e.  $< 1$ , 1–5,  $> 5$  ng oestradiol/ $10^6$  granulosa cells/3 h, respectively) and time (i.e. 0–6 h, 12–24 h, 36–48 h) after PG treatment ( $P > 0.25$ ; contingency table analysis), whereas for ++ ewes, a significant relationship was found ( $P < 0.05$ ; contingency table analysis). In ++ ewes 76% of the non-atretic follicles ( $n = 33$ ) sampled at 0–6 h after PG contained granulosa cells with aromatase activity  $\geq 1$  ng oestradiol/ $10^6$  cells/3 h, whereas at 12–24 ( $n = 27$ ) and 36–48 ( $n = 23$ ) h after PG, 37% and 43% respectively of the follicles had this level of activity. In the F+ ewes 73% of the non-atretic follicles ( $n = 37$ ) at 0–6 h after PG had aromatase activity  $> 1$  ng oestradiol/ $10^6$  cells/3 h whereas at 12–24 ( $n = 24$ ) h and 36–48 ( $n = 26$ ) h after PG 58% and 76% respectively of the follicles had granulosa cells with that level of activity.

There were significant relationships in F+ and ++ ewes between aromatase activity in granulosa cells and the health of the follicle (Table 2). For ewes of both genotypes, none of the granulosa cell populations in atretic follicles had aromatase activity  $> 5$  ng oestradiol/ $10^6$  cells/3 h. For non-atretic or atretic follicles, the proportions with different levels of activity were independent of genotype (F+ ewes,  $P > 0.05$ ; ++ ewes,  $P > 0.025$ ; contingency table analysis; Table 2).

There was a significant effect of genotype on follicle diameter with respect to aromatase activity in granulosa cells from non-atretic follicles (Text-fig. 1b). When follicles had reached 2–2.5 mm in

Table 2. Contingency table showing influence of follicular health on aromatase activity in granulosa cells

Genotype	Follicular health	Aromatase activity (ng oestradiol/10 <sup>6</sup> cells/3 h)		
		<1	1-5	>5
F+	Non-atretic*	26	21	34
	Atretic†	34	11	0
++	Non-atretic*	38	19	21
	Atretic†	48	10	0

\* 43/81 (53.1%) and 43/78 (55.1%) of the respective values from the F+ and ++ non-atretic follicles were from cells pooled from more than one follicle.

† 40/45 (88.9%) and 47/58 (81.0%) of the respective values from the F+ and ++ atretic follicles were from cells pooled from more than one follicle.

There were significant relationships in F+ and ++ ewes between aromatase activity in granulosa cells and the health of the follicle (F+ ewes,  $P < 0.001$ ; ++ ewes,  $P < 0.001$ ).

diameter, aromatase activity was already significantly higher in F+ than in ++ ewes (Text-fig. 1b). In F+ ewes, peak aromatase activity was recorded in granulosa cells from 4-4.5 mm follicles, whereas in ++ ewes it was not reached until the follicles attained a diameter of 5-5.5 mm.

#### Androstenedione output from LH-stimulated thecal tissue from F+ and ++ ewes

Histological examination of the purity of theca interna studied *in vitro* indicated that  $51 \pm 3\%$  (s.e.m.,  $n = 41$ ) of the tissue was theca interna with the major contaminant being theca externa and/or stroma with a residual contamination of red blood cells and granulosa cells. For each 5  $\mu$ m section of theca, the geometric mean number (and 95% confidence limits) of granulosa cell contamination was 2 (0-4) cells ( $n = 41$ ). The androstenedione outputs from the theca preparations were not corrected for tissue purity or androstenedione content at the initiation of the perfusion experiments.

There was no significant effect of time after PG treatment (i.e. 0-6 h, 12-24 h, 36-48 h) on the number of thecal preparations from non-atretic follicles which secreted high ( $\geq 21$  ng) or low ( $< 21$  ng) androstenedione/10 mg tissue/3 h quantities of steroid ( $P > 0.10$ ; contingency table analysis). For each genotype, there was no significant effect of follicular diameter (i.e. 1-3 mm vs  $\geq 3.5$  mm) on LH-stimulated thecal output of androstenedione. The respective androstenedione outputs as geometric means (and 95% confidence limits) from theca of 1-3 mm diameter and  $\geq 3.5$  mm diameter follicles in F+ ewes were 31 (23-41) (N = 13 ewes) and 37 (17-52) ng/10 mg tissue/3 h (N = 14) and in ++ ewes the respective thecal androstenedione outputs were 27 (16-46) (N = 12 ewes) and 40 (26-60) ng/10 mg tissue/3 h (N = 19) (both genotypes;  $P > 0.1$ , Student's *t* test on log-transformed means).

The thecal androstenedione outputs for ewes of each genotype after pooling the data with respect to follicular diameter and time after PG treatment are summarized in Table 3. The outputs of thecal androstenedione from non-atretic follicles for ++ and F+ ewes were at least 2-fold greater than those from atretic follicles ( $P < 0.05$ ), 7-fold greater than from thecal externa tissue ( $P < 0.01$  for F+ and ++ theca) and ~10-fold greater than those present in the tissue at 0 h. However, there was no effect of genotype on the ability of LH-stimulated theca to secrete androstenedione.

**Table 3.** Androstenedione output (ng/10 mg theca, geometric means and 95% confidence limits) from perfused thecal tissue from F+ and ++ ewes

Thecal tissue (genotype)	Follicular status	Time (h)	Androstenedione output at 0 h or over 3 h	No. of ewes
Interna (F+/++ pooled)	Non-atretic	0	3 (2-5) <sup>a,b</sup>	15
Externa (F+/++ pooled)	Non-atretic	3	5 (3-8) <sup>c,d</sup>	7
Interna (F+/++ pooled)	Atretic	3	13 (8-21) <sup>e,f</sup>	15
Interna (F+)	Non-atretic	3	38 (30-48) <sup>a,c,e</sup>	21
Interna (++)	Non-atretic	3	37 (27-50) <sup>b,d,f</sup>	21

Numbers sharing a common superscript are significantly different from one another: <sup>a,b,c,d,e</sup>  $P < 0.01$ ; <sup>f</sup>  $P < 0.05$ .

#### Steroid-secretion rates in F+ and ++ ewes

The secretion rates of androstenedione, testosterone and oestradiol at 0-6 h and 12-48 h after PG treatment were pooled according to whether the steroids originated from ovaries containing a dominant follicle or from the contralateral ovary. A dominant follicle was one containing  $\geq 50$  ng oestradiol/ml. Compared to 0-6 h, the secretion rates from ovaries with a dominant follicle at 12-48 h were significantly higher for oestradiol (F+ ewes,  $P < 0.05$ ; ++ ewes,  $P < 0.05$ ) but there were no significant changes for androgens (Table 4). Irrespective of genotype, the secretion rates of all three steroids from the contralateral ovary at 0-6 h were either not different or significantly higher than those at 12-48 h after PG. For ewes of both genotypes, the mean secretion rates of all steroids at 12-48 h from dominant follicles were higher than those from the contralateral ovary although these were not significant for androstenedione or testosterone in the ++ ewes. When the steroid-secretion rates at 0-6 or 12-48 h from ovaries with a dominant follicle or the contralateral ovary were compared between genotypes no significant differences were noted.

**Table 4.** Steroid secretion rates (geometric means and 95% confidence limits) [no. of ewes]

Steroid	Time after PG (h)	Steroid secretion rates (ng/min)			
		F+ ewes		++ ewes	
		Ovary with a dominant follicle*	Contralateral ovary	Ovary with a dominant follicle	Contralateral ovary
Androstenedione	0-6	5.1 (2.1-12.3) [8]	5.3 <sup>c</sup> (3.9-7.1) [6]	4.0 (2.2-7.1) [7]	3.3 (1.5-7.1) [7]
	12-48	8.1 <sup>a</sup> (5.3-12.1) [12]	1.0 <sup>a,e</sup> (0.5-2.2) [6]	4.9 (2.4-9.8) [12]	1.7 (0.9-3.2) [12]
Testosterone	0-6	0.9 (0.4-1.8) [8]	1.4 (0.6-3.5) [6]	1.2 (0.6-2.2) [7]	1.5 (0.8-2.8) [7]
	12-48	1.8 <sup>b</sup> (1.0-3.3) [12]	0.3 <sup>b</sup> (0.1-0.6) [6]	1.1 (0.6-2.1) [12]	0.5 (0.3-0.9) [12]
Oestradiol	0-6	0.6 <sup>f</sup> (0.4-1.2) [8]	0.4 (0.2-0.8) [6]	0.8 <sup>g</sup> (0.6-1.2) [7]	0.4 (0.2-0.7) [7]
	12-48	2.2 <sup>c,f</sup> (1.4-3.3) [12]	0.4 <sup>c</sup> (0.2-0.7) [6]	2.5 <sup>d,g</sup> (1.5-4.3) [12]	0.3 <sup>d</sup> (0.2-0.5) [12]

\* A dominant follicle was one with  $\geq 50$  ng oestradiol/ml follicular fluid (i.e. an 'oestrogenic' follicle). Follicles in the contralateral ovary were not 'oestrogenic'. Numbers sharing a common superscript are significantly different from one another. <sup>f,g</sup>  $P < 0.05$ ; <sup>a,b,c,d,e</sup>  $P < 0.01$ .

At 12–36 h after PG treatment, F+ and ++ ewes each contained at least one ovary with an ‘oestrogenic’ follicle (i.e.  $\geq 50$  ng oestradiol/ml follicular fluid). At other times after PG treatment some of the ewes had no ‘oestrogenic’ follicles. The F+ ewes contained 2.8-fold more ‘oestrogenic’ follicles than did ++ ewes but the mean follicular diameter in F+ ewes was on average 1.8 mm smaller ( $P < 0.01$ ) and there were  $3.7 \times 10^6$  fewer granulosa cells ( $P < 0.01$ ) respectively than in the ++ ewes. (Table 5). However, the total number of granulosa cells in the ‘oestrogenic’ follicles from F+ and ++ ewes were similar (i.e. F+ ewes,  $55.8 \times 10^6$ ; ++ ewes,  $60.6 \times 10^6$ , calculated from Table 5). Over the period 12–36 h after PG, the oestradiol concentrations in the ‘oestrogenic’ follicles from F+ and ++ ewes were not significantly different. The respective geometric mean (and 95% confidence limits) concentrations were 131 (102–169) and 164 (115–234) ng/ml. Moreover, the oestradiol secretion rates between the two genotypes were also not significantly different (Table 5).

**Table 5.** Number and diameter of ‘oestrogenic’ follicles\* and the number of granulosa cells in F+ and ++ ewes, together with the oestradiol secretion rate from ovaries containing ‘oestrogenic’ follicles at 12–36 h after PG treatment

Ewes	Ovaries with ‘oestrogenic’ follicles	‘Oestrogenic’ follicles			Oestradiol secretion rate (ng/min)	
		No.	Diam. (mean $\pm$ s.e.m., mm)	Granulosa cell no. (mean $\pm$ s.e.m. $\times 10^{-6}$ )		
++	9	10	11	$5.3^a \pm 0.2$	$5.5^b \pm 0.5$	$3.3 \pm 0.9$
F+	9	13	31	$3.5^a \pm 0.2$	$1.8^b \pm 0.2$	$3.4 \pm 0.9$

\* Follicle containing  $\geq 50$  ng oestradiol/ml follicular fluid.

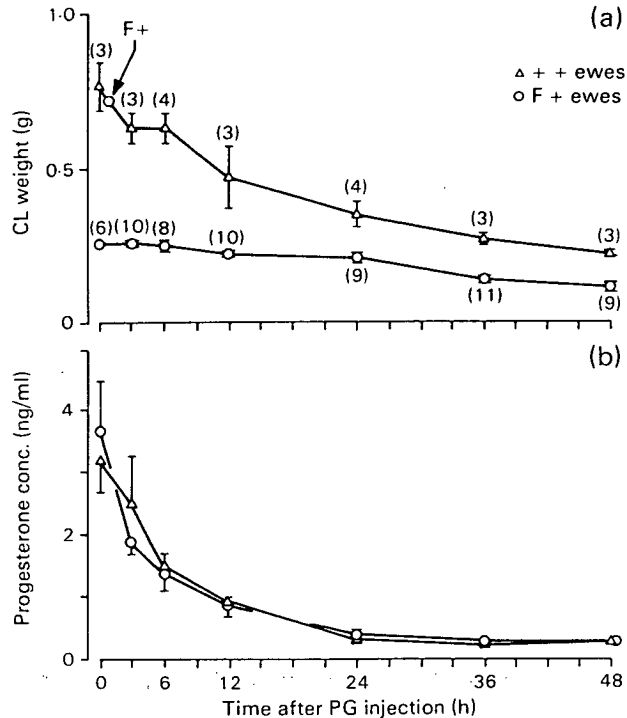
Numbers sharing a common superscript are significantly different from one another:  $P < 0.01$ .

At 48 h after PG treatment, the mean ( $\pm$  s.e.m.) diameter of the presumptive preovulatory follicles in F+ and ++ ewes was  $4.4 \pm 0.5$  mm (3 ewes), and  $7.3 \pm 0.3$  mm (3 ewes) respectively, and the respective mean numbers of granulosa cells in these follicles were  $2.1 \pm 0.2 \times 10^6$  and  $6.5 \pm 0.8 \times 10^6$ .

#### *Corpus luteum (CL) numbers, weight and function in ++ and F+ ewes*

Based on CL number, the ovulation rate in the cycle under study was  $3.3 \pm 0.25$  (s.e.m.) for F+ ewes ( $N = 21$ ) and  $1.10 \pm 0.07$  (s.e.m.) for ++ ewes ( $N = 21$ ). In ++ ewes, there were 19 animals with one CL and 2 with two CL. In F+ ewes, the number of animals with 1, 2, 3, 4, 5 and 6 CL was 1, 3, 9, 5, 2 and 1 respectively. The mean CL weights and plasma progesterone concentrations for F+ and ++ ewes are summarized in Text-fig. 2(a). From 0 to 6 h after PG treatment, there was no significant change in CL weight or diameter (data not shown) for either genotype (++ animals, 10 CL; F+ animals, 24 CL). The mean weights and diameters of CL tissue over this time interval would therefore be representative of CL during the mid-luteal phase (i.e. Day 10) of the oestrous cycle. At this time, the mean ( $\pm$  s.e.m.) CL weights and diameters in ++ ( $N = 9$ ) and F+ ( $N = 8$ ) ewes were  $0.69 \pm 0.04$  g and  $0.25 \pm 0.01$  g respectively ( $P < 0.01$ ; Student’s *t* test) and  $11.3 \pm 0.05$  mm and  $7.8 \pm 0.3$  mm respectively ( $P < 0.01$ ).

After PG-induced luteolysis on Day 10 of the oestrous cycle there were no significant differences at 0, 3, 6, 12, 24 or 48 h in progesterone concentrations in ++ compared to F+ ewes (Text-fig. 2b). In the one F+ animal which had one CL, the CL was atypical of those present in the other F+ ewes at 0 h (see Text-fig 2a); its weight was 0.74 g whereas the range of CL weights ( $n = 6$ ) for the other two F+ ewes was 0.24–0.27 g. The plasma progesterone concentration in this animal was 1.6 ng/ml and this value was the lowest recorded in any of the F+ and ++ ewes at this time.



**Text-fig. 2.** The mean CL weights per genotype (a) and mean plasma progesterone concentrations per genotype (b) at different times after PG injection in ewes previously segregated as being heterozygous carriers (F+) or non-carriers (++) of a major gene influencing the ovulation rate. Vertical bars = s.e.m. (3 animals per point). The numbers in parentheses in (a) refer to the number of CL in the 3 animals at each time. The CL weight from one of the 3 F+ ewes at 0 h was excluded from the group mean because it was 3-fold heavier than that of any other CL from F+ animals; this animal had only one CL which was more typical of a ++ ewe (see arrow).

### Discussion

The major finding of this study was that ovarian follicular development in Booroola  $\times$  Romney ewes with an ovulation rate of  $\geq 3$  (F+ ewes) differed from that in Booroola  $\times$  Romney ewes with an ovulation rate of  $< 3$  (++) ewes). However, the difference between the genotypes was not due to differences in the number of antral follicles ( $\geq 1$  mm diam.) or the number of non-atretic follicles but was the result of the maturation of follicles in the F+ ewes at a smaller diameter. Ovarian follicles in F+ ewes developed an ability to synthesize oestradiol and reached preovulatory size at smaller diameters than in ++ ewes. The maturation of the follicle in F+ ewes at a smaller follicular diameter is presumably directly related to the earlier synthesis of oestradiol (McNatty, 1982). In rat, cow, sheep and human ovaries, granulosa cells are the major source of oestradiol (Moor, 1977; Hillier, 1981; McNatty *et al.*, 1984a, b) principally because granulosa cells are the richest source of aromatase activity. Induction and/or activation of aromatase activity in granulosa cells is critically dependent on FSH stimulation (Hillier, van Hall, van den Boogaard, de Zwart & Keyzer, 1982). Perhaps, therefore, the greater sensitivity of F+ ewes to PMSG (Piper *et al.*, 1982; Kelly *et al.*, 1983) is evidence to support the notion that granulosa cells in F+ ewes are more sensitive to FSH than are cells from ++ ewes. Consistent with this view is the finding that the

frequency of follicles with aromatase activity did not change after PG-induced luteolysis in F+ ewes whereas a significant reduction in frequency occurred in ++ ewes. Ewes of both genotypes may experience a similar reduction of about 15–40% in the plasma concentrations of FSH for up to 24 h with the decline in FSH starting about 12 h after the injection of PG (K. P. McNatty, unpublished data). A drop in FSH of this magnitude may well have contributed to the reduction in the number of follicles with aromatase activity in the ++ ewes which, unlike the F+ ewes, may be relatively insensitive to FSH stimulation.

There was no evidence from the present study to indicate that theca interna tissue in F+ ewes was more sensitive to LH stimulation than that in ++ ewes with respect to androstenedione production, but more studies are needed with different doses of LH. Nevertheless, the output of thecal androstenedione per unit mass of tissue did not differ with respect to follicular size for ewes of either genotype. Since the LH pulse frequency (Scaramuzzi & Radford, 1983; K. P. McNatty, unpublished data), the intrafollicular concentrations of testosterone, and the number of non-atretic follicles in the F+ ewes described herein did not exceed those in ++ ewes (Table 1), it seems unlikely that LH and/or the level of androstenedione synthesis are critical determinants of the high ovulation rates in F+ ewes.

In non-atretic follicles from F+ ewes there were about one-third as many granulosa cells as in follicles from ++ ewes. On a per cell basis, the peak output of oestradiol from granulosa cells from F+ ewes was similar to that from cells of ++ ewes. Therefore, the finding that the oestradiol secretion rate from ovaries of F+ ewes with ~3 'oestrogenic' follicles was similar to that from ovaries of ++ ewes with 1 'oestrogenic' follicle is consistent with the hypothesis that a granulosa-cell mass from 3 F+ follicles is needed to generate the same quantity of oestradiol as that produced by 1 ++ follicle (see also Baird, Ralph, Seamark, Amato & Bindon, 1982). Whether this particular quantity of oestradiol (i.e. 4–5 µg/24 h) is rate limiting with respect to oestrous behaviour and ovulation in both genotypes is unknown.

In F+ ewes the CL were only 0.69 times the diameter and 0.39 times the weight of those in the ++ ewes. This is consistent with the view that they originated from smaller preovulatory follicles (see McNatty, 1979, for review). The finding of a similar decline in the rate of progesterone secretion after PG-induced luteolysis in ewes of both genotypes (Text-fig. 2b), together with similar androgen and oestradiol secretion rates during the follicular phase, reinforces the view that the fundamental difference in F+ and ++ ewes is concerned with the differential rates of maturation of the small antral follicles.

It has been reported that the putative gene effect is not always expressed at puberty or in sequential oestrous cycles (Davis & Kelly, 1983). In the present study, one particular ewe was noted to have had a regular annual ovulation rate of 1 until the age of 6 years at which time she ovulated 3 follicles and was thereafter classified as an F+ ewe. Subsequently, in the cycle under study, her single CL was typical of that for a ++ ewe (see Text-fig. 2b) yet she also contained three 3 mm follicles with granulosa cells having an aromatase activity of 18 ng oestradiol/10<sup>6</sup> cells/3 h which was typical of that found in an F+ ewe (see Text-fig. 1b).

In conclusion, these data suggest that the putative gene effect is manifested in the ovary in small (≤2.5 mm diam.) follicles which results in their maturation at a smaller diameter (i.e. 3–5 mm) than for those in ++ ewes (i.e. ≥5 mm diameter). As a consequence, the development of 3 preovulatory follicles in F+ ewes may be necessary to provide a cell mass capable of producing the same quantity of oestradiol as that from one preovulatory follicle in ++ ewes.

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## Gonadotrophin-stimulated cyclic AMP production by granulosa cells from Booroola × Romney ewes with and without a fecundity gene

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**Summary.** The influence of follicular size and health on FSH and LH stimulation of cAMP production by granulosa cells *in vitro* was studied in cells from Booroola × Romney ewes, with (F+) and without (++) a fecundity gene. The granulosa cells were obtained 0-48 h after the initiation of luteolysis on Day 10 of the oestrous cycle by cloprostenol. The highest mean amounts of cAMP produced by granulosa cells challenged with FSH or LH were not significantly different between the genotypes. However, they were achieved using granulosa cells from follicles > 3-4 mm in diameter in F+ ewes but from follicles > 4 mm in diameter in ++ ewes. Follicles may thus attain ovulatory maturity at a smaller diameter in F+ ewes than in ++ ewes. Granulosa cells from most atretic follicles gave a poor cAMP response to FSH or LH, compared to cells from non-atretic follicles. Granulosa cell responsiveness to FSH was independent of the time the cells were recovered after cloprostenol treatment in F+ ewes, but not in ++ ewes. Cellular responsiveness to LH was independent of time for sheep of both genotypes. There was a significant positive relationship for sheep of both genotypes between the level of aromatase activity in granulosa cells and cellular responsiveness to FSH and LH.

### Introduction

The Booroola Merino ewe and its crossbreeds are some of the most prolific sheep known. The high fecundity of the Booroola ewe can be attributed to the presence of a major gene(s) influencing ovulation rate; up to 10 or 11 ovulations being recorded for individual ewes (Bindon & Piper, 1981; Davis, Montgomery, Allison, Kelly & Bray, 1982; Piper & Bindon, 1982). The physiological basis of the high ovulation rate of these sheep is uncertain. It may be a consequence of higher concentrations of follicle-stimulating hormone (FSH) being present in the blood, and/or a greater sensitivity to FSH at the ovarian level, relative to ewes without the fecundity gene(s) (Cummins, O'Shea, Bindon, Lee & Findlay, 1983; Bindon *et al.*, 1984; Kelly, Owens, Crosbie, McNatty & Hudson, 1984; Robertson, Ellis, Foulds, Findlay & Bindon, 1984). Genetic differences in ovarian sensitivity to FSH, which might affect follicular maturation, could originate in the granulosa cells because these are the target cells for FSH action in the ovary. As follicles mature, the granulosa cells acquire receptors for luteinizing hormone (LH) (Webb & England, 1982). Thus, any differences between the genotypes in sensitivity to FSH and follicular development may be reflected in granulosa cell responsiveness to FSH and LH. Both FSH and LH, when interacting with their specific plasma membrane receptors on granulosa cells, stimulate production of adenosine 3',5'-monophosphate (cAMP), which mediates the intracellular actions of these gonadotrophins (Weiss, Seamark, McIntosh & Moor, 1976; Weiss, Armstrong, McIntosh & Seamark, 1978). Measurement of cAMP production by granulosa cells challenged *in vitro* with FSH and LH therefore provides a convenient index of their responsiveness to gonadotrophins. The purpose of this study was to compare the

responsiveness to FSH and LH of granulosa cells from follicles of different size and health obtained throughout the preovulatory period from Booroola  $\times$  Romney ewes with and without the fecundity gene(s). In this way it was hoped to gain further insight into the underlying mechanism(s) which differentiate high and low fecundity Booroola ewes.

### Materials and Methods

*Sheep.* Three Booroola genotypes have been defined on the basis of maximum ovulation rates from repeated observations (Davis *et al.*, 1982). These are homozygous carriers (FF) with maximum ovulation rates  $\geq 5$ , heterozygous carriers (F+) with maximum ovulation rates of 3 or 4 and non-carriers (++) with maximum ovulation rates of 1 or 2. This study was restricted to a comparison of 21 heterozygous carriers with 21 non-carriers using Booroola  $\times$  Romney ewes aged 6–7 years. The ewes, and their life-time ovulation-rate and lambing records, were generously provided by Dr R. Kelly, Mr G. Davis, Ms J. Armstrong and Mr J. Owens of the Invermay Agricultural Research Centre, Mosgiel, New Zealand. The ewes were transported to Wallaceville Animal Research Centre for the present study. They were grazed on open pasture, and run with a vasectomized ram fitted with a marking harness to detect oestrous activity. The ovulation rates and follicular activity and steroidogenic ability of these sheep have been reported elsewhere (McNatty *et al.*, 1985).

*Recovery of granulosa cells.* On Day 10 of the oestrous cycle (Day 0 day of oestrus) the ewes were injected intramuscularly with 125  $\mu$ g cloprostenol (ICI-Tasman Vaccine Laboratories, New Zealand) to induce luteolysis. Ovariectomy was performed 0, 3, 6, 12, 24, 36 and 48 h later (3 F+ and 3 ++ ewes at each time). (Oestrus occurs about 48 h after cloprostenol treatment.) Immediately after ovariectomy, the corpora lutea and all antral follicles  $\geq 1.0$  mm in diameter were individually dissected from each pair of ovaries into sterile Medium 199 with Earle's salts (Eagle, 1959), supplemented with HEPES buffer (20 mM), gentamicin (50  $\mu$ g/ml; Gibco, Grand Island, New York, U.S.A.), sodium heparin (50 i.u./ml; Weddel Pharmaceuticals Ltd, London, U.K.) and 1.0% bovine serum albumin (Fraction V; Sigma Chemical Co., St Louis, MO, U.S.A.) (Medium A). After recording the follicular diameter and examining the thecal vasculature, each follicle was incised to release its contents. The released follicular fluid was examined for the presence or absence of debris and aspirated through a fine-bore capillary tube. The internal face of the follicle wall was washed gently and repeatedly with 2 ml Medium A and the released clumps of granulosa cells were dispersed by pipetting several times through a finely drawn Pasteur pipette. The oocyte was isolated and assessed subjectively as being healthy or degenerate as previously described (McNatty *et al.*, 1983). The follicle wall was washed several times more, and the total number of granulosa cells in the pooled washings was counted using a haemocytometer. The colour of the theca interna was noted.

*Follicle classification.* Each follicle was classified as non-atretic or atretic on the basis of its morphological appearance. Follicles considered to be non-atretic were those with: visible thecal capillaries when viewed at  $\times 10$  magnification under a dissecting microscope, no debris in the follicular fluid, an oocyte of healthy appearance,  $\geq 26\%$  of the maximum number of recoverable granulosa cells for a follicle of a given size (McNatty *et al.*, 1982, 1984) and a pink to red theca interna. Follicles were considered to be atretic when one or more of these 5 criteria was not applicable.

*Determination of granulosa cell responsiveness to gonadotrophins, and oestradiol-17 $\beta$  synthetase (aromatase) activity.* To obtain sufficient granulosa cells from each pair of ovaries to establish replicate determinations of cellular responsiveness to gonadotrophins and cellular ability to synthesize oestradiol-17 $\beta$  (aromatase activity), it was often necessary to pool cells from follicles of a

similar size and classification, particularly with small and atretic follicles. Each pool was regarded as a single sample of cells. After collecting the granulosa cells, and pooling when appropriate, each sample of cells was split into 2 fractions, one to study cellular responsiveness to gonadotrophins by measuring cAMP production, the other to study aromatase activity. The cells were centrifuged at 200 *g* for 10 min and resuspended in Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin (DBS-BSA) for determining the cAMP response to gonadotrophins, or in Medium A devoid of sodium heparin (Medium B) for determination of aromatase activity. Aliquants of each were taken for determination of cell number by haemocytometer counts. To determine responsiveness to gonadotrophins, aliquants of granulosa cells ( $150 \times 10^3$  cells) in 0.5 ml DBS-BSA were dispensed into a series of 10  $\times$  75 mm plastic test-tubes. Ovine FSH (NIADDK-oFSH-15) or ovine LH (NIADDK-oLH-23) was added in 0.5 ml DBS-BSA to give a final concentration of gonadotrophin ranging from 0 to 1  $\mu$ g/ml. The tubes were capped and incubated at 37°C for 1 h in a shaking water bath before being transferred to an 80°C water bath for 15 min. Some tubes were transferred directly to the 80°C bath so that the endogenous cAMP content of the cells at zero time could be determined. All the tubes were frozen (-20°C) until assayed for cAMP by radioimmunoassay. Preliminary studies showed that cAMP production by granulosa cells incubated at 37°C was constant for 2 h.

For determination of aromatase activity, aliquants of cells (60 to 600  $\times 10^3$ ) in 0.5 ml Medium B were pipetted into 10  $\times$  75 mm plastic tubes containing 0.5 ml of a solution of testosterone (2  $\mu$ g/ml) in Medium B. The tubes were gassed with 5% CO<sub>2</sub> in air, capped and incubated for 3 h at 37°C in a shaking water bath. At the end of the incubation, the tubes were snap frozen to -70°C. Subsequently the tubes were thawed, centrifuged for 15 min at 1500 *g* and the supernatants were assayed for oestradiol-17 $\beta$ . Preliminary studies indicated that the aromatase reaction was constant for the first 3 h for granulosa cells from all atretic and most non-atretic follicles. However, with cells from some large, non-atretic follicles the reaction was only constant for the first 2 h. Therefore, the aromatase activity of cells from some large, non-atretic follicles may be underestimated by up to 20%. A 3-h incubation was preferable to 2 h, however, to ensure that the low amounts of oestradiol-17 $\beta$  produced by cells from small and/or atretic follicles could be accurately measured. Using substrate concentrations > 1  $\mu$ g testosterone/ml to increase oestradiol-17 $\beta$  production by these cells was not practical because it produced unacceptably high blanks in the oestradiol-17 $\beta$  radioimmunoassay.

*cAMP assay.* Ethanol (1 ml) was added to 0.1 ml of the sample for cAMP determination. Precipitated protein was pelleted by centrifugation at 2000 *g* for 15 min and the supernatant was poured off and evaporated to dryness under a stream of nitrogen. The residue was taken up in 0.05 M-sodium acetate buffer (pH 6.2) and aliquants were assayed for cAMP using <sup>125</sup>I-labelled cAMP radioimmunoassay kits (New England Nuclear, Boston, U.S.A.); the acetylation step was included (Harper & Brooker, 1975). Tritiated cAMP was used to monitor the recovery of cAMP after ethanolic extraction and the recovery was routinely > 90%. cAMP concentrations were normalized with respect to recovery and number of granulosa cells in the incubation, and the results were expressed as pmol cAMP/10<sup>6</sup> cells. The intra- and inter-assay coefficients of variation were 8% and 14% respectively.

*Oestradiol-17 $\beta$  assay.* Aliquants of granulosa cell incubation media were assayed directly, without extraction, for oestradiol-17 $\beta$  using a specific radioimmunoassay described previously (McNatty, Gibb, Dobson, Thurley & Findlay, 1981; McNatty *et al.*, 1982). Steroids showing > 1% cross-reactivity with the oestradiol-17 $\beta$  antiserum (WA-27) were oestrone (7.3%) oestriol (1.4%) and oestradiol-17 $\alpha$  (1.4%). The sensitivity of the assay (per tube) was 5 pg. The intra- and inter-assay coefficients of variation were < 11%.

*Statistics.* The data were analysed statistically using analysis of variance in conjunction with Newman-Keuls multiple range test, Student's *t* test or  $\chi^2$  as appropriate. When heterogeneity of

variance was indicated by Bartlett's test, the data were transformed to logarithms to equalize the variances before statistical analysis. In these instances, the data have been presented as geometric means together with 95% confidence limits. The level of significance was set at  $P < 0.05$ .

### Results

#### *Ovulation rate and number of follicles in F+ and ++ ewes*

The mean ovulation rate of each genotype was  $1.1 \pm 0.1$  (s.e.m.) for ++ ewes and  $3.3 \pm 0.3$  for F+ ewes ( $n = 21$ ). Table 1 shows the distribution of follicles in each genotype with respect to follicular diameter. The ovaries of F+ ewes contained significantly fewer follicles of  $> 2-3$  mm ( $P < 0.05$ ) and  $> 5$  mm ( $P < 0.001$ ) diameter than did ++ ewes, and significantly more ( $P < 0.05$ ) of  $> 3-4$  mm diameter. In ewes of both genotypes, the number of follicles at each diameter was independent of the time after cloprostenol treatment.

Table 1. Number of follicles per ewe with respect to follicular diameter and genotype (F+/++)

Follicular diam. (mm)	No. of follicles	
	++	F+
1 to 2	$35.5 \pm 3.2$	$34.9 \pm 2.3$
> 2 to 3	$6.1 \pm 0.9^*$	$3.5 \pm 0.5$
> 3 to 4	$0.9 \pm 0.2^*$	$1.8 \pm 0.3$
> 4 to 5	$0.9 \pm 0.2$	$0.9 \pm 0.2$
> 5	$0.8 \pm 0.1^*$	$0.1 \pm 0.1$

Values are arithmetic mean  $\pm$  s.e.m. for  $n = 21$ .

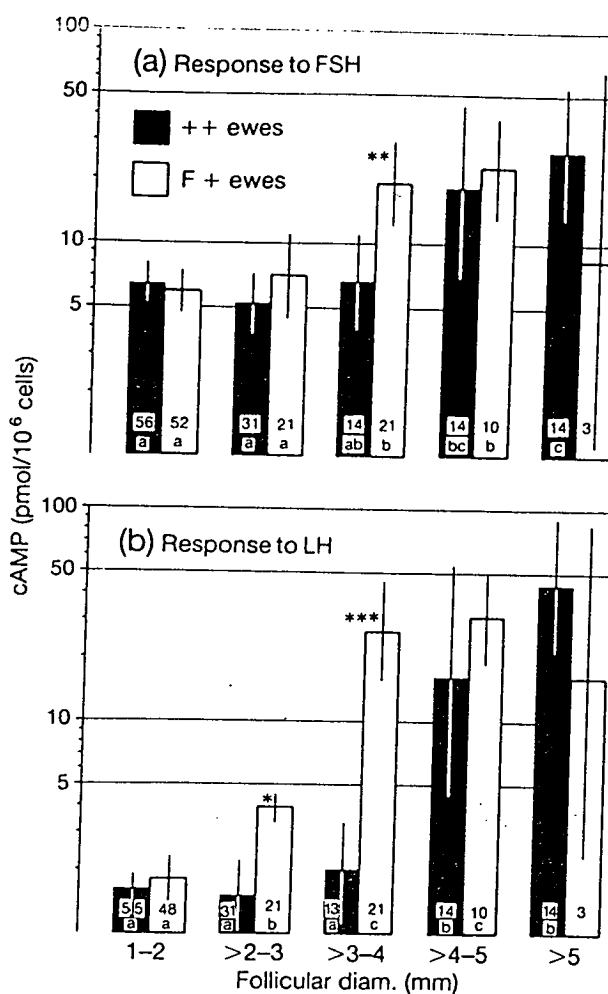
\*Significantly different from value for follicles of corresponding diameter in other genotype (Student's *t* test).

#### *Influence of genotype and follicular diameter on FSH and LH stimulation of cAMP production by granulosa cells*

The endogenous cellular concentration of cAMP at zero time did not differ significantly from the amount produced after incubation for 1 h at 37°C in the absence of gonadotrophin; the combined overall mean  $\pm$  s.d. value was  $1.3 \pm 1.2$  pmol cAMP/ $10^6$  cells ( $n = 771$ ). Granulosa cells challenged with FSH or LH were considered responsive if the amount of cAMP produced was at least two standard deviations above this overall mean value (i.e.  $> 3.7$  pmol cAMP/ $10^6$  cells).

Text-figure 1 (a & b) shows the influence of genotype and follicular diameter on cAMP production by granulosa cells challenged with FSH (100 ng/ml) or LH (100 ng/ml). In F+ ewes, the highest mean response to FSH ( $\sim 20$  pmol cAMP/ $10^6$  cells) was achieved with granulosa cells from follicles  $> 3-4$  mm in diameter (Text-fig. 1a). A comparable mean response in ++ ewes required the use of cells from follicles  $> 4$  mm in diameter. The maximum mean response to LH was also achieved with granulosa cells from follicles  $> 3-4$  mm in diameter in F+ ewes (Text-fig. 1b). In ++ ewes it again required granulosa cells from  $> 4$  mm diameter to achieve a comparable mean response. In follicles  $\leq 4$  mm in diameter, in ++ ewes, a response to LH occurred in only 9 of the 99 observations.

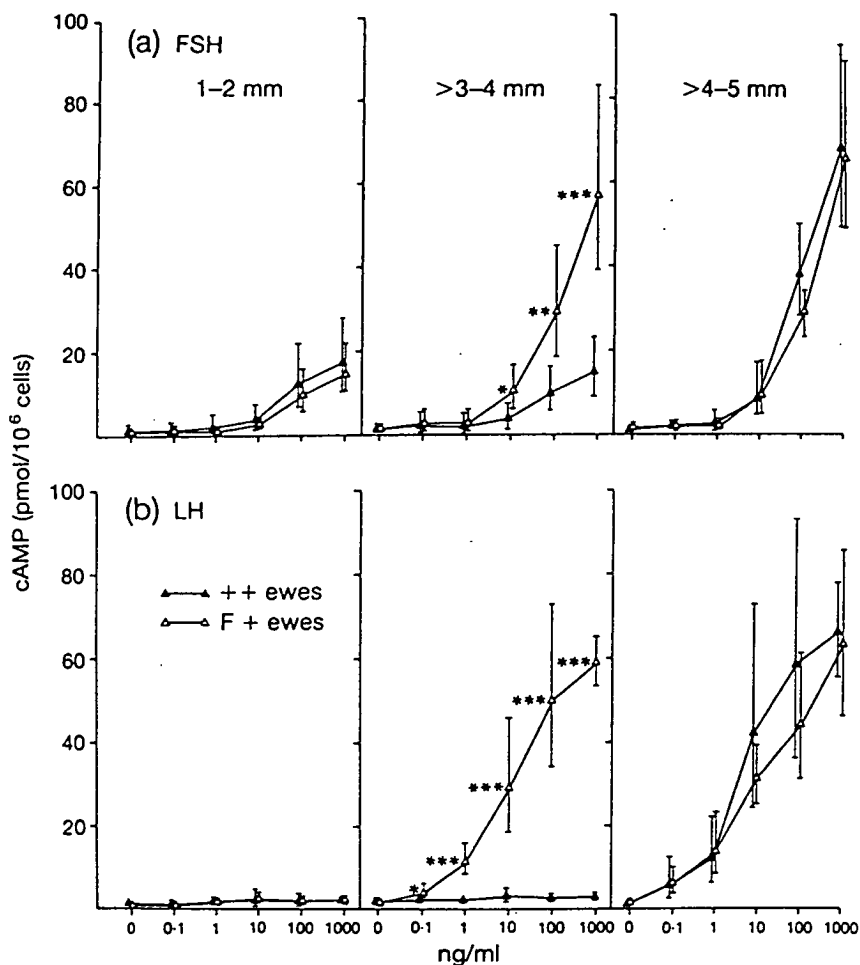
Text-figure 2 shows the effect of increasing concentrations of FSH and LH on cAMP production by granulosa cells from follicles of 3 different diameters. There was no significant difference ( $P > 0.05$ ) between the genotypes in the response of the granulosa cells from follicles of 1-2 mm or  $> 4-5$  mm diameter to the different concentrations of FSH and LH. In  $> 3-4$  mm diameter follicles, mean cAMP production by granulosa cells from F+ ewes was significantly higher than the mean production by cells from ++ ewes when challenged with  $\geq 10$  ng FSH/ml or  $\geq 0.1$  ng LH/ml. Within each genotype there was an increase in the sensitivity of the granulosa cells to FSH



**Text-fig. 1.** Influence of genotype (F+ or ++) and follicular diameter on in-vitro cAMP production by granulosa cells challenged with (a) FSH (100 ng/ml) and (b) LH (100 ng/ml). Values are geometric means of the number of observations indicated in each bar with 95% confidence limits indicated by the vertical lines. Mean values with different letters within the same genotype are significantly different ( $P < 0.05$ , analysis of variance in conjunction with Newman-Keuls multiple range test on logarithmically transformed data). Asterisks indicate significant differences between genotypes in mean values for follicles of the same diameter ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , Student's  $t$  test on logarithmically transformed data). The data from F+ ewes for follicles  $> 5$  mm in diameter were not included in the statistical analysis because of the limited number of datum points.

challenge as follicular diameter increased, indicated by a shift to the left in the dose-response curves. For example, in ++ ewes, granulosa cells from follicles  $> 3-4$  mm in diameter had to be challenged with 100 ng FSH/ml to cause a mean cAMP response comparable to that achieved with just 10 ng FSH/ml by cells from follicles  $> 4-5$  mm in diameter (Text-fig. 2). A similar increase in sensitivity occurred in F+ ewes as follicular diameter increased from 1-2 mm to  $> 3-4$  mm. For cells from ewes of both genotypes, 10 ng FSH/ml and 0.1 ng LH/ml was the minimum concentration capable of stimulating granulosa cell cAMP production.

The above effects of genotype and follicular diameter on cAMP production by granulosa cells stimulated with gonadotrophins are unlikely to be a consequence of differences in cellular phospho-



Text-fig. 2. Effect of increasing concentrations of (a) FSH and (b) LH on cAMP production by granulosa cells from follicles of diameter 1–2 mm, > 3–4 mm and > 4–5 mm from F+ and ++ ewes. Values are geometric means with 95% confidence limits indicated by the vertical lines for  $n = 5$ . Asterisks indicate significant differences between genotypes in mean cAMP production in response to the same dose of gonadotrophin (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Student's  $t$  test on logarithmically transformed data).

diesterase activity. Inhibition of its activity by inclusion of 3-isobutyl-1-methylxanthine (500  $\mu\text{M}$ ) in the granulosa cell incubations increased the response to gonadotrophin stimulation approximately 2-fold in all instances regardless of follicular diameter or ewe genotype.

*Influence of time after cloprostenol treatment on FSH and LH stimulation of cAMP production by granulosa cells*

Analysis by  $\chi^2$  of the data in Table 2 shows that the time after cloprostenol treatment significantly influenced the amount of cAMP produced by granulosa cells challenged with FSH (100 ng/ml) in ++ ewes ( $P < 0.025$ ) but not in F+ ewes ( $P > 0.05$ ). In ++ ewes, a lower proportion of the granulosa cell samples obtained 12–24 h (22%) and 36–48 h (34%) after cloprostenol treatment produced  $\geq 10$  pmol cAMP/ $10^6$  cells when challenged with FSH, compared with cells



Table 2. Frequency table showing the influence of time after cloprostenol treatment on FSH (100 ng/ml) and LH (100 ng/ml) stimulation of cAMP production by granulosa cells

Genotype	Time after cloprostenol treatment (h)	cAMP production (pmol/10 <sup>6</sup> cells)				
		FSH-treated cells			LH-treated cells	
		< 10	≥ 10–< 20	≥ 20	< 20	≥ 20
++	0–6	24	17	13	42	11
	12–24	29	6	2	32	5
	36–48	25	6	7	34	4
F+	0–6	23	9	8	30	9
	12–24	20	4	7	23	8
	36–48	23	5	8	25	8

Table 3. Frequency table showing the influence of follicular health on FSH (100 ng/ml) and LH (100 ng/ml) stimulation of cAMP production by granulosa cells

Genotype	Follicle type	cAMP production (pmol/10 <sup>6</sup> cells)					
		FSH-treated cells			LH-treated cells		
		≤ 4	> 4–≤ 10	> 10	≤ 4	> 4–≤ 25	> 25
++	Non-atretic	0	7	20	7	6	14
	Atretic	38	38	26	90	8	2
F+	Non-atretic	3	7	20	5	12	13
	Atretic	23	34	20	49	16	8

obtained 0–6 h (56%) after treatment. Granulosa cell production of cAMP in response to LH was independent of time after cloprostenol treatment in F+ and ++ ewes ( $P > 0.05$ ,  $\chi^2$  analysis).

*Influence of follicular health on FSH and LH stimulation of cAMP production by granulosa cells from F+ and ++ ewes*

Analysis by  $\chi^2$  of the data in Table 3 indicates that for both genotypes follicular health significantly influenced the amount of cAMP produced by granulosa cells stimulated with FSH or LH ( $P < 0.001$ ). Elevated amounts of cAMP were produced by a higher proportion of granulosa cell samples from non-atretic than from atretic follicles. (Bias in the  $\chi^2$  analysis of the LH data for ++ ewes was avoided by combining the data in the two columns showing frequencies for cAMP production  $> 4$  pmol/10<sup>6</sup> cells in response to LH.)

*Relationship between granulosa cell cAMP production in response to stimulation with FSH or LH, and cellular aromatase activity*

For both genotypes, there was a significant positive relationship ( $P < 0.001$ ,  $\chi^2$  analysis) between the ability of granulosa cells to metabolize testosterone to oestradiol-17 $\beta$  (aromatase activity) and the amount of cAMP produced by the cells in response to challenge with FSH or LH (Table 4). Bias in the  $\chi^2$  analyses was avoided by adding together the number of granulosa cell samples in the two columns for cAMP production  $\geq 10$  pmol/10<sup>6</sup> cells in response to FSH, or  $\geq 4$  pmol/10<sup>6</sup> cells in response to LH.

Table 4. Frequency table showing the relationship between cellular aromatase activity and granulosa cell cAMP production stimulated by FSH (100 ng/ml) or LH (100 ng/ml)

Hormone	Aromatase activity (ng oestradiol- $17\beta$ /10 <sup>6</sup> cells)	cAMP production (pmol/10 <sup>6</sup> cells)					
		++ ewes			F+ ewes		
		<10	$\geq 10$ -<20	$\geq 20$	<10	$\geq 10$ -<20	$\geq 20$
FSH	<1	66	13	2	41	6	1
	$\geq 1$ -<20	15	16	5	20	16	10
	$\geq 20$	0	0	13	0	0	12
LH	<4	70	9	0	41	8	0
	$\geq 4$ -<50	21	10	2	19	31	0
	$\geq 50$	0	0	11	1	3	9

### Discussion

The present study demonstrates that, in Booroola  $\times$  Romney ewes, granulosa cell responsiveness to FSH and LH (in terms of cAMP production *in vitro*) is influenced by genotype, follicular diameter and health and time after administration of a luteolytic dose of the PGF-2 $\alpha$  analogue, cloprostenol.

Although FSH is essential for antral follicle development, the later stages of follicular maturation in sheep occur when plasma concentrations of FSH are falling (Baird, Swanston & McNeilly, 1981). For sheep of both genotypes granulosa cells became more sensitive to FSH as follicular diameter increased due to a shift in the dose-response curve to FSH (Text-fig. 2). This may be important in ensuring that final maturation of follicles can occur in spite of declining plasma FSH concentrations. Large ovulatory follicle(s) may be protected from the deleterious effects of low plasma FSH concentrations by the increased sensitivity to FSH of the granulosa cells, thereby permitting the follicle(s) to continue to respond to FSH and so continue development. Follicles not having acquired this increased sensitivity to FSH, when plasma FSH levels decline, would probably undergo atresia due to lack of FSH. Indeed, only 25% of the granulosa cell samples from atretic follicles produced high amounts of cAMP ( $> 10$  pmol/10<sup>6</sup> cells) in response to FSH, compared to 70% of those from non-atretic follicles (Table 3). The factors controlling the formation of FSH receptors coupled to adenylate cyclase (i.e. functional receptors) in granulosa cells is poorly understood, although FSH itself and steroids may all be involved (Richards, 1979). Granulosa cell responsiveness to FSH was independent of time after treatment with cloprostenol in F+ ewes, but not in ++ ewes (Table 2). Perhaps differences between the genotypes in plasma FSH concentrations during the preovulatory period (Bindon *et al.*, 1984) may account, in part, for this genotypic difference in granulosa cell responsiveness to FSH.

Studies in the rat indicate that a major action of FSH on granulosa cells, which is thought to be mediated through cAMP, is the induction/activation of the aromatizing enzymes necessary for oestradiol-17 $\beta$  biosynthesis (Hillier, 1981; Wang, Hsueh & Erickson, 1982). A similar action of FSH in sheep is still not proven. In ewes of both genotypes there was a significant relationship between granulosa cell responsiveness to FSH and aromatase activity (Table 4). The highest levels of aromatase activity ( $\geq 20$  ng oestradiol-17 $\beta$ /10<sup>6</sup> cells) were only observed in granulosa cells very responsive to FSH, i.e. producing  $\geq 20$  pmol cAMP/10<sup>6</sup> cells. This suggests that the aromatase activity of granulosa cells may be regulated, at least in part, by the capacity of cells to produce cAMP in response to FSH. Some granulosa cell populations that had a low aromatase activity were still able to produce substantial amounts of cAMP when stimulated with FSH (Table 4). Perhaps in

these instances, although the FSH receptors were coupled to adenylate cyclase, the adenylate cyclase was uncoupled from the aromatase enzyme system. A significant positive relationship also existed between granulosa cell aromatase activity and cellular responsiveness to LH (Table 4). It is uncertain whether the increase in aromatase activity may be a cause (Richards, 1979) or a possible consequence (McNeilly, Fraser & Baird, 1984) of the increased cellular responsiveness to LH.

Follicles containing granulosa cells very responsive to LH were found in ewes of both genotypes at all of the times studied, in the luteal and follicular phase (Table 2). However, the response to LH was dependent upon the health (Table 3) and diameter (Text-figs 1 & 2) of the follicle; granulosa cells very responsive to LH were found most frequently in large, non-atretic follicles. The acquisition by granulosa cells, during follicular maturation, of LH receptors coupled to adenylate cyclase is thought to be essential for the follicle to respond properly to the ovulatory LH surge and differentiate into a functional corpus luteum (Henderson, 1979). The degree of maturation of a follicle may therefore be assessed by the responsiveness of its granulosa cells to LH. In the present study, the highest mean production of cAMP in response to an LH stimulus occurred in granulosa cells from follicles of > 3–4 mm diameter in F+ ewes. In contrast, in ++ ewes, it required cells from follicles > 4 mm diameter to attain a similar mean production of cAMP (Text-fig. 1b). Granulosa cells also acquire maximum aromatase activity in follicles of smaller diameter in F+ ewes than in ++ ewes (McNatty *et al.*, 1985). This may be a consequence of the maximum response of granulosa cells to FSH also being acquired at a smaller diameter (Text-fig. 1a). Collectively, these findings suggest that follicles may achieve ovulatory maturity at a smaller diameter in F+ ewes than in ++ ewes. This may account for the difference in ovulation rate between the two genotypes (McNatty *et al.*, 1985). In ++ ewes, with a mean ovulation rate of  $1.1 \pm 0.1$ , ovulatory maturity may not be attained until follicles are > 4 mm in diameter. Ovulation rate is then restricted by the fact that there are usually only 1 or 2 follicles of this diameter in each pair of ovaries (Table 1; McNatty *et al.*, 1985). Because follicles in F+ ewes (mean ovulation rate  $3.3 \pm 0.3$ ) can achieve ovulatory maturity at a smaller diameter than those of ++ ewes, there are more follicles in F+ ovaries which may be available to ovulate (Table 1; McNatty *et al.*, 1985), hence the ovulation rate can be higher than that of ++ ewes. The notion of follicles ovulating at a smaller diameter in F+ ewes is consistent with the observation that the corpora lutea of F+ ewes are smaller than those of ++ ewes (Kelly *et al.*, 1984; McNatty *et al.*, 1985). Why follicles in F+ ewes should attain ovulatory maturity at a smaller diameter than those of ++ ewes is not known. It may be a consequence of follicles maturing faster in F+ ewes because they are exposed to higher concentrations of circulating FSH (Robertson *et al.*, 1984), and/or because the fecundity gene(s) influences some ovarian intracellular rate-limiting step which controls the rate of follicular maturation.

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## Differences in ovarian activity between Booroola × Merino ewes which were homozygous, heterozygous and non-carriers of a major gene influencing their ovulation rate

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**Summary.** Differences in the function and composition of individual ovarian follicles were noted in Booroola Merino ewes which had previously been segregated on at least one ovulation rate record of  $\geq 5$  (FF ewes,  $N=15$ ), 3–4 (F+ ewes,  $N=18$ ) or  $< 3$  (++) ewes,  $N=18$ ).

Follicles in FF and F+ ewes produced oestradiol and reached maturity at a smaller diameter than in ++ ewes. In FF ( $N=3$ ), F+ ( $N=3$ ) and ++ ( $N=3$ ) ewes, the respective mean  $\pm$  s.e.m. diameters for the presumptive preovulatory follicles were  $3.4 \pm 0.3$ ,  $4.1 \pm 0.2$  and  $6.8 \pm 0.3$  mm and in each of these follicles the respective mean  $\pm$  s.e.m. numbers of granulosa cells ( $\times 10^6$ ) were  $1.8 \pm 0.3$ ,  $2.2 \pm 0.3$  and  $6.6 \pm 0.3$ . During a cloprostenol-induced follicular phase, the oestradiol secretion rates from FF ewes with  $4.8 \pm 0.4$  'oestrogenic' follicles, F+ ewes with  $3.2 \pm 0.2$  'oestrogenic' follicles and ++ ewes with  $1.5 \pm 0.02$  'oestrogenic' follicles were not significantly different from one another. Moreover, the mean total numbers of granulosa cells from the 'oestrogenic' follicles from each genotype were identical, namely  $5.4 \times 10^6$  cells. Irrespective of genotype the mean weight of each corpus luteum was inversely correlated to the ovulation rate ( $R = 0.91$ ,  $P < 0.001$ ).

Collectively, these findings support the notion that the maturation of  $\geq 5$  follicles in FF ewes and 3–4 follicles in F+ ewes may each be necessary to provide a follicular-cell mass capable of producing the same quantity of oestradiol as that from 1–2 preovulatory follicles in ++ ewes.

### Introduction

The Booroola Merino is one of the most prolific sheep breeds in the world (Bindon, 1984). The exceptional prolificacy of the Booroola has been attributed to a major gene(s) which influences its ovulation rate (Bindon & Piper, 1981; Davis, Montgomery, Allison, Kelly & Bray, 1982; Piper & Bindon, 1982). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative gene(s) have tentatively been segregated on the basis of at least one ovulation rate recording of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982).

In recent studies on F+ and ++ Booroola × Romney ewes, marked genotypic differences were noted in both the function and composition of similar sized ovarian follicles but not in the total numbers of antral follicles (Henderson, Kieboom, McNatty, Lun & Heath, 1985; McNatty *et al.*, 1985a). These studies also showed that, in F+ ewes, antral follicles (2–4.5 mm diam.) were more sensitive to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and produced more oestradiol compared to similar sized follicles from ++ ewes. During a cloprostenol-induced follicular phase, the granulosa-cell number and secretion rate of oestradiol from ovaries of F+

ewes containing 3 presumptive preovulatory follicles were both similar to those from one such follicle in ++ ewes. Subsequently, follicles in F+ ewes reached ovulatory size at a smaller diameter and transformed into smaller corpora lutea than those in ++ ewes.

At present, little is known about the composition, steroidogenic capacities and size of the largest non-atretic follicles in FF ewes. The aim of this study was to examine some of these aspects in FF Booroola × Merino ewes and to see how they compared with those in F+ and ++ ewes.

### Materials and Methods

*Animals and procedures.* Using the criteria of Davis *et al.* (1982), 15 Booroola × Merino ewes (6–8 years of age) were classified as FF and 18 Booroola × Merino ewes (6–8 years of age) were classified as F+ on the basis of 2–4 previous annual ovulation rate recordings of  $\geq 5$  (FF) or 3–4 (F+). Another 18 Booroola × Merino ewes (4–5 years of age) with 2–4 previous annual recordings of ovulation  $< 3$  were classified as non-carriers (++) of the putative fecundity gene.

On Day 10 (time 0) of the oestrous cycle (oestrus = Day 0), all but 3 ewes from each genotype were injected with cloprostenol (125 µg s.c.; Coopers Animal Health Laboratories, Upper Hutt, New Zealand). Previous studies with this experimental regimen in Romney ewes and in ++ and F+ Booroola × Romney ewes have shown that ovarian oestradiol secretion immediately preceding the preovulatory LH surge coincides with the emergence of 'oestradiol-17β enriched' follicles about 12 h after cloprostenol treatment (McNatty, 1982; McNatty *et al.*, 1982, 1985a; unpublished data). Moreover, these and other studies (Baird, Ralph, Seamark, Amato & Bindon, 1982) have shown that the number of oestradiol-17β-enriched follicles from 12 to 36 h after cloprostenol treatment correlated with the ovulation rate of the sheep breed in question. At time 0 (uninjected ewes) and at intervals thereafter (6, 12, 24, 36 and 48 h) ovarian (10–20 ml from both left and right ovarian veins) and peripheral (50 ml) venous blood was collected from 3 FF, 3 F+ and 3 ++ ewes anaesthetized with thiopentone sodium (Intraval; May & Baker, Wellington, New Zealand); the only exception to this was at 6 h after cloprostenol injection when no FF ewes were studied. During ovarian venous blood collection the rate of blood flow was also measured (McNatty, Dobson, Gibb, Kieboom & Thurley, 1981b). Immediately after the blood had been collected, the ovaries of each animal were removed and all follicles  $\geq 1$  mm in diameter and CL were dissected and their diameters measured to the nearest 0.5 mm as previously described (McNatty *et al.*, 1985a).

*Blood samples.* All blood samples were centrifuged at 4000 g at 4–6°C for 20 min within 30 min of collection, and the plasma samples stored at –20°C until assayed.

*Follicle classification.* For the purpose of this study, a non-atretic follicle was defined as that which contained a vascularized theca interna, no debris in follicular fluid,  $\geq 25\%$  of the maximum number of granulosa cells for a given follicle size and a healthy-looking oocyte. An atretic follicle was so defined if one or more of the above criteria were not satisfied. The validity of this method of classification has been established (McNatty *et al.*, 1985a). In the present study, the maximum number of granulosa cells recorded in 1, 2, 3, 4, 5, 6 and 7 mm diameter follicles from ++ ewes was 0.9, 2.2, 2.9, 3.8, 6.3 and 7.5 × 10<sup>6</sup> cells respectively. In F+ ewes, the maximum number in 1, 2, 3, 4 and 5 mm diameter follicles was 0.8, 2.2, 2.3, 3.5 and 3.6 × 10<sup>6</sup> cells respectively. In FF ewes the maximum number in 1, 2, 3 and 4 mm diameter follicles was 0.6, 1.3, 2.0 and 2.9 × 10<sup>6</sup> cells respectively.

*The granulosa cell aromatase assay.* For each ewe, the granulosa cells from individual follicles were pooled with respect to follicle size (1–1.5 mm, 2–2.5 mm, 3–3.5 mm diameter, etc.) and follicle health (atretic or non-atretic). The cells were then washed and resuspended in Medium A (i.e. Medium 199 containing sodium bicarbonate (0.85 g/l), Earle's salts, L-glutamine (0.1 g/l), Hepes

buffer (20 mM) and 1% BSA (w/v)) to a cell concentration of  $40\text{--}200 \times 10^3$  cells per 0.15 ml; cell viability was not established. Subsequently, 0.15 ml aliquants of the above cell preparations were incubated in a 96-well microtest plate (Nunc, Kanstrup, Denmark) with 0.15 ml of Medium A containing 2000 ng testosterone/ml for 3 h at 37°C in an humidified (100%) incubator gassed with 5% CO<sub>2</sub> in air. Aromatase activity was calculated from the oestradiol content of the supernatant after the cells plus medium were centrifuged and separated at the end of the incubation. The oestradiol content was estimated at time 0 but was always negligible. Under these conditions, the rate of oestradiol formation was constant for the first 3 h.

*Theca interna perfusions.* To determine the ability of theca interna to secrete androstenedione, samples of theca interna (10–25 mg) were perfused *in vitro* using the method described in detail by McNatty *et al.* (1984, 1985a). An aliquant of the theca was also homogenized in 1 ml ethanol and its endogenous steroid content determined. Another small portion was fixed in a 10% formalin solution for histological examination to test the purity of the tissue before perfusion. The theca was perfused at 37°C for 4 h at a flow-rate of 1.4 ml/min with 12-min fractions being collected. LH (NIH-LH-S24; 8 ng/ml) was introduced into the perfusion medium for 20 min after the tissue had first been perfused for 1 h. At the end of the 4-h period, the tissue was fixed for histological examination and the medium from each tube was stored at –20°C until assayed for androstenedione by a specific radioimmunoassay (McNatty *et al.*, 1984). In this study, all thecae were recovered from non-atretic follicles. For each ewe, all the thecae from non-atretic follicles only, irrespective of size, were pooled to ensure that sufficient tissue was available for the study (i.e. at least 10 mg thecal tissue per perfusion column); the viability of the thecal cells was not determined. In a previous study we showed that the thecal androstenedione output from F+ and ++ ewes was not influenced by follicular diameter ( $\geq 1$  mm diameter; McNatty *et al.*, 1985a). The androstenedione output was expressed as the cumulative output (ng) per 10 mg theca interna over the 3 h period during and after LH stimulation.

*Hormone assays.* All steroids were measured using previously published RIA procedures (McNatty, Gibb, Dobson, Thurley & Findlay, 1981a; McNatty *et al.*, 1985a). The ovarian steroid secretion rates were calculated from a knowledge of haematocrit, blood flow and steroid concentration (McNatty *et al.*, 1981a, b).

Details of the working solutions and specifications of the progesterone (WA-26), androstenedione (WA-965), testosterone (WA-36) and oestradiol-17 $\beta$  (WA-27) antisera are provided elsewhere (McNatty *et al.*, 1981a, 1984).

The detection limit of the steroids in follicular fluid was 1 ng/ml and those of progesterone and oestradiol in plasma were 200 and 1 pg/ml respectively. The intra- and inter-assay coefficients of variation for all the above steroid assays were <8% and <15% respectively.

*Statistical procedures.* The data on numbers of follicles, or granulosa cells, hormones in follicular fluid or plasma, aromatase activity in granulosa cells and androstenedione output from thecal tissues were first normalized by log-transformation and the values averaged where appropriate for each animal. Thereafter the data were subjected to analysis of variance and Neuman–Keuls multiple range test when comparing the overall means between genotypes. The effects of cloprostenol on the above parameters were subjected to contingency table analysis.

## Results

### *Ovarian follicular activity*

There was no significant effect of time after cloprostenol treatment (i.e. 0–6 h, 12–24 h, 36–48 h) on the mean proportion of non-atretic follicles ( $\geq 1$  mm diam.) in ovaries of ++, F+ or FF ewes



**Table 1.** Follicle numbers with respect to genotype, follicular diameter and follicular health (values are geometric means (and 95% confidence limits))

Follicle diameter (mm)	Follicle number	Genotype		
		++	F+	FF
Total ( $\geq 1$ mm)	Total	32.0 (28.4,35.9)	30.8 (24.2,39.4)	38.0 (30.8,47.0)
	Non-atretic	16.9 (14.8,19.4)	14.5 (11.2,18.8)	17.7 (14.0,22.2)
1-1.5	Total	19.0 (15.8,23.0)	22.3 (16.6,29.9)	27.2 (21.0,35.2)
	Non-atretic	7.2 (5.3,9.8)	8.6 (7.8,9.4)	9.8 (6.8,13.9)
2-2.5	Total	8.3 (5.8,11.8)	4.7 (3.3,6.5)	5.5 (3.7,7.9)
	Non-atretic	5.4 (3.9,7.4)	2.4* (1.5,3.4)	3.2* (2.0,4.9)
3-3.5	Total	1.3 (0.7,2.1)	1.4 (0.8,2.2)	2.5 (1.4,4.0)
	Non-atretic	0.9* (0.4,1.5)	0.9* (0.4,1.6)	2.1 (1.2,3.4)
4-4.5	Total	0.5 (0.2,1.0)	1.2 (0.7,1.9)	0.5 (0.1,1.1)
	Non-atretic	0.4* (0.1,0.8)	1.2 (0.8,1.8)	0.5* (0.1,1.1)
$\geq 5$	Total	0.9 (0.8,1.0)	0.1** (0,0.2)	0**
	Non-atretic	0.8 (0.6,1.0)	0**	0**

Numbers marked \*, \*\* are significantly less ( $P < 0.05$ ;  $P < 0.01$ , respectively; ANOVA) than unmarked numbers in the same line.

( $P > 0.25$ ; contingency table analysis; data for the above time frames were pooled). Nor was there a significant effect of genotype on the total number of follicles  $\geq 1$  mm diameter (i.e. irrespective of time after cloprostenol treatment) (Table 1). The total numbers as well as numbers of non-atretic follicles for each genotype with respect to follicular diameter are also summarized in Table 1. There were similar numbers of small follicles (1-1.5 mm diam.) for all genotypes with respect to the total population and also with respect to the non-atretic population. As follicles enlarged beyond 1.5 mm in diameter, genotypic differences in the geometric mean numbers of non-atretic follicles became apparent. Essentially, none of the follicles in FF or F+ ewes reached 5 mm or more in diameter. In FF and F+ ewes, the largest non-atretic follicles were between 2 and 4.5 mm in diameter whereas in ++ ewes, they ranged between 5 and 7.5 mm in diameter.

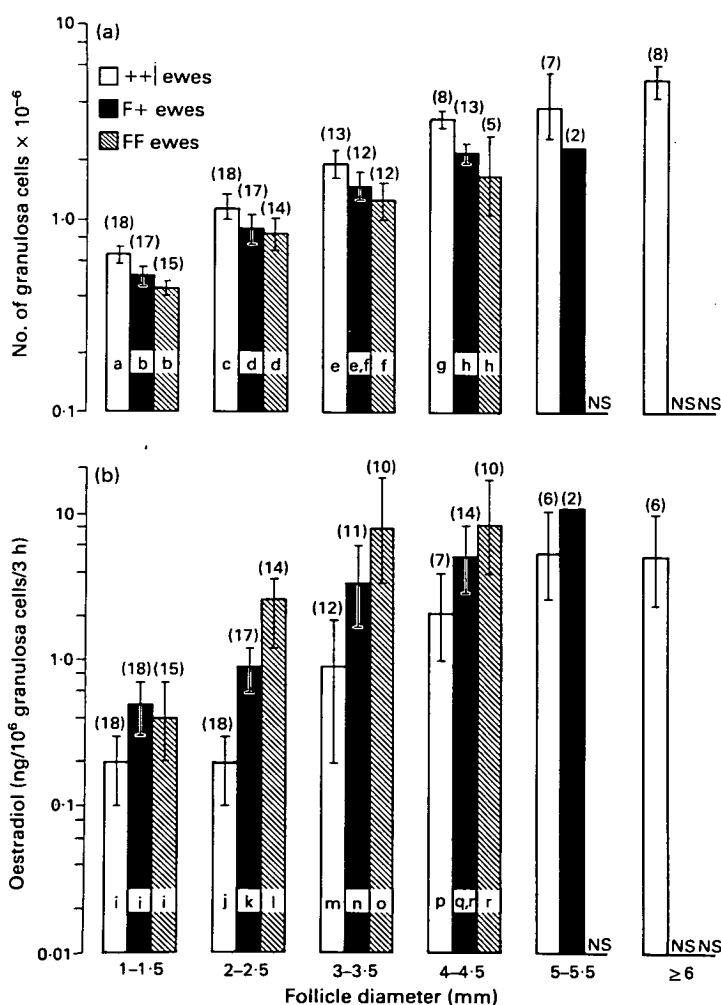
#### *Effect of genotype on the number of granulosa cells*

These data for non-atretic follicles are summarized in Fig. 1(a). For all diameters from 1 to 4.5 mm there were significantly more cells in follicles of ++ than in FF ewes, with the cell numbers in F+ ewes in between.

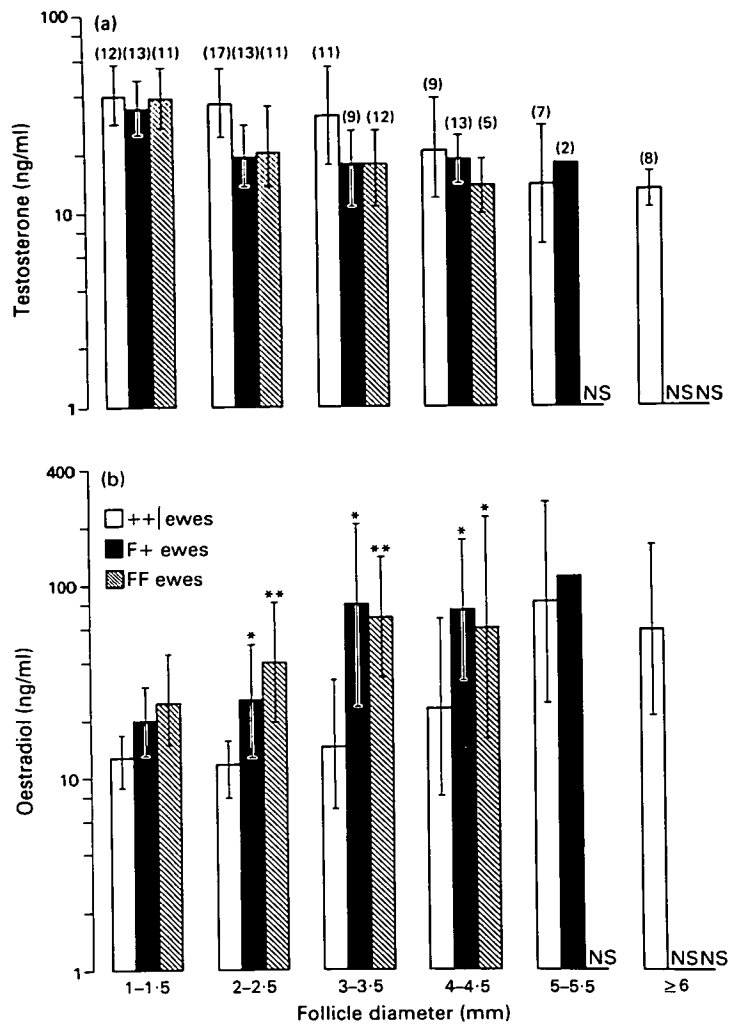
*Influence of genotype on follicular fluid concentrations of testosterone and oestradiol*

There were no significant differences between atretic and non-atretic follicles in the follicular concentrations of testosterone in the ewes of all the genotypes. The percentages of non-atretic follicles ( $\geq 1$  mm diam.) from each genotype with high testosterone concentrations ( $\geq 25$  ng/ml; see Fig. 2) are shown in Table 2. These percentages increased with time after cloprostenol injection for F+ and ++ but not for FF follicles ( $P < 0.01$ ; contingency table analysis).

For oestradiol in follicular fluid, there was a significant effect in F+ and FF but not ++ ewes between the percentages of non-atretic follicles ( $\geq 1$  mm diam.) with low ( $< 50$  ng/ml; see Fig. 2) and high ( $\geq 50$  ng/ml) concentrations and time (0–6 h, 12–24 h and 36–48 h) after cloprostenol injection (FF ewes,  $P < 0.05$ ; F+ ewes,  $P < 0.01$ ; contingency table analysis; Table 2).



**Fig. 1.** The number of granulosa cells (a) and aromatase activity in granulosa cells (b) from different-sized non-atretic follicles of Booroola Merino ewes previously segregated as homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing ovulation rate. NS = no samples. Results are geometric means with vertical bars representing 95% confidence limits. Numbers in parentheses refer to number of ewes from which granulosa cells were studied. c vs d, n vs o, p vs r, m vs n, k vs l, p vs q all  $P < 0.05$ ; a vs b, e vs f, g vs h, j vs k, j vs l, m vs o,  $P < 0.01$  (ANOVA in conjunction with Neuman-Keuls test).



**Fig. 2.** The concentrations of testosterone (a) and oestradiol (b) in follicular fluid from different-sized non-atretic follicles of Booroola Merino ewes previously segregated as homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing ovulation rate. NS = no samples. Results are geometric means with vertical bars representing 95% confidence limits. Numbers in parentheses refer to number of ewes from which the concentrations of testosterone and oestradiol were measured. \* $P < 0.05$  compared to ++ ewes, \*\* $P < 0.01$  compared to ++ ewes.

The concentrations of testosterone and oestradiol in follicular fluid of non-atretic follicles with respect to genotype and follicle diameter are summarized in Fig. 2. There was no significant difference in concentrations of testosterone between the genotypes at each follicular diameter (ANOVA). However, for all genotypes, the overall testosterone concentrations decreased with increasing follicular diameter. In ++ ewes, the testosterone concentrations in  $\geq 6$  mm diameter follicles were significantly lower than in 1-1.5 mm follicles ( $P < 0.05$ , ANOVA). In FF and F+ ewes, the respective testosterone concentrations in 3-3.5 and 4-4.5 mm diameter follicles were significantly lower than in 1-1.5 follicles (FF ewes,  $P < 0.025$ ; F+ ewes,  $P < 0.05$ ; ANOVA).

**Table 2.** Percentages of non-atretic follicles with high concentrations of testosterone ( $\geq 25$  ng/ml) or oestradiol ( $\geq 50$  ng/ml) in follicular fluid with respect to genotype and time after cloprostenol injection

Hormone	Genotype	Time after cloprostenol injection		
		0-6 h	12-24 h	36-48 h
Testosterone	++	38 (79)†	54 (56)	73 (79)
	F+	33 (84)	35 (74)	67 (92)
	FF	56 (32)	49 (61)	47 (58)
Oestradiol	++	11 (76)†	15 (33)	33 (57)
	F+	21 (39)	43 (28)	57 (35)
	FF	26 (27)	49 (41)	60 (54)

† Values in parentheses refer to the number of follicles studied.

**Table 3.** Influence of genotype and follicular health on level of aromatase activity (ng/10<sup>6</sup> cells/3 h) in granulosa cells

Genotype	Follicular health (no. of follicles)	% Follicles with aromatase activity		
		1 <	1-5 >	5
++	Non-atretic (70)	60	27	13
	Atretic (42)	77	23	0
F+	Non-atretic (61)	41	36	23
	Atretic (41)	95	5	0
FF	Non-atretic (46)	33	30	37
	Atretic (23)	91	9	0

For oestradiol in follicular fluid there were some differences between the genotypes at follicle sizes greater than 1 mm in diameter. In 2-2.5 and 3-3.5 mm follicles the oestradiol concentrations were significantly higher in the FF and F+ ewes compared to those in the ++ ewes (2-2.5 mm, FF vs ++,  $P < 0.01$ ; F+ vs ++,  $P < 0.05$ ; 3-3.5 mm, FF vs ++,  $P < 0.01$ ; F+ vs ++,  $P < 0.05$ ; ANOVA). In 4-4.5 mm follicles, the oestradiol concentrations in FF ewes were significantly higher than those in ++ ewes ( $P < 0.05$ ) whereas those in F+ ewes were similar to those in FF ewes but higher ( $P < 0.05$ ) than those in ++ ewes. In FF, F+ and ++ ewes the mean oestradiol concentrations reached their highest values in 3-4.5 mm, 3-5.5 mm and  $\geq 5$  mm diameter follicles respectively.

Irrespective of follicular diameter, the geometric means (and 95% confidence limits) of oestradiol in atretic follicles from FF, F+ and ++ ewes were 5 (4,6), 5 (4,6) and 4 (3,5) ng/ml respectively.

#### *Influence of genotype on aromatase activity in granulosa cells*

There was no significant relationship between the frequency of non-atretic follicles with low or medium to high levels of aromatase activity *in vitro* (i.e.  $< 1$ ,  $\geq 1$  ng oestradiol/10<sup>6</sup> granulosa cells/3 h respectively) and time (i.e. 0-6 h, 12-24 h, 36-48 h) after cloprostenol treatment (FF, F+ and ++ ewes all  $P < 0.25$ , contingency table analysis). There were significant relationships in FF, F+ and ++ ewes between the level of aromatase activity ( $< 1$ , 1-5,  $> 5$  ng oestradiol/10<sup>6</sup>

cells/3 h) in granulosa cells and the health of the follicle (non-atretic vs atretic; FF,  $P < 0.001$ ; F+,  $P < 0.01$ ; ++,  $P < 0.05$ ; Table 3). None of the granulosa cell populations in atretic follicles from any of the genotypes had aromatase activity  $> 5$  ng oestradiol/ $10^6$  cells/3 h.

Irrespective of genotype and follicular health there was a highly significant correlation between aromatase activity in granulosa cells and the concentration of oestradiol in follicular fluid ( $R = 0.88$ ,  $n = 163$  follicles,  $P < 0.001$ ). This relationship could be expressed by the equation in  $y = 1.06 \ln x - 3.5$  where  $y$  = aromatase activity in ng/ $10^6$  cells/3 h and  $x$  = concentration of oestradiol in follicular fluid in ng/ml.

There was a significant influence of genotype on follicular diameter with respect to aromatase activity in granulosa cells from non-atretic follicles (Fig. 1b). When follicles reached 2–2.5 mm in diameter, aromatase activity was already significantly higher in FF than in F+ ewes ( $P < 0.001$ ) which in turn was significantly higher than in ++ ewes ( $P < 0.01$ ). In FF ewes, the highest values for aromatase activity were recorded in granulosa cells from 3–4.5 mm diameter follicles whereas in F+ and ++ ewes peak activity was reached when follicles were 3–5.5 mm and  $\geq 5$  mm in diameter respectively.

#### *Androstenedione output from LH-stimulated thecal tissue from FF, F+ and ++ ewes*

Histological analysis of the dissected tissue showed that  $62 \pm 3\%$  (s.e.m.,  $n = 51$ ) of the material was authentic theca interna. The major contaminant was theca externa and/or stroma with a residual contamination of red blood cells and granulosa cells (i.e.  $\leq 4$  granulosa cells per  $5 \mu\text{m}$  section).

Regardless of genotype, the geometric mean androstenedione contents (and 95% confidence limits) in theca interna and theca externa at the time of isolation were 1 (1,3) ng/10 mg theca interna ( $N = 24$  ewes) and 1 (0.7,1.3) ng/10 mg theca externa ( $N = 13$  ewes) respectively. After perfusion, the androstenedione outputs were not corrected for tissue purity or androstenedione content at time of recovery. There was no evidence of any androstenedione being synthesized by perfused theca externa during and/or after stimulation with LH. In contrast, theca interna tissue produced a level of androstenedione which was substantially more than was present in the tissue at time 0 h. For FF ( $N = 14$  ewes), F+ ( $N = 16$ ) and ++ ewes ( $N = 18$ ), the geometric mean cumulative androstenedione outputs (and 95% confidence limits) during and after LH stimulation were 55 (40,74), 47 (33,66) and 48 (37,63) ng/10 mg theca interna/3 h respectively. There was no effect of genotype on the ability of LH-stimulated theca to secrete androstenedione. Moreover, irrespective of genotype, there was no effect of time after cloprostenol treatment (i.e. 0–6 h, 12–24 h, 36–48 h) on the number of thecal preparations from non-atretic follicles which secreted high ( $\geq 40$  ng) or low ( $< 40$  ng androstenedione/10 mg tissue/3 h) quantities of steroid ( $P > 0.1$ , contingency table analysis). A threshold of 40 ng/10 mg theca interna was chosen because about 50% of the values were above this value.

#### *Relationships between the number of 'oestrogenic' follicles, follicular diameter, granulosa-cell number, oestradiol secretion rate and genotype*

At 12–36 h after cloprostenol injection, all the FF ( $N = 9$ ), F+ ( $N = 9$ ) and all but one of the ++ ewes ( $N = 8$ ) each contained at least one ovary with an 'oestrogenic' follicle (i.e.  $\geq 50$  ng oestradiol/ml follicular fluid). At other times after cloprostenol treatment some of the ewes had no 'oestrogenic' follicles (see also McNatty *et al.*, 1982, 1985a). For ++ ewes at 12, 24 and 36 h respectively after cloprostenol injection (3 ewes/time) 1, 2 and 1 ewes had two 'oestrogenic' follicles, 1, 1 and 2 ewes had one 'oestrogenic' follicle and 1 of the animals at 12 h had no 'oestrogenic' follicles. For F+ ewes at 12, 24 and 36 h respectively after cloprostenol injection 1, 0 and 2 had four 'oestrogenic' follicles, 2, 2 and 1 had three 'oestrogenic' follicles and 0, 1 and 0 had two such follicles. For the FF ewes at 12, 24 and 36 h respectively after cloprostenol, 0, 1 and 0 had 6 'oestro-

genic' follicles, 2, 1 and 3 had 5 'oestrogenic' follicles and 1, 1 and 0 had 3 or 4 such follicles. The data summarizing the number and diameter of 'oestrogenic' follicles and the number of granulosa cells in FF, F+ and ++ ewes, together with the oestradiol secretion rate from ovaries containing 'oestrogenic' follicles at 12–36 h after cloprostenol treatment are shown in Table 4. On average, the FF and F+ ewes contained 3.2-fold and 2.1-fold more 'oestrogenic' follicles respectively than did the ++ ewes. However, the respective mean follicular diameters in FF and F+ ewes were 2.2 mm and 1.8 mm smaller (both  $P < 0.01$ ) than those in ++ ewes. In addition, the respective mean number of granulosa cells in FF and F+ ewes were  $2.7 \times 10^6$  and  $2.1 \times 10^6$  fewer than in ++ ewes ( $P < 0.01$  for F+ and FF ewes vs ++ ewes) (Table 4). However, when the number of 'oestrogenic' follicles was multiplied by the number of granulosa cells in each of these follicles for each genotype, then the total number of cells in the 'oestrogenic' follicles from each genotype was identical (Table 4). Moreover, the oestradiol secretion rates either from the ovaries containing 'oestrogenic' follicles or from both ovaries from each ewe with respect to genotype were not significantly different (Table 4). Over the 12–36 h period after cloprostenol treatment the geometric mean (and 95% confidence limits) oestradiol concentrations in peripheral plasma were 9.8 (7.8, 12.3), 10.8 (7.6, 15.3) and 10.8 (8.1, 14.3) pg/ml for ++, F+ and FF ewes respectively; these values were not significantly different from one another (ANOVA).

**Table 4.** Number and diameter of 'oestrogenic' follicles\* and the number of granulosa cells in FF, F+ and ++ ewes together with the oestradiol secretion-rate† at 12–36 h after cloprostenol injection

Genotype	Ewes		'Oestrogenic' follicles			
	No.	No./ewe	Diam. (mm)	Granulosa cell no./follicle ( $\times 10^{-6}$ )	Total granulosa cell no./ewe ( $\times 10^{-6}$ )	Oestradiol secretion rate (ng/min)§
++‡	8	1.5 ± 0.2 <sup>a</sup>	5.1 ± 0.3 <sup>a</sup>	3.8 ± 0.4 <sup>a</sup>	5.4 ± 0.4	3.2 (1.6, 5.8)
F+	9	3.2 ± 0.2 <sup>b</sup>	3.3 ± 0.2 <sup>b</sup>	1.7 ± 0.1 <sup>b</sup>	5.4 ± 0.4	4.5 (3.0, 6.5)
FF	9	4.8 ± 0.3 <sup>c</sup>	2.9 ± 0.1 <sup>b</sup>	1.1 ± 0.05 <sup>c</sup>	5.4 ± 0.5	3.0 (1.9, 4.5)

Values are mean ± s.e.m.

\* Follicle containing  $\geq 50$  ng oestradiol/ml follicular fluid.

† Oestradiol secretion rate = total output from left and right ovaries.

‡ One ewe excluded because no 'oestrogenic' follicles were present.

§ Geometric means with 95% confidence limits.

For each column a vs b,  $P < 0.01$ ; a vs c,  $P < 0.01$ ; b vs c,  $P < 0.025$  (ANOVA).

At 48 h after cloprostenol treatment, the respective mean ( $\pm$  s.e.m.) diameters of the presumptive preovulatory follicles in FF, F+ and ++ ewes were  $3.4 \pm 0.3$  mm,  $4.1 \pm 0.2$  mm and  $6.8 \pm 0.3$  mm (FF vs ++,  $P < 0.01$ ; FF vs F+,  $P < 0.05$ ; F+ vs ++,  $P < 0.05$ , ANOVA). Moreover, the respective mean numbers of granulosa cells in these follicles were  $1.8 \pm 0.3 \times 10^6$ ,  $2.2 \pm 0.3 \times 10^6$  and  $6.6 \pm 0.3 \times 10^6$  (FF vs ++,  $P < 0.01$ ; FF vs F+,  $P < 0.05$ ; F+ vs ++,  $P < 0.01$ , ANOVA). At 48 h after cloprostenol treatment, the median (and range) of oestradiol secretion rates (i.e. the sum of secretion rates from the left plus right ovaries) were 1.2 (1.0, 28.7), 3.1 (0.5, 5.6) and 6.9 (3.2, 12.9) ng/min for the ++, F+ and FF ewes respectively (3 ewes/genotype). Moreover the median (and range) of 'oestrogenic' follicles per ewe were 1 (0, 1), 3 (0, 4) and 5 (3, 6) for the ++, F+ and FF ewes respectively (3 ewes/genotype). The corresponding median (and range) of oestradiol concentrations in plasma were 4.5 (2.2, 14.0), 12.2 (7.2, 12.3) and 13.1 (7.4, 17.5) pg/ml for ++, F+ and FF ewes respectively.

*Corpus luteum (CL) numbers, weight and function in FF, F+ and ++ ewes*

The respective mean ( $\pm$  s.e.m.) ovulation rates in FF, F+ and ++ ewes for the cycle under study were  $5.1 \pm 0.4$  (N = 15 ewes),  $2.8 \pm 0.1$  (18 ewes) and  $1.2 \pm 0.1$  (18 ewes). The numbers of animals with 1, 2, 3, 4, 5, 6, 7 and 8 CL were 0, 1, 0, 4, 5, 3, 0 and 2 respectively for the FF ewes, 0, 5, 12, 1, 0, 0, 0 and 0 respectively for the F+ ewes and 15, 3, 0, 0, 0, 0, 0 and 0 respectively for the ++ ewes. Between 0 and 12 h after cloprostenol treatment, there was no significant change in CL weight for any of the genotypes (i.e.  $P > 0.1$ , Students *t* test for each genotype). Since cloprostenol was injected on Day 10 of the oestrous cycle, the weights of the CL over the first 12 h of cloprostenol treatment would, therefore, be representative of those during the mid-luteal phase of the cycle. The respective mean ( $\pm$  s.e.m.) CL weights in FF, F+ and ++ ewes were  $0.16 \pm 0.01$  (N = 6 ewes),  $0.25 \pm 0.02$  (9 ewes) and  $0.53 \pm 0.03$  g (9 ewes); FF vs F+ vs ++;  $P < 0.01$ , ANOVA). Irrespective of genotype in Booroola ewes the ovulation rate had a significant influence on the CL weight. For example, in the aforementioned 24 animals (FF, F+ and ++), the ovulation rates of 1, 2, 3, 4, 5, 6, 7 and 8 were inversely correlated to the respective mean CL weights of  $0.54 \pm 0.03$  (N = 8 ewes),  $0.30 \pm 0.04$  (6),  $0.20 \pm 0.007$  (5),  $0.18$  (2), no sample,  $0.16$  (1), no sample and  $0.14$  (2) g. The correlation is expressed by the equation  $1/y = 0.01 + 1.73x$  where  $x =$  CL weight in g, and  $y =$  the ovulation rate ( $R = 0.91$ , N = 24,  $P < 0.001$ ).

With respect to time after cloprostenol injection, the respective mean ( $\pm$  s.e.m.) concentrations of progesterone in plasma for FF, F+ and ++ ewes were:  $4.0 \pm 0.6$ ,  $1.3 \pm 0.2$  and  $1.4 \pm 0.2$  ng/ml at 0 h;  $1.0 \pm 0.1$ ,  $0.6 \pm 0.02$  and  $0.6 \pm 0.2$  ng/ml after 24 h;  $0.20 \pm 0.05$ ,  $0.14 \pm 0.02$  and  $0.12 \pm 0.02$  ng/ml after 36 h and  $0.10 \pm 0.03$ ,  $0.09 \pm 0.02$  and  $0.10 \pm 0$  after 48 h (N = 3 ewes/genotype per time after cloprostenol injection). At 0 h, the mean weight of CL tissue in FF, F+ and ++ ewes was  $0.99 \pm 0.07$ ,  $0.59 \pm 0.05$  and  $0.53 \pm 0.04$  g (N = 3 sheep per genotype; FF vs F+,  $P < 0.02$ ; FF vs ++,  $P < 0.02$ , ANOVA).

### Discussion

The aim of the present study was to compare aspects of follicular development in FF Booroola  $\times$  Merino ewes with those found in F+ and ++ Booroola  $\times$  Merino ewes. It could be argued that some of the observed differences between FF or F+ ewes and ++ ewes were attributable to the age differences of the animals since the FF/F+ ewes were 6–8 years old and the ++ animals 4–5 years old. However, this is unlikely as the ++ Booroola  $\times$  Merino animals in this study have follicle numbers, granulosa cell numbers, oestradiol and testosterone concentrations in follicular fluid and levels of aromatase activity in granulosa cells which are similar to those reported for 6–8-year-old Booroola  $\times$  Romney ++ animals in an earlier study (McNatty *et al.*, 1985a). Moreover Driancourt, Cahill & Bindon (1985) report that the number of antral follicles in 2-year-old Booroola  $\times$  Merinos did not differ from that in 8-year-old animals.

These results show that ovarian follicular function in FF ewes differs in some respects from that in F+ ewes which in turn differs from that in ++ animals. The proportions of follicles with high concentrations of testosterone ( $\geq 25$  ng/ml) and oestradiol ( $\geq 50$  ng/ml) in follicular fluid at different times after cloprostenol injection were related to genotype (Table 2). Also, in most instances, the levels of oestradiol in follicular fluid and aromatase activity in granulosa cells were significantly higher in non-atretic 2–4.5 mm diameter follicles from F-gene carriers (FF and/or F+ ewes) compared to those from ++ ewes (Figs 1 & 2). These findings together with those showing the influence of follicular health on aromatase activity (Table 3) confirm and extend those of an earlier study which examined the above variables in non-atretic and atretic follicles from F+ and ++ Booroola ewes (McNatty *et al.*, 1985a). When comparing FF and F+ ewes, the levels of oestradiol in follicular fluid did not differ between the genotypes over any range of follicular diameters from 1 mm to 4.5 mm (Fig. 2). However, high concentrations of oestradiol (i.e.  $\geq 50$  ng/ml) were often

reached in 2–2.5 mm diameter follicles in FF ewes (i.e. in 40% of non-atretic follicles; data not shown) which was a less common occurrence in the other genotypes (i.e. in 17% and 2% in non-atretic follicles from F+ and ++ ewes respectively). This tendency to synthesize large amounts of oestradiol in small sized follicles in FF compared to F+ ewes was evident when granulosa cell aromatase activity was examined. Aromatase activity was significantly higher in cells from non-atretic 2–2.5 and 3–3.5 mm follicles from FF ewes than in those from F+ ewes.

Another major difference between F-bearing ewes and ++ animals was the number of granulosa cells present in follicles of similar size. The cell numbers in FF or F+ ewes were always lower ( $P < 0.05$ ) than those in ++ ewes over all follicle sizes that could be compared (i.e. 1–4.5 mm diameter). There was a tendency for the cell numbers to be lower in FF ewes compared to F+ ewes but the differences were never statistically different for the pooled data. However, when the cell numbers in oestrogen-secreting follicles at 12–36 h after a cloprostenol-induced follicular phase for FF and F+ ewes were examined, a significant difference in cell numbers between these two genotypes was evident ( $P < 0.025$ , Table 4).

In this study the numbers of granulosa cells in the 'oestrogenic' and/or presumptive pre-ovulatory follicles of F-bearing ewes although similar to those in our previous report (McNatty *et al.*, 1985a) were very much higher than those of Driancourt *et al.* (1985) who in turn reported higher mean values than did Baird *et al.* (1982). In the present study and that of Baird *et al.* (1982), the freshly isolated cells were counted by haemocytometer after treatment of the ewes with cloprostenol. A possible explanation of discrepancy between the studies might be related to the efficiency of cell recovery from the follicle wall. In our study, recovery of granulosa cells was routinely in excess of 90% since histological sections of the follicle wall showed  $\leq 4$  granulosa cells/5  $\mu\text{m}$  section. In the study of Driancourt *et al.* (1985), the number of cells was calculated from histological sections of ovaries of ewes previously primed with exogenous progesterone. The cell numbers were determined from a knowledge of the mean cellular density and the estimated area of the follicle occupied by the granulosa cells. Thus differences between our study and that of Driancourt *et al.* (1985) are likely to be due to the hormone pretreatment of the animals and/or the methods of cell quantification.

The collective evidence from this and previous studies (Henderson *et al.*, 1985; McNatty *et al.*, 1985a) suggests that the ovulation-rate differences between the Booroola genotypes (FF, F+ and ++ ewes) are not due to absolute differences in the sizes of the antral follicle pool (Table 1; Driancourt *et al.*, 1985) as has been inferred for other breeds of sheep (Lahlou-Kassi & Mariana, 1984). Instead, the ovulation rate differences are more likely to be a consequence of follicles in F-bearing ewes being more sensitive to gonadotrophin stimulation compared to similar size follicles in ++ ewes with respect to cAMP synthesis (Henderson *et al.*, 1985). This fundamental difference between the genotypes appears to be established before and/or during antrum formation since basal cAMP concentrations in 0.1–0.5 mm diameter follicles of FF and F+ ewes are higher than in ++ ewes (McNatty, Kieboom, McDiarmid, Heath & Lun, 1985b). Although the reasons for these differences in gonadotrophin sensitivity are not known, they are likely to be ultimately responsible for the genotypic differences described herein, namely in the numbers of atretic and non-atretic follicles (Table 1), steroid synthesis (Fig. 2), granulosa cell number (Fig. 1) and the diameter of mature (i.e. oestrogen-secreting) follicles 12–48 h after cloprostenol injection (Table 4 and 'Results'; see also Driancourt *et al.*, 1985). Consequently, follicles in FF ewes reach maturity when between 2 and 4.5 mm in diameter, whereas in F+ ewes they are more likely to be between 3 and 4.5 mm in diameter; in FF and F+ ewes, follicles  $\geq 5$  mm diameter were rarely found (Table 1). In contrast, in ++ ewes, mature follicles were  $\geq 4$  mm in diameter and most commonly  $\geq 5$  mm in diameter (see also Henderson *et al.*, 1985; McNatty *et al.*, 1985a). This difference in size of the follicles at maturity is probably an important factor in determining the ovulation-rate differences between the genotypes since the smaller the follicle is at maturity, the greater the pool of potentially ovulatory follicles. In FF ewes, the total (and non-atretic) number of follicles  $\geq 2$  mm in diameter varied from 5.2 to 13.0 (3.3 to 9.4), in F+ ewes the total (and non-atretic) number of



follicles  $\geq 3.0$  mm varied from 1.5 to 4.1 (1.2–3.6), whereas in ++ ewes the number of follicles  $\geq 4$  mm varied from 1 to 2 (0.7 to 1.8). The actual number of non-atretic (see Table 1) as well as ovulatory follicles within the above size ranges for each genotype is probably influenced by the temporal fluctuations ( $\sim 5$ –20%) in plasma FSH concentrations before, during or after luteolysis (McNatty *et al.*, 1985c).

In the present study, the mean numbers of 'oestrogenic' follicles in FF, F+ and ++ ewes at 12–36 h after cloprostenol injection were similar to the mean ovulation rates for the same ewes from the previous cycle. However, despite the differences in the numbers of oestrogenic follicles in each genotype, the oestradiol secretion rates for each of the genotypes were similar (Table 4). As with our previous findings for F+ and ++ ewes (McNatty *et al.*, 1985a), the equivalent rates of oestradiol secretion at 12–36 h after cloprostenol injection for the three genotypes are almost certainly related to the fact that the total population of granulosa cells in the 'oestrogenic' follicles were identical. It could therefore be argued that the maturation of  $\geq 5$  preovulatory follicles in FF ewes and 3–4 such follicles in F+ ewes may be necessary to provide a cell mass capable of producing the same quantity of oestradiol as that from 1 or 2 such follicles in ++ ewes. At 48 h after cloprostenol injection, the median plasma oestradiol concentrations and the median oestradiol secretion rate in FF ewes was  $\sim 3$  and  $\sim 6$ -fold higher respectively than the corresponding values in ++ ewes, whereas those for F+ ewes were in between. Moreover, at this time all ovaries of FF ewes ( $N = 3$ ) contained 'oestrogenic' follicles whereas this was not the case for the F+ ( $N = 3$ ) and ++ ewes ( $N = 3$ ). These findings might suggest that ovarian oestradiol secretion continues for a longer period in FF ewes than in F+ and ++ ewes, but this will require confirmation with larger numbers of animals per genotype.

Thecal tissue is a major source of androstenedione in the sheep ovary (McNatty *et al.*, 1985a). In the present study, the theca interna was the sole source of this steroid, with neither the theca externa, stroma (McNatty *et al.*, 1985a) or granulosa cells (data not shown) contributing appreciable levels of this steroid *in vitro*. Using a 20-min LH pulse of 8 ng/ml, no significant effect of genotype on androstenedione could be demonstrated. Indeed the cumulative (3 h) androstenedione output after an 8 ng/ml LH pulse was no different from that observed from thecae from F+ and ++ ewes that had been subjected to a 20-min LH pulse of 200 ng/ml (McNatty *et al.*, 1985a). The LH receptor characteristics in the theca interna (i.e. the equilibrium dissociation constant and maximum binding capacity) do not differ between F+ and ++ genotypes (L. E. Kieboom & K. P. McNatty, unpublished data). Also, the number of thecal LH receptors in a 4–5 mm follicle, from a ewe of either genotype, which contains about 5 mg theca interna (wet weight), was estimated to be about  $1 \times 10^{11}$ . If it is assumed that this receptor number is not altered substantially over a 20-min perfusion interval, then a 20-min LH pulse of 8 ng/ml (i.e. at 1.4 ml/min) may still be an excessively high dose of LH. Thus, to answer the question as to whether genotypic differences exist with respect to thecal sensitivity to LH, it may be necessary to reassess the androstenedione response to lower doses of LH than have so far been attempted. Nevertheless, the lack of difference in the follicular fluid concentrations of androstenedione (McNatty *et al.*, 1985a), or of testosterone (Fig. 2), which is a major metabolite of androstenedione, do not indicate that the theca is an important contributor to the differences in oestradiol concentration that have been observed between the genotypes.

In FF ewes, the CL were only 0.30 times the weight of those in ++ ewes, whereas in F+ ewes the CL were 0.47 times those in ++ ewes. These findings are consistent with the view that the CL in FF and F+ ewes originated from smaller preovulatory follicles. The finding of an inverse linear correlation between ovulation rate and CL weight is also consistent with the negative correlation between the number of putative preovulatory follicles and their number of granulosa cells (Driancourt *et al.*, 1985). Moreover, it is consistent with the view that for Booroola ewes, at least, the higher the ovulation rate, the smaller the diameter the preovulatory follicles are likely to be at ovulation. On Day 10 of the oestrous cycle, the plasma concentration of progesterone was significantly higher in the FF ewes than in the F+ or ++ ewes. This difference may be due in part to the total CL mass in the FF ewes being twice that in F+ or ++ ewes. It is not known whether the

progesterone concentrations in FF ewes are consistently higher than those in the other genotypes; this will need to be determined in another study.

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## <sup>125</sup>I-labelled hCG binding characteristics in theca interna and other tissues from Romney ewes and from Booroola × Romney ewes with and without a major gene influencing their ovulation rate

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**Summary.** Specific receptors for <sup>125</sup>I-labelled hCG in ovarian follicle wall were located in the theca interna. No specific binding of <sup>125</sup>I-labelled hCG was found in theca externa and/or stromal tissue. The kinetics of <sup>125</sup>I-labelled hCG binding to theca interna followed second order kinetics with calculated association rate constants ( $k_a \pm$  s.d.) of  $1.57 \pm 0.16 \times 10^6$  and  $0.57 \pm 0.02 \times 10^6$  litres mol<sup>-1</sup> sec<sup>-1</sup> at 37°C and 22°C respectively. Dissociation of specifically bound <sup>125</sup>I-labelled hCG from theca interna was minimal at 37°C and 22°C. The binding of <sup>125</sup>I-labelled hCG to theca interna could be displaced with PMSG, FSH-P and sheep LH but other sheep pituitary hormones and LH-releasing hormone showed little or no cross-reaction.

The calculated binding capacities ( $B_{max}$ ) and equilibrium dissociation constants ( $K_d$ ) for <sup>125</sup>I-labelled hCG binding to theca interna did not differ between Romney ewes and Booroola × Romney ewes with and without the fecundity (F) gene on Day 10 of the oestrous cycle, during anoestrus or at 36 h after an injection of cloprostenol on Day 10 of the oestrous cycle. When the data for Day 10 and anoestrus were pooled, the median (range)  $B_{max}$  and  $K_d$  values in non-atretic follicles ( $\geq 3$  mm diameter) were 12.0 (5.1-23.5) fmol/mg protein and 0.10 (0.05-0.16) nM respectively. At 36 h after cloprostenol injection the respective median (range)  $B_{max}$  and  $K_d$  values in non-atretic follicles ( $\geq 3$  mm diam.) increased to 46.9 (28.4-70.3) fmol/mg protein and 0.23 (0.13-0.65) nM respectively. In corpora lutea the hCG binding characteristics were similar in all the above breeds/genotypes. On Day 10 of the cycle, the mean  $B_{max}$  but not the mean  $K_d$  value was significantly higher ( $P < 0.01$ ) than the corresponding value at 36 h after cloprostenol injection. In granulosa cells, from follicles of  $\geq 5$  mm diameter of Romney and Booroola × Romney (+ +) ewes and from follicles of  $\geq 3$  mm diameter of Booroola × Romney (F +) ewes, the hCG binding characteristics were similar. In granulosa cells from smaller sized follicles from the above breeds/genotypes, no specific hCG binding was noted.

### Introduction

The ovarian follicle wall is the major source of androgen synthesis in the sheep ovary (Moor, 1977; McNatty *et al.*, 1985a). In the sheep as well as in other species, the synthesis of androgen (i.e. androstenedione and/or testosterone) is regulated, at least in part, by luteinizing hormone (LH; McCracken, Uno, Goding, Ichikawa & Baird, 1969; Baird, Swanston & Scaramuzzi, 1976; McNatty *et al.*, 1984; McNatty *et al.*, 1985a). Specific <sup>125</sup>I-labelled human chorionic gonadotrophin (hCG) binding has been demonstrated in the sheep follicle wall in several reports (Carson, Findlay, Burger & Trounson, 1979; McNatty, 1982; Webb & England, 1982). LH and hCG are

known to share a common receptor (Lee & Ryan, 1973; Dufau & Catt, 1978) and these hormones were equipotent at stimulating androstenedione synthesis from perfused thecal tissue from cow ovaries (Henderson, Kieboom, McNatty, Lun & Heath, 1984). However, despite knowledge of the existence of hCG/LH receptors in the follicle wall of sheep ovaries, the maximum binding capacities ( $B_{max}$ ), equilibrium dissociation constants ( $K_d$ ) and association ( $k_a$ )/dissociation ( $k_d$ ) rate constants for these receptors are unknown.

In most sheep breeds, the mean ovulation rate (i.e. as judged by the number of corpora lutea formed per oestrous cycle) normally varies between 1 and 2. In contrast, ewes of the Booroola genotype may sometimes have a mean ovulation rate between 3 and 6. These particular animals are thought to contain a major gene(s) which influences their ovulation rate (Davis, Montgomery, Allison, Kelly & Bray, 1982; Piper & Bindon, 1982). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative fecundity gene have tentatively been segregated on the basis of at least one ovulation rate recording of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). There is evidence to suggest that the ovulation rate differences between Booroola genotypes may be attributable to differences in the sensitivity of ovarian follicles to the gonadotrophins, follicle-stimulating hormone (FSH) and LH (Piper, Bindon, Curtis, Cheers & Nethery, 1982; Kelly, Owens, Crosbie, McNatty & Hudson, 1983; Henderson, Kieboom, McNatty, Lun & Heath, 1985). For example, granulosa cells from follicles (3–4 mm diam.) in F+ ewes synthesize significantly more adenosine cyclic 3',5'-monophosphate in response to LH or FSH than cells from similar sized follicles in ++ ewes. However, the characteristics of the FSH and LH receptors in ovaries of Booroola ewes with or without the F gene have not been described.

The purpose of this study was to examine the characteristics of  $^{125}\text{I}$ -labelled hCG binding to theca interna tissue from Romney ewes and Booroola  $\times$  Romney ewes with or without the F gene and to compare these with binding to other ovarian tissues.

## Materials and Methods

### *Animals*

The Romney ewes (40–60 kg) were parous animals 2–3 years of age. The Booroola  $\times$  Romney ewes were between 6 and 9 years of age. All the Booroola ewes were culls from breeding flocks at the Invermay Agricultural Research Centre, Mosgiel, New Zealand. These animals were known to be parous but their weights at the time of the study were unknown. The Booroola ewes classified as F+ (N = 67) had had at least 3 annual ovulation rate recordings of 3 or 4 whereas those classified as ++ (N = 67) had repeated annual ( $\geq 3$ ) ovulation-rate recordings of 1 or 2. The ovaries of ewes studied during anoestrus were recovered during November, December and January, whereas those studied during the breeding season were recovered during May, June, July and August. All ovaries during the breeding season were recovered on Day 10 of the oestrous cycle (Day 0 = oestrus) or 36 h after a s.c. injection of 125  $\mu\text{g}$  cloprostenol (Coopers Animal Health Laboratories, Upper Hutt, New Zealand) which was given on Day 10 of the cycle.

### *Preparation of ovarian tissues for binding studies*

*Granulosa cells.* All follicles ( $\geq 1$  mm diam.) were dissected from Romney (N = 20) and Booroola  $\times$  Romney (F+, N = 20; ++, N = 20) ewes, and classified as atretic or non-atretic as previously described (McNatty *et al.*, 1985a). Briefly, non-atretic follicles were those containing a vascularized theca interna, were devoid of debris in the follicular fluid, had  $\geq 25\%$  of the maximum number of granulosa cells for a given follicular diameter and contained a healthy-looking oocyte. An atretic follicle was one for which one or more of the above criteria was not satisfied. The maximum number of granulosa cells in follicles from Romney and Booroola  $\times$  Romney ewes with and with-

out the fecundity (F) gene are reported elsewhere (McNatty, 1982; McNatty *et al.*, 1985a). The isolated cells from each follicle in dissection medium (minimum essential medium plus Earle's salts plus 1% (w/v) bovine serum albumin) were pooled according to follicle diameter (i.e. 1–2.5 mm, 3–4.5 mm or  $\geq 5$  mm) and follicle health. Thereafter they were centrifuged at 450 *g* at 4–6°C for 20 min and the cellular pellet resuspended in Tris buffer [i.e. 0.05 M-Tris-HCl buffer containing 0.1 M-sucrose and 5 mM-MgCl<sub>2</sub> (pH 7.5)] to a final concentration of 4–10  $\times 10^6$  cells/ml. The cell suspensions were added in 0.5 ml aliquants to assay tubes, capped and stored at –70°C until the binding studies were performed. Since it was necessary to pool granulosa cells from 10–20 ewes to obtain sufficient points for a Woolf plot, we studied the hCG binding characteristics at only one stage of the oestrous cycle.

*Thecal and luteal tissue.* Thecae internae were separated from thecae externae as well as the granulosa cells as has been described in detail for the cow ovary (McNatty *et al.*, 1984). Routine histological examination of the purity of thecae internae from non-atretic follicles indicated that 64  $\pm$  2% (s.e.m.; 64 pools of thecae) of the tissue was theca interna with the major contaminant being theca externa and/or stroma with a residual contamination of red blood cells and granulosa cells (< 4 granulosa cells per 5  $\mu$ m section of thecae). Three pools of thecae internae from atretic follicles were studied. Histological examination of the purity of these pools indicated that 35, 40 and 42% of the respective preparations were theca interna with the major contaminant being theca externa/stroma; no granulosa cells were observed. The thecae externae were totally devoid of theca interna and granulosa cells. The stromal tissue was a residual tissue devoid of corpora lutea, corpora albicantia, the ovarian cortex, the major ovarian blood vessels and follicles  $\geq 0.1$  mm in diameter.

Normally the entire thecae internae from all non-atretic follicles ( $\geq 1$  mm diam.) from one ewe totalled 6–25 mg (wet weight). Therefore, to undertake a single Woolf study it was necessary to pool thecae from the ovaries of 3–20 ewes. The pooled thecae were homogenized by hand with a glass/glass Potter-Elvehjem homogenizer in Tris buffer (see above). The homogenates were filtered through several layers of sterile surgical gauze and aliquants of the filtrate were stored at –70°C until used for the binding studies. Unless stated otherwise the thecae internae were recovered from non-atretic follicles (McNatty *et al.*, 1985a). Homogenates of pools of luteal tissue were prepared as above. Aliquants of each preparation kept for binding studies were assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

#### *HCG iodination and <sup>125</sup>I-labelled hCG binding studies*

HCG (CR121, 13 450 i.u./mg) was iodinated to a specific activity of 30–50 Ci/ $\mu$ g using lactoperoxidase/H<sub>2</sub>O<sub>2</sub> (Miyachi, Vaitukaitis, Nieschlag & Lipsett, 1972). The specific activity of <sup>125</sup>I-labelled hCG was calculated as described by Bolton (1977). The hCG (5  $\mu$ g) was iodinated using 0.5 mCi Na<sup>125</sup>I (Amersham, U.K.) and the products were purified on a column of Sephadex G-75 (20  $\times$  1 cm).

For the <sup>125</sup>I-labelled hCG binding studies aliquants of tissue homogenates or granulosa cells (100–1000  $\mu$ g protein) were incubated in polystyrene tubes (12  $\times$  75 mm) with <sup>125</sup>I-labelled hCG (0.5–50 ng) in a final volume of 1 ml Tris buffer containing 0.5% egg albumin (Sigma Chemical Co., St Louis, MO, U.S.A.). Incubations were performed at 22 or 37°C for up to 40 h. The receptor-bound <sup>125</sup>I-labelled hCG was separated from free <sup>125</sup>I-labelled hCG by centrifuging the tubes at 4000 *g* for 30 min at 4°C. The supernatant containing free <sup>125</sup>I-labelled hCG was removed by aspiration. The pellets containing the bound <sup>125</sup>I-labelled hCG were washed with ice-cold incubation buffer and the radioactivity in the pellets was determined after a second centrifugation at 4000 *g*. Non-specific binding was determined by co-incubating tissue homogenates or cells with excess unlabelled gonadotrophin (i.e. 10 i.u. gonadotrophin-LH, Paines & Byrne Ltd, Greenford, U.K.); this was equivalent to a 300-fold excess of unlabelled hCG. Dose-response studies indicate

that 10 i.u. gonadotrophin-LH was about twice the minimum amount necessary to displace the maximum amount of  $^{125}\text{I}$ -labelled hCG (5 ng). The amount of specifically bound  $^{125}\text{I}$ -labelled hCG was calculated by subtracting non-specific binding from the total amount of  $^{125}\text{I}$ -labelled hCG bound. Because of the limited amounts of theca interna, datum points were generated from duplicate (i.e. about 60% of the samples) or single incubation studies. Analysis of the duplicates in binding studies showed the coefficient of variation between them to be uniformly below 8%.

With the exception of the equilibrium binding studies all other studies on the characteristics of the hCG/LH receptor were made from tissues of Romney ewes.

#### *The Woolf plots and analysis of results*

From the equilibrium binding studies, the specific binding data were fitted to the Woolf equation, i.e.  $\frac{F}{B} = \frac{K_d}{B_{\max}} + \left(\frac{1}{B_{\max}}\right) F$  (Haldane, 1957), where  $F$  = the concentration of free hormone and  $B$  = the concentration of bound hormone. The advantages of using the Woolf plot instead of the Scatchard plot are discussed by Keightley & Cressie (1980). The respective s.d. of the  $B_{\max}$  and  $K_d$  were derived from the s.d. of the slope and intercept of the Woolf plot (Davis, Thompson & Pardue, 1978). The binding characteristics ( $B_{\max}$ ,  $K_d$ ) with respect to follicle size, reproductive status or genotype were examined by Wilcoxon rank sum test, Student's  $t$  test or analysis of variance.

## Results

#### *Characteristics of $^{125}\text{I}$ -labelled hCG binding to theca interna*

Specific  $^{125}\text{I}$ -labelled hCG binding to theca interna varied with the concentration of  $^{125}\text{I}$ -labelled hCG and the amount of thecal tissue in the incubations. With 4.5 mg of theca interna homogenate per tube, the specific binding of 0.5, 2.5 and 5.0 ng  $^{125}\text{I}$ -labelled hCG (i.e. 5 ng = 277 100 c.p.m.) was, respectively, 1681, 6839 and 10 878 c.p.m. With 1 mg theca interna homogenate per tube, the specific binding of 0.5, 2.5 and 5.0 ng  $^{125}\text{I}$ -labelled hCG was respectively 1032, 3887 and 2163 c.p.m. These data were obtained after performing duplicate incubations at 22°C for 20 h.

The specific binding of  $^{125}\text{I}$ -labelled hCG was also a time- and temperature-dependent process (Fig. 1). At 37°C specific binding increased rapidly for the first 2 h, but thereafter proceeded more slowly to reach a maximum at 20 h. After 40 h incubation at 37°C, the binding was unchanged relative to that at 20 h. Specific binding at 22°C proceeded more slowly than at 37°C with the maximum binding about 20% less than that achieved at 37°C.

The specific binding of  $^{125}\text{I}$ -labelled hCG as shown in Fig. 1 followed second-order kinetics.

From the equation  $\ln \left[ \frac{b(a-x)}{a(b-x)} \right] = k_a t$  where the initial hormone concentration, receptor concentration, hormone-receptor complex concentration and time are represented by the terms  $a$ ,  $b$ ,  $x$  and  $t$  respectively, the association rate constants ( $k_a \pm \text{s.d.}$ ) were calculated to be  $1.57 \pm 0.16 \times 10^6$  and  $0.57 \pm 0.02 \times 10^6$  litres  $\text{mol}^{-1} \text{sec}^{-1}$  at 37°C and 22°C respectively. The  $k_a$  values at 37 or 22°C were obtained from regression lines fitted through 6 or 7 datum points over the first 90 (at 37°C) or 120 min (at 20°C).

The dissociation of specifically bound  $^{125}\text{I}$ -labelled hCG from ovine theca interna appeared to be minimal. To test for dissociation, theca interna (0.3 mg protein) was preincubated with  $^{125}\text{I}$ -labelled hCG (5 ng) for 20 h at 22°C. The bound  $^{125}\text{I}$ -labelled hCG was pelleted by centrifugation as described in the 'Materials and Methods'. The washed pellet was then resuspended and reincu-

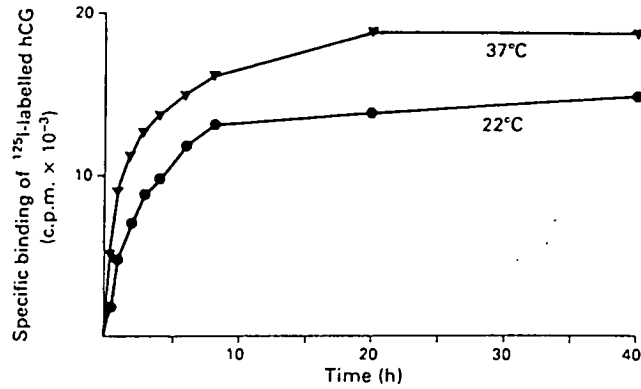


Fig. 1. Time dependence of specific  $^{125}\text{I}$ -labelled hCG (5 ng/tube) binding to sheep theca interna tissue (0.3 mg protein/tube) at 22 and 37°C. Values are means of duplicates.

bated in 1 ml Tris-HCl buffer (0.05 M, pH 7.5) at 37 or 22°C for up to 6 h. At 37°C, 6% of the label had dissociated after 15 min and 8% had dissociated after 6 h. At 22°C, 4% of the label had dissociated after 15 min and 6% after 6 h.

#### Specificity studies

The binding of  $^{125}\text{I}$ -labelled hCG (1 ng) to sheep theca interna could be displaced with equimolar amounts of ovine LH (oLH: NIH-LH-S23) or hCG (CR121) (Fig. 2). Ovine FSH (NIH-FSH-S11) showed ~0.01% cross-reaction with hCG but luteinizing hormone-releasing hormone (Peninsular Laboratories Inc., Belmont, California, U.S.A.), ovine growth hormone (NIH-GH-S10), ovine prolactin (NIH-P1-S12), ovine thyroid-stimulating hormone (NIH-TSH-S6) showed no ability to displace  $^{125}\text{I}$ -labelled hCG at the concentrations tested. The gonadotrophic preparations PMSG (NICHD-PMSG) and FSH-P (Burns-Biotec Laboratories Inc., Omaha, Nebraska, U.S.A.) over the respective ranges of 0.1–100 units and 0.001–10 mg were effective in displacing  $^{125}\text{I}$ -labelled hCG from the theca interna receptor. For example, 1 unit PMSG and 0.005 mg FSH-P showed 25% and 100% cross-reaction respectively with  $^{125}\text{I}$ -labelled hCG.

#### Equilibrium binding studies

Binding of  $^{125}\text{I}$ -labelled hCG to sheep theca interna as a function of  $^{125}\text{I}$ -labelled hCG concentration is shown in Fig. 3(a). Incubations were performed at 22°C for 20 h to achieve equilibrium. Typically a linear plot was obtained when the specific binding data were fitted to the Woolf equation (Keightley & Cressie, 1980; Fig. 3b). The calculated binding capacity ( $B_{\text{max}}$ ) and equilibrium dissociation constant ( $K_d$ ) from the Woolf plot were 23.5 fmol/mg protein and 0.10 nM respectively.

The calculated  $B_{\text{max}}$  and  $K_d$  values from Woolf plots of specific  $^{125}\text{I}$ -labelled hCG binding to theca interna from Romney ewes and Booroola × Romney ewes with respect to follicular health and reproductive status are shown in Table 1. All values were obtained from 6–9 datum points on the Woolf plots with linear correlation coefficients in excess of 0.9. Regardless of reproductive status and genotype, specific hCG/LH receptors were present in theca interna of small (1–2.5 mm diam.), medium (3–4.5 mm diam.) and large ( $\geq 5$  mm diam.) follicles. Moreover, they were also detectable in atretic follicles although the  $B_{\text{max}}$  values were low. In contrast, no specific binding was found in any of the theca externa or stromal tissues. Irrespective of follicle size and health, the  $B_{\text{max}}$  and  $K_d$  values for theca interna on Day 10 (i.e. pooled across genotypes) were not different from



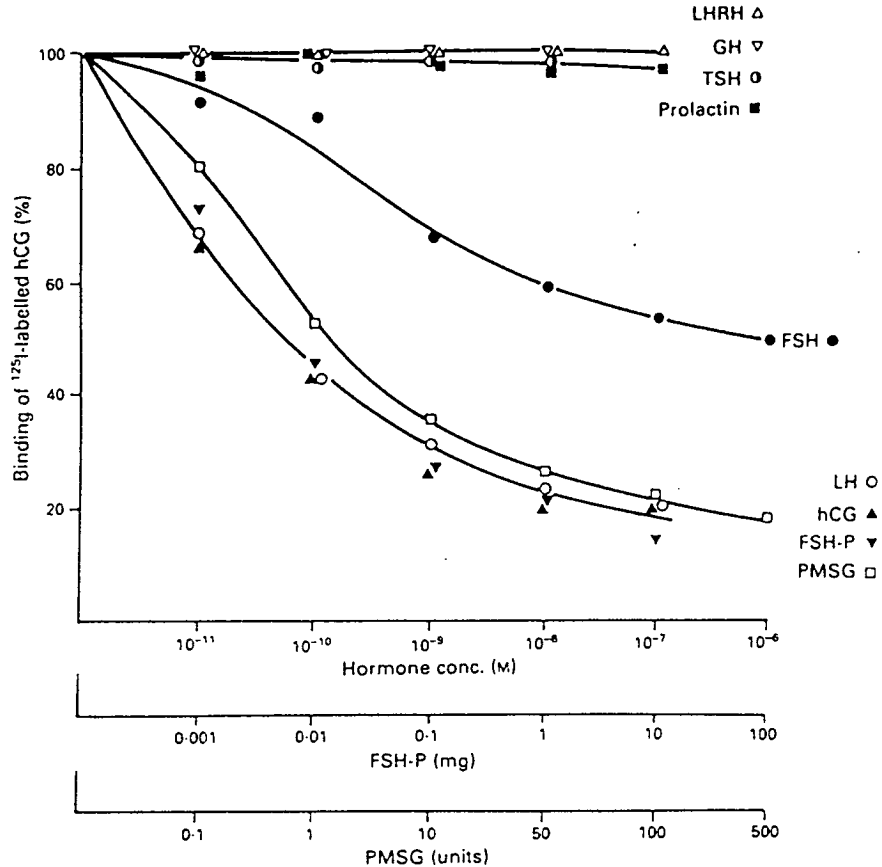


Fig. 2. Displacement of <sup>125</sup>I-labelled hCG binding by increasing amounts of unlabelled hormones. Binding in the absence of unlabelled hormone was regarded as 100%. FSH-P is a material of unspecified (species) origin and purity. Accordingly, the FSH-P scale differs from that of other hormones. Likewise the data for PMSG (NICHD-PMSG) was plotted on a scale which is unrelated to those of the other hormones. Each point is the mean of duplicate measurements.

those found during anoestrus (i.e. also pooled across genotypes;  $P > 0.05$ , Wilcoxon rank sum test). Since all the studies were based on one or two pools of tissue with respect to genotype, reproductive status and/or follicle health, it is not possible to establish whether the apparent difference in  $B_{max}$  and  $K_d$  values in the 3–4.5 mm diameter follicles in Booroola × Romney F+ ewes between Day 10 and anoestrus is statistically significant. When the hCG/LH receptor characteristics in non-atretic follicles for Day 10 and anoestrus were combined with respect to genotype, they were found not to be genotypically different (ANOVA). For all genotypes, the median (range)  $B_{max}$  and  $K_d$  values in non-atretic 1–2.5 mm follicles (anoestrus + Day 10 pooled,  $n = 4$ ) were 6.4 (5.1–9.5) fmol/mg protein and 0.10 (0.05–0.16) nM respectively. In the medium- to large-sized follicles ( $\geq 3$  mm diam.) the respective median (range)  $B_{max}$  and  $K_d$  values in non-atretic follicles (anoestrus + Day 10 pooled regardless of genotype,  $n = 9$ ) were 12.0 (5.5–23.5) fmol/mg protein and 0.10 (0.06–0.16) nM. When the above pooled (anoestrus + Day 10)  $B_{max}$  and  $K_d$  values for theca interna in 1–2.5 mm diameter non-atretic follicles were compared to those in  $\geq 3$  mm diameter non-atretic follicles by the Wilcoxon rank sum test, they were not significantly different. Collectively, however, these values were much lower than those recorded at 36 h after cloprostenol

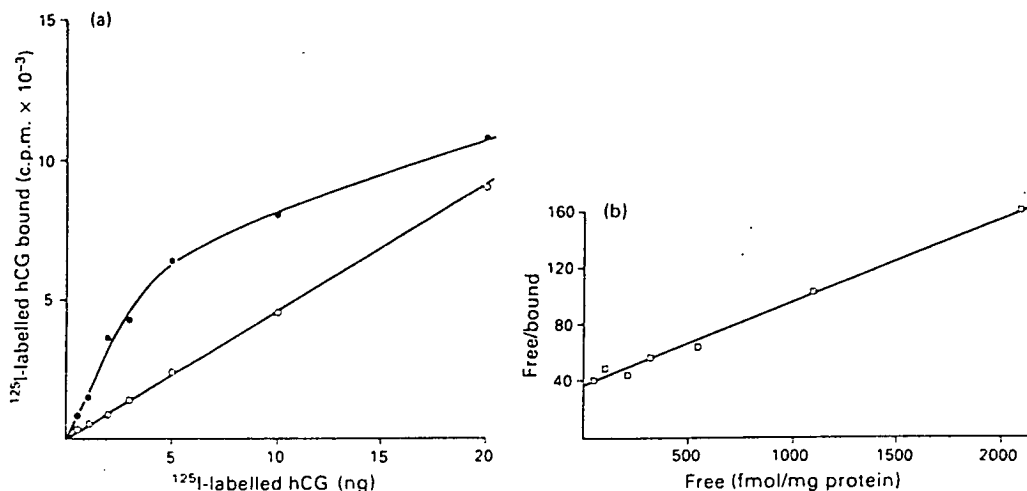


Fig. 3. (a) Specific (●—●) and non-specific (○—○) binding of  $^{125}\text{I}$ -labelled hCG to theca interna tissue with respect to  $^{125}\text{I}$ -labelled hCG concentration. Thecae internae were recovered from 3–4.5 mm non-atretic follicles from F+ Booroola  $\times$  Romney ewes during anoestrus. (b) Woolf plot of the above data for specific  $^{125}\text{I}$ -labelled hCG binding to theca interna.

injection. At this time the respective median (range)  $B_{\max}$  and  $K_d$  values in non-atretic follicles ( $\geq 3$  mm diam.) over all genotypes were 46.9 (28.4–70.3) fmol/mg protein and 0.23 (0.13–0.65) nM respectively. The median  $B_{\max}$  and  $K_d$  values in non-atretic  $\geq 3$  mm diameter follicles over all genotypes at 36 h after cloprostenol were both significantly higher ( $P < 0.01$ ; Wilcoxon rank sum test) than the corresponding values in non-atretic  $\geq 3$  mm diameter follicles during anoestrus and Day 10.

The characteristics of the hCG/LH receptor in sheep luteal tissue and in granulosa cells with respect to breed (genotype), reproductive status and follicle health are shown in Tables 2 and 3. On Day 10 of the cycle the  $B_{\max}$  and  $K_d$  values in luteal tissue (Table 2) were similar for all breeds (genotypes). Moreover, these  $B_{\max}$  values were 5- to 26-fold higher than those found at 36 h after cloprostenol injection. When the  $B_{\max}$  values on Day 10 were pooled across the genotypes (mean  $\pm$  s.e.m. =  $38.5 \pm 5.2$  fmol/mg protein, Table 2) and compared to the pooled values at 36 h after cloprostenol injection (mean  $\pm$  s.e.m. =  $2.9 \pm 0.5$  fmol/mg protein, Table 2), the mean on Day 10 was significantly higher than at 36 h after cloprostenol injection ( $P < 0.01$ , *t* test). However, when the  $K_d$  values on Day 10 (mean  $\pm$  s.e.m. =  $0.44 \pm 0.05$ ; Table 2) and at 36 h after cloprostenol injection (mean  $\pm$  s.e.m. =  $0.25 \pm 0.03$ ; Table 2) were compared across the genotypes, the respective means were not significantly different from one another ( $P > 0.05$ , *t* test).

In granulosa cells (Table 3), the characteristics of the hCG/LH receptor were similar in Romney and Booroola  $\times$  Romney (++) ewes for follicles  $\geq 5$  mm diameter. Moreover, in large follicles from Romney ewes there was no effect of follicle health on the binding characteristics. In Booroola  $\times$  Romney F+ ewes, the largest follicles present are normally between 3 and 4.5 mm diameter. The characteristics of the hCG/LH receptor in granulosa cells of these follicles were similar to those in the larger-sized follicles of Romney or Booroola  $\times$  Romney (++) ewes. Likewise in the F+ ewes, the binding characteristics in the granulosa cells from 3–4.5 mm diameter follicles were independent of follicular health. No specific binding in granulosa cells from 1–4.5 mm diameter follicles of Romney ewes or Booroola  $\times$  Romney ++ ewes was observed. Likewise no specific binding in granulosa cells from 1–2.5 mm diameter follicles of Booroola  $\times$  Romney F+ ewes was noted.

Table 1. Characteristics of the hCG/LH receptor in ovarian theca interna of sheep with reference to genotype, follicle health and reproductive status

Breed (genotype)	Reproductive status	Follicle diam. (mm)	Follicle health	$B_{max}$ (fmol/mg protein)	$K_d$ (nM)
Romney	Day 10	$\geq 5$	NA†	$8.0 \pm 2.3$	$0.15 \pm 0.04$
	Anoestrus	$\geq 5$	NA	$14.0 \pm 0.4$	$0.10 \pm 0.01$
	36 h after PG	$\geq 5$	NA	$70.3 \pm 19.5$	$0.65 \pm 0.06$
	Day 10	3-4.5	NA	$5.5 \pm 0.4$	$0.10 \pm 0.01$
	Day 10	3-4.5	NA	$5.7 \pm 0.6$	$0.07 \pm 0.02$
	Day 10	1-2.5	NA	$7.4 \pm 3.4$	$0.16 \pm 0.06$
	Day 10	1-2.5	NA	$5.1 \pm 0.4$	$0.06 \pm 0.01$
Booroola × Romney (++)	Day 10	$\geq 5$	NA	$12.0 \pm 0.8$	$0.11 \pm 0.01$
	Anoestrus	$\geq 5$	NA	$14.7 \pm 0.4$	$0.10 \pm 0.01$
	36 h after PG‡	$\geq 5$	NA	$64.6 \pm 22.2$	$0.23 \pm 0.05$
	36 h after PG‡	$\geq 5$	NA	$42.3 \pm 3.4$	$0.13 \pm 0.02$
	Anoestrus	$\geq 5$	A	$4.3 \pm 0.3$	$0.06 \pm 0.03$
	Anoestrus	3-4.5	NA	$23.5 \pm 0.5$	$0.10 \pm 0.01$
	Anoestrus	3-4.5	A	$2.8 \pm 0.3$	$0.04 \pm 0.02$
	Anoestrus	1-2.5	NA	$9.5 \pm 0.9$	$0.05 \pm 0.03$
Booroola × Romney (F+)	Day 10	3-4.5	NA	$5.6 \pm 1.5$	$0.06 \pm 0.03$
	Anoestrus	3-4.5	NA	$17.8 \pm 0.6$	$0.16 \pm 0.01$
	36 h after PG‡	3-4.5	NA	$28.4 \pm 3.8$	$0.21 \pm 0.04$
	36 h after PG‡	3-4.5	NA	$46.9 \pm 9.1$	$0.29 \pm 0.04$
	Anoestrus	3-4.5	A	$0.9 \pm 0.2$	$0.01 \pm 0.007$
	Anoestrus	1-2.5	NA	$5.3 \pm 0.6$	$0.14 \pm 0.03$

Values are mean  $\pm$  s.d. Each  $B_{max}$  and  $K_d$  value was obtained from a pool of thecae from 3-20 ewes. Each  $B_{max}$  and  $K_d$  s.d. was derived from the respective s.d. of the slope and intercept of the Woolf plot. No specific binding was found in theca externa (N = 6 pools, 2 from Romney, 2 from F+ and 2 from ++ ewes).

† NA = non-atretic, A = atretic.

‡ 125  $\mu$ g cloprostenol (a PGF-2 $\alpha$  analogue) was injected s.c. on Day 10 of the oestrous cycle (Day 0 = day of oestrus).

Table 2. Characteristics of the hCG/LH in luteal tissue of sheep with reference to breed, genotype and reproductive status

Breed (genotype)	Reproductive status	$B_{max}$ (fmol/mg protein)	$K_d$ (nM)
Romney	Day 10	$23.4 \pm 2.1$	$0.36 \pm 0.03$
	Day 10	$55.4 \pm 9.3$	$0.67 \pm 0.04$
	36 h after PG†	$2.4 \pm 0.6$	$0.32 \pm 0.12$
Booroola × Romney (++)	Day 10	$38.9 \pm 2.6$	$0.32 \pm 0.03$
	Day 10	$38.9 \pm 1.5$	$0.48 \pm 0.02$
	36 h after PG	$4.1 \pm 1.2$	$0.24 \pm 0.14$
Booroola × Romney (F+)	Day 10	$52.4 \pm 2.8$	$0.44 \pm 0.03$
	Day 10	$22.1 \pm 1.5$	$0.35 \pm 0.02$
	36 h after PG	$2.1 \pm 0.2$	$0.19 \pm 0.02$

Values are mean  $\pm$  s.d. Each  $B_{max}$  and  $K_d$  value was obtained from a pool of luteal tissue recovered from the same 3-20 ewes described in Table 1. The  $B_{max}$  s.d. and  $K_d$  s.d. were derived from the respective s.d. of the slope and intercept of the Woolf plot.

† 125  $\mu$ g cloprostenol (as prostaglandin F<sub>2 $\alpha$</sub>  analogue, PG) was injected s.c. on Day 10 of the oestrous cycle (Day 0 = day of oestrus).

Table 3. Characteristics of the hCG/LH receptor in granulosa cells of sheep with reference to genotype, follicle diameter and health†

Breed (genotype)	Follicle diam. (mm)	Follicle health	B <sub>max</sub> (fmol/mg protein)	K <sub>d</sub> (nM)
Romney	≥ 5	NA	55.0 ± 8.7	0.10 ± 0.01
	≥ 5	A	66.6 ± 19.8	0.10 ± 0.03
	3-4.5	NA	No specific binding*	
	1-2.5	NA		
Booroola × Romney (++)	≥ 5	NA	61.1 ± 3.7	0.10 ± 0.01
	3-4.5	NA	No specific binding*	
	1-2.5	NA		
Booroola × Romney (F+)	3-4.5	NA	55.0 ± 14.9	0.13 ± 0.01
	3-4.5	A	40.0 ± 4.6	0.14 ± 0.02
	1-2.5	NA	No specific binding*	

Values are mean ± s.d. Each B<sub>max</sub> and K<sub>d</sub> value was obtained from a pool of granulosa cells recovered from atretic or non-atretic follicles from 10-30 ewes. The B<sub>max</sub> s.d. and K<sub>d</sub> s.d. were derived from the respective s.d. of the slope and intercepts of the Woolf plot.

\* Two pools per breed (genotype) were examined.

† All ewes were at Day 10 of the oestrous cycle.

### Discussion

This study demonstrates that specific receptors for <sup>125</sup>I-labelled hCG in the sheep follicle wall are located in the theca interna. The absence of hCG/LH receptors in theca externa and/or stromal tissue and the inability of LH to stimulate androstenedione synthesis in the aforementioned tissues when perfused *in vitro* (McNatty *et al.*, 1985a) suggests that the theca interna is probably the sole source of LH-stimulated androstenedione synthesis in the sheep ovary. Granulosa cells in this species synthesize little, if any, androgen *de novo* (Moor, 1977).

The finding that the binding of hCG to theca interna was time- and temperature-dependent is consistent with that for cow ovaries (Henderson *et al.*, 1984). At equilibrium, the maximum binding of <sup>125</sup>I-labelled hCG to the theca interna receptor was lower at 22 than at 37°C. This was also noted to be the case for the bovine theca interna (Henderson *et al.*, 1984) but reasons why such differences occurred are obscure. The finding that the kinetics of <sup>125</sup>I-labelled hCG binding to theca interna obeyed a second order rate equation is also consistent with previous results for hCG/LH binding to granulosa cells, theca interna and luteal tissue in other species (Gospodarowicz, 1973; Lee & Ryan, 1973; Rao, 1974; Stouffer, Tyrey & Schomberg, 1976; Cameron & Stouffer, 1982; Henderson *et al.*, 1984). The binding of <sup>125</sup>I-labelled hCG to theca interna was not readily reversible and very slow relative to that observed for <sup>125</sup>I-labelled hCG and <sup>125</sup>I-labelled LH dissociation from granulosa cells (Stouffer *et al.*, 1976) and luteal tissue (Gospodarowicz, 1973; Rao, 1974; Cameron & Stouffer, 1982). It was not possible to determine accurately the dissociation rate constant (k<sub>d</sub>) from the present experiment.

The present studies show that ovine LH is equipotent with hCG with respect to binding to the hCG/LH receptor. Also that the receptor is highly specific for hCG/LH, with the major cross-reacting material being ovine FSH at a level of ~0.01% relative to that of hCG. Of interest was the finding that FSH-P and PMSG were very effective in displacing <sup>125</sup>I-labelled hCG from the theca interna LH receptor. With regard to PMSG, the FSH:LH ratio of partly purified serum extracts from pony mares has been estimated to be 1.08 (range 0.87-1.30; Stewart, Allen & Moor, 1976). The FSH:LH ratio in the PMSG preparation (NICHED) used in the present study was unknown, as we did not test the FSH bioactivity. In the pig, PMSG exhibited a higher affinity for the LH than for the FSH testis receptor (Combarrous, Hennen & Ketelslegers, 1978). For different batches of FSH-P, Monniaux, Chupin & Saumande (1983) estimated that the FSH:LH ratio varied between

0.2 and 0.5. It therefore seems likely that the effectiveness of PMSG and FSH-P in displacing  $^{125}\text{I}$ -labelled hCG from the thecal LH receptor can be attributed to LH contamination in the preparation (i.e. FSH-P) or to the LH-like activity that is intrinsic to the preparation (i.e. PMSG). The results from the present study suggest that when PMSG or FSH-P are used to stimulate ovulation rate in sheep these compounds are also likely to exert a marked influence on the level of thecal steroidogenesis.

The present results indicate that the LH receptor characteristics (i.e.  $B_{\max}$ ,  $K_d$ ) in theca interna do not vary substantially between the sheep genotypes studied herein. It therefore seems reasonable to suggest that the changes observed in any one of the genotypes with respect to follicle diameter, reproductive status or follicle health are probably typical for all the genotypes studied. In the non-atretic follicles, there was no significant difference in the hCG/LH-binding characteristics on Day 10 of the cycle compared to anoestrus. During anoestrus, but not on Day 10, there was a tendency for the  $B_{\max}$  values to be higher in follicles 3–4.5 mm and/or  $\geq 5$  mm in diameter compared to those in 1–2.5 mm follicles; whether this difference is of physiological significance will require further investigation. Irrespective of follicular diameter, the binding characteristics in theca interna from atretic follicles were lower than those in theca interna from similar-sized non-atretic follicles. However, these lower values may be more apparent than real since the proportion of theca interna in the atretic preparations was lower than that in the non-atretic samples (see 'Materials & Methods' for theca interna purity). It is therefore possible that the theca LH receptor remains an integral part of the cell right up to the time that cellular degeneration occurs. However, whether or not this is so, LH was unable to stimulate androstenedione synthesis in theca interna from atretic follicles *in vitro* (McNatty *et al.*, 1985a) despite the existence of at least some LH receptors in these tissues (Table 1). This finding is consistent with that for cow ovary in which early atresia was characterized by a loss in thecal steroidogenic activity which preceded any loss in LH-induced cAMP synthesis and LH receptor loss (Henderson *et al.*, 1984; McNatty, Lun, Heath, Kieboom & Henderson, 1985b).

In contrast to the LH binding characteristics in theca interna on Day 10 or during anoestrus, there was a marked increase in the  $B_{\max}$  (4.5-fold) and  $K_d$  (2.3-fold) values in theca interna of large non-atretic follicles ( $\geq 3$  mm diam.) at 36 h after cloprostenol injection. These changes in hCG/LH binding characteristics coincide with a major reduction in  $B_{\max}$  (> 85%) and  $K_d$  (~ 50%) values in the corpus luteum compared to those values on Day 10 of the cycle. The increase in hCG/LH binding capacity of theca interna during a cloprostenol-induced follicular phase is consistent with the results of earlier studies showing a greater uptake of  $^{125}\text{I}$ -labelled hCG by the follicle wall of presumptive preovulatory ovine follicles around the time of the preovulatory LH surge (Webb & England, 1982). During the follicular phase, there is a marked reduction in luteal mass, luteal blood flow (Moor, Hay, Seamark, 1975) and hCG/LH receptor capacity (Table 2; Diekman, O'Callaghan, Nett & Niswender, 1978) with a concomitant increase in thecal LH receptor capacity (Table 1), thecal blood flow (Moor *et al.*, 1975) and possibly also in the mass of thecal tissue of the presumptive preovulatory follicles, which collectively confirms the notion that the theca interna is a major target tissue for LH action during the follicular phase (McNatty, 1982; Webb & England, 1982).

During the luteal phase of the oestrous cycle (i.e. Day 10) granulosa cells in the largest follicles from each sheep genotype displayed similar hCG/LH receptor characteristics. Moreover, these LH receptor characteristics also resembled those in luteal tissue and theca interna 36 h after cloprostenol treatment. It is not known whether these hCG/LH binding properties in granulosa cells differ from those in similar-sized follicles during anoestrus or from presumptive preovulatory follicles during the follicular phase. However, the data of Webb & England (1982) suggest that the  $B_{\max}$  value may be greater in granulosa cells of presumptive preovulatory follicles than in those observed during the luteal phase.

The present results are consistent with those of Henderson *et al.* (1985) who showed that granulosa cells in follicles from F+ ewes develop an LH responsiveness with respect to cAMP synthesis

at a smaller diameter (i.e. 3–4.5 mm diam.) compared to that for cells from follicles from ++ ewes (i.e.  $\geq 5$  mm diam.). In the present study the hCG/LH binding characteristics in granulosa cells from the largest atretic follicles were similar to those in cells from the largest non-atretic follicles. This is consistent with the findings of Henderson *et al.* (1985) who showed that granulosa cells from large atretic follicles in sheep were capable of producing cyclic AMP after exposure to LH. However, as atretic follicles invariably have a reduced population of granulosa cells (McNatty, 1982), the LH binding capacity with respect to the total granulosa cell population is likely to be severely reduced. This interpretation is consistent with the earlier findings of McNatty (1982).

In conclusion, the present studies demonstrate that the hCG/LH receptor in the sheep follicle wall is located exclusively in the theca interna and that the binding characteristics of this receptor are similar to those of the hCG/LH receptor in granulosa cells and in luteal tissue. Moreover, it is evident that the binding characteristics of the hCG/LH receptor in theca interna, granulosa cells and luteal tissue of Romney ewes and Booroola  $\times$  Romney ewes with and without the fecundity gene are similar.

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## Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate

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**Summary.** The mean plasma concentrations of FSH and LH were significantly higher in FF ewes than in ++ ewes with those for F+ animals being consistently in between. These gene-specific differences were found during anoestrus, the luteal phase and during a cloprostenol-induced follicular phase, suggesting that the ovaries of ewes with the F-gene are more often exposed to elevated concentrations of FSH and LH than are the ovaries of ewes without the gene.

The gene-specific differences in LH secretion arose because the mean LH amplitudes were 2–3 times greater in FF compared to ++ ewes with the LH amplitudes for F+ ewes being in between. The LH pulse frequencies were similar. In these studies the pulsatile nature of FSH secretion was not defined.

The pituitary contents of LH during the luteal phase, were similar in all genotypes whereas for FSH they were significantly higher in the F-gene carriers compared to ++ ewes. The pituitary sensitivity to exogenous GnRH (0.1, 0.5, 5.0 and 25 µg i.v.) was related to genotype. Overall the LH responses to GnRH were lower in FF ewes than in ++ ewes with the results for the F+ ewes being in between. The FSH responses to all GnRH doses in the FF genotype were minimal (i.e. <2-fold). In the other genotypes a >2-fold response was noted only at the highest GnRH dose (i.e. 25 µg). Treatment of FF and F+ but not ++ ewes with GnRH eventually led to a reduced FSH output, suggesting that the pituitary responses to endogenous GnRH were being down-regulated in the F-gene carriers whereas this was not the case in the non-carriers.

Collectively these data confirm that peripheral plasma and the pituitary together with the ovary are compartments in which F-gene differences can be observed. In conclusion, these findings raise the possibility that F-gene-specific differences may also extend to the hypothalamus and/or other regions of the brain.

### Introduction

High-fecundity Booroola ewes contain a major gene(s) (F) which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative gene have been segregated on the basis of at least one ovulation rate recording of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). The endocrine basis for the high ovulation rate in Booroola ewes has been the subject of several recent reports. It has been established that F-gene carriers have ovarian follicles with higher tissue levels of adenosine cyclic 3',5'-monophosphate

(cAMP) compared to those in similar-sized follicles from non-carriers (++) (McNatty *et al.*, 1986a). Also, ovarian follicles in F-gene carriers are more sensitive than those in ++ ewes to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) with respect to cAMP synthesis (Henderson *et al.*, 1985; McNatty *et al.*, 1986a). Furthermore, in F-gene carriers, granulosa cells synthesize oestradiol, acquire functional LH receptors and the follicles ovulate at a smaller diameter than do those in ++ Booroola ewes (Henderson *et al.*, 1985; McNatty *et al.*, 1985b, 1986c). However, despite these differences at the ovarian level, it is not known whether the principal site(s) of F-gene expression are in the ovary or elsewhere.

It seems reasonable to expect that the anterior pituitary might be an important, if not principal, site of F-gene expression (Bindon, 1984) since FSH is known to exert a major influence on follicular viability, the level of oestradiol synthesis and the ovulation rate (Wright *et al.*, 1981; McNatty *et al.*, 1985a; Henderson *et al.*, 1987). Support for this notion has come from the studies of Robertson *et al.* (1984) and Bindon (1984), who showed that the pituitary FSH contents of Booroola Merinos were higher than in non-Booroola Merino controls. Consistent with these findings are those showing that the plasma concentrations of FSH in 30-day-old Booroola lambs were higher than in similarly aged non-Booroola lambs (Findlay & Bindon, 1976). In contrast, however, the plasma values of FSH in 60- and 90-day-old lambs did not differ between Booroola and non-Booroola Merinos. In mature ewes, the plasma and urine concentrations of FSH in Booroola Merinos have sometimes been found to differ from those in control Merinos (Bindon, 1984; Bindon *et al.*, 1985). However, these authors have also reported that the FSH values were often not different between the Booroola genotypes or between Booroola and control ewes, indicating some uncertainty as to whether FSH has an important role in influencing the ovulation rates of the F-gene carriers. With regard to LH there has been no evidence to indicate gene-specific differences (i.e. FF vs F+ vs ++) in the basal LH concentrations or in the number of LH pulses in ewes during the breeding season (Bindon, 1984).

The aims of this study were to measure the plasma concentrations of FSH and LH in FF, F+ and ++ Booroola Merino ewes during anoestrus and/or at different times during the oestrous cycle in an attempt to confirm or refute the notion that the FSH but not LH concentrations differed between the genotypes. In addition, the pituitary contents of FSH and LH, and the influences of gonadotrophin-releasing hormone (GnRH) in FF, F+ and ++ ewes were assessed.

### Materials and Methods

Booroola Merino ewes born in 1973–1976, which had been assigned F+ or FF genotypes based on 4–5 laparoscopies according to the criteria of Davis *et al.* (1982), were mated with progeny tested FF or F+ Merino rams. The resultant female progeny that were used in the present experiments were examined laparoscopically each year on 4 or 5 occasions and then assigned to FF, F+ or ++ groups according to ovulation rates. All progeny of FF × FF crosses were found to have had at least one ovulation rate  $\geq 5$  and were included in the FF group. For all FF × F+ and F+ × F+ crosses the number and type of progeny with each phenotype was consistent with the genotypes expected from those matings (see Davis *et al.*, 1982). Sixteen of the ++ group were offspring of a progeny-tested ++ Merino-type Booroola ram mated with Merino ewes; all had ovulation rates of 1 or 2. The FF, F+ and ++ animals in this study were between 6 and 9 years of age and of proven parity.

*Experiment 1* was designed to examine the hourly patterns of FSH and LH secretion as well as the pulsatile nature of LH secretion (at 10-min intervals) before and after a cloprostenol (125 µg s.c.; Coopers Animal Health, Upper Hutt, N.Z.) injection in FF (N = 12), F+ (N = 9) and ++ (N = 12) ewes on Day 10 of the oestrous cycle (May 1984). On the day before blood sampling, the ewes were penned indoors and each was fitted with an intrajugular cannula. When blood sampling began, the animals were bled (2.5 ml) via the jugular cannulae once hourly for 66 consecutive hours as well as for 10-min intervals from -6 to 0 h before or 6 to 12 h and 24 to 30 h after cloprostenol injection. Cloprostenol was injected after the first 6 h of sampling. Oestrous activity was recorded in all ewes by using two vasectomized rams with marking harnesses which were introduced to the ewes 24 h after cloprostenol injection. At 7 days after the end of the intensive blood sampling regimen, all animals were subjected to laparoscopy to determine their ovulation rate. The blood samples which were taken at -6, 0, 6, 18, 24 and 36 h were also assayed for progesterone to investigate the patterns of luteolysis in the different genotypes.

*Experiment 2* was designed to examine the FSH concentrations during anoestrus ( $N = 10$  ewes/genotype; during January 1984) as well as during the luteal phase (i.e. Days 6 to 10) of the oestrous cycle ( $N = 35$  ewes/genotype; during April, 1984). The FF, F+ and ++ ewes were blood sampled via an intrajugular cannula (2 ml/collection) once hourly for 6 consecutive hours. The absence of corpora lutea in the anoestrous ewes was confirmed by laparoscopy 2 days before blood sampling began and also by the fact that the plasma progesterone values were  $<0.5$  ng/ml on the day of blood sampling for FSH.

*Experiment 3* was carried out to compare the pituitary FSH and LH contents in FF ( $N = 10$ ), F+ ( $N = 7$ ) and ++ ( $N = 7$ ) ewes slaughtered on Day 10 of the oestrous cycle (i.e. between April and July, 1984). Each freshly dissected pituitary gland was weighed and approximately 50% of the gland ( $\sim 0.4$ – $0.5$  g) was then finely cut with scissors and homogenized in a 1 mM aqueous solution of EDTA (5 ml) at room temperature. Subsequently, 0.5 ml aliquants of the homogenate were added to  $12 \times 75$  mm plastic tubes containing an equivalent volume of a solution containing EDTA (1 mM), 0.5% (w/v) egg white and 30  $\mu$ l bacitracin (0.02 M). This method of extraction was based on that reported by McIntosh & McIntosh (1983a) for LH. However, the efficiency of this procedure for both LH and FSH was not assessed in our laboratory. These tubes were capped, and frozen to  $-20^\circ\text{C}$  until assayed for LH and FSH by specific RIA.

*Experiment 4* was designed to test the pituitary responsiveness of anoestrous (February, 1984) FF, F+ and ++ Booroola ewes to 0, 0.1, 0.5, 5.0 or 25  $\mu$ g GnRH (i.v.; Peninsular Laboratories Inc., Belmont, CA, U.S.A.; 2 ewes/genotype/dose). All animals were fitted with intrajugular cannulae the day before blood sampling began. For each ewe, 7 consecutive hourly blood samples were collected (2.5 ml/collection) before the GnRH injection. Thereafter the animals were bled every 10 min for the first hour after the GnRH injection and then every subsequent hour for another 8 h. The plasmas from the hourly blood samples were assayed for both FSH and LH which was also measured in the 10-min plasma samples. The absence of corpora lutea in the anoestrous ewes was confirmed by laparoscopy 2 days before GnRH treatment and by the presence of  $<0.5$  ng progesterone/ml on the day of GnRH treatment.

*Blood samples.* All blood samples were centrifuged at 4000 g at room temperature for 20 min within 30 min of collection and the plasma samples were stored at  $-20^\circ\text{C}$  until assayed.

### Hormone assays

*Progesterone.* The radioimmunoassay procedure was identical to that described by McNatty *et al.* (1981). The antiserum (WA-26) was raised in an ovariectomized ewe against progesterone-11 $\alpha$ -hemisuccinate conjugated to bovine serum albumin and used at an initial dilution of 1:8000. Major cross-reacting steroids in the assay were 11 $\alpha$ -hydroxyprogesterone (120%), 11 $\beta$ -hydroxyprogesterone (25%), 20 $\alpha$ -dihydroprogesterone (3.5%) and androstenedione (0.45%). The minimum detectable level of progesterone was 0.15 ng/ml. The intra- and interassay coefficients of variation were 10.6 and 14.0% respectively.

*FSH.* The radioimmunoassay kit was that supplied by The National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, Maryland, U.S.A. The ovine (o) FSH for iodination was NIAMDD-oFSH-1-1, the oFSH reference preparation was NIAMDD-oFSH-RP-1 (biopotency  $75 \times$  NIH-FSH-S1) and the oFSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113). At a final FSH antiserum dilution of 1:80 000 this homologous assay had a working range of 0.01 to 5 ng per assay tube. The volume of plasma which was assayed was 0.1 ml and each sample was assayed in duplicate. When assaying the pituitary homogenates for FSH, the homogenates were serially diluted 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560 in assay buffer (0.01 M-phosphate-buffered saline + 2% normal rabbit serum, pH = 6.8) and 50  $\mu$ l aliquants at each dilution in quadruplicate were assayed for FSH content. The intra- and interassay coefficients of variation were 6.3 and 9.6%.

*LH.* The radioimmunoassay for LH was identical to that described by McNatty *et al.* (1981). The LH antiserum was raised in a rabbit against NIH-LH-S11 and used at an initial dilution of 1:40 000. The antiserum exhibited low cross-reactivity reactions with NIH-P-S12 (0.09%), NIH-TSH-S8 (2.4%), NIH-GH-S11 (0.4%) and NIH-FSH-S10 (0.4%). The volume of plasma which was assayed was 0.2 ml and each sample was assayed in duplicate. When assaying the pituitary homogenates for LH, the homogenates were serially diluted 1/1000, 1/2000, 1/4000, 1/8000, 1/16 000, 1/32 000 in assay buffer (0.01 M phosphate buffer + 2% normal rabbit serum, pH 6.8) and 100  $\mu$ l aliquants were assayed for LH content. The minimum detectable level of LH was 0.3 ng/ml plasma. The intra- and interassay coefficients of variation were  $<10$  and  $<13\%$  respectively.

### Data presentation and statistical analysis

When the raw data were normally distributed (i.e. as assessed from the N-score on Minitab), the results are expressed as means  $\pm$  s.e.m., but when not normally distributed the results were either normalized by log transformation and presented as geometric means (and 95% confidence limits) or presented as medians (and 95% confidence limits). The only exception to this was for Fig. 1(a) in which the results are shown as means  $\pm$  s.e.m. to enhance the visual presentation even though the data were analysed non-parametrically. Since all the experiments involved ewes between 6 and 9 years of age, the influence of age was tested but none was noted. Therefore, in all of the experiments (i.e. 1–4), the results for each genotype were pooled irrespective of age of the animals.

The FSH, LH and progesterone data between genotypes were compared by analysis of variance (ANOVA) in conjunction with the Neuman-Keuls test (Exps 1, 2 & 3), or by the Kruskal-Wallis test (Exp. 1). It is stressed that all genotypic differences were assessed against the between-animal data. The effects of the different genotypes (FF, F+ and ++ ewes) with respect to doses of GnRH on FSH and LH secretion (i.e. Exp. 4) were tested as follows. For each ewe, the mean FSH or LH value preceding the GnRH injection was obtained (i.e. the Xpre value). Thereafter each LH concentration over every 10 min for the first hour after GnRH injection, and for every subsequent hour for the remaining 8 h, was divided by the Xpre concentration to obtain a scaled value. Scaled values from each hourly FSH concentration after GnRH injection were similarly obtained. The scaled maximum values as well as the scaled areas encompassed by the LH and FSH concentrations after GnRH injection were examined by two-way ANOVA.

The episodic LH data (Exp. 4) were analysed by the Pulsar program of Merriam & Wachter (1982). The LH peaks were identified from the Pulsar program using the values of 3.8, 2.6, 1.9, 1.5 and 1.2 for G(1), G(2), G(3), G(4) and G(5) respectively. The assay s.d. terms for Pulsar were obtained as follows. The s.d. of replicate LH standards equivalent to 0.3, 0.4, 0.6, 0.8, 1.5, 3.3, 4.0, 4.5, 5.0, 6.0, 8.0 and 10.0 ng/ml (10 replicates/dose) were determined. The s.d. of each of the above values was then fitted to the corresponding mean values using least squares regression. This gave constant and linear values for the Pulsar program of 2.6 and 9.8 respectively; no quadratic term was needed. The smoothing time in the program was 12 h. The mean LH values described herein are equivalent to the 'smoothed mean' values from Pulsar (i.e. the baseline values after all the peak samples were omitted). The amplitude of each LH peak was the difference between the peak value and the aforementioned mean LH value.

## Results

### *Hormone concentrations before and after a cloprostenol-induced follicular phase with respect to Booroola genotype (Exp. 1)*

The mean  $\pm$  s.e.m. ovulation rates in the FF (N = 12), F+ (N = 9) and ++ (N = 12) ewes after the cloprostenol-induced follicular phase were  $4.5 \pm 0.3$ ,  $2.8 \pm 0.1$  and  $1.3 \pm 0.1$  respectively.

**Progesterone.** At -6, 0, 6, 18, 24 and 36 h from cloprostenol injection, the respective mean ( $\pm$  s.e.m.) plasma concentrations of progesterone were  $2.5 (\pm 0.3)$ ,  $2.3 (\pm 0.3)$ ,  $1.2 (\pm 0.1)$ ,  $0.6 (\pm 0.1)$ ,  $0.4 (\pm 0.06)$  and  $0.2 (\pm 0.03)$  ng/ml for the ++ ewes (N = 12),  $2.7 (\pm 0.2)$ ,  $2.7 (\pm 0.3)$ ,  $1.1 (\pm 0.1)$ ,  $0.7 (\pm 0.1)$ ,  $0.4 (\pm 0.08)$  and  $0.2 (\pm 0.06)$  ng/ml for the F+ ewes (N = 9) and  $2.8 (\pm 0.2)$ ,  $2.9 (\pm 0.4)$ ,  $1.2 (\pm 0.1)$ ,  $0.7 (\pm 0.09)$ ,  $0.5 (\pm 0.04)$  and  $0.3 (\pm 0.03)$  ng/ml for the FF ewes (N = 12).

**FSH.** These data are summarized in Fig. 1(a). The overall mean  $\pm$  s.e.m. values before cloprostenol injection were  $2.4 \pm 0.3$ ,  $1.6 \pm 0.2$  and  $1.4 \pm 0.2$  ng/ml respectively for the FF, F+ and ++ ewes. The mean value for the FF ewes (N = 12) was significantly higher than that for the F+ ewes (N = 9;  $P < 0.05$ ) and for the ++ ewes (N = 12;  $P < 0.05$ ) with the values for the F+ and ++ ewes being not different from one another. For all genotypes, the mean FSH concentrations declined after the cloprostenol injection to reach basal values about 14 h later. The FSH concentrations obtained from the start of blood sampling until the rams were introduced were fitted to a regression line. The median (and 95% confidence limits) of the intercepts of these regression lines for FF, F+ and ++ ewes were  $2.0 (1.7, 3.4)$ ,  $1.5 (1.1, 2.0)$  and  $1.5 (0.9, 1.7)$  ng/ml respectively; the median FSH value for the FF ewes was significantly higher than those for F+ and ++ ewes (both  $P < 0.05$ ; Kruskal-Wallis test). The median (and 95% confidence limits) of the slopes of the regression lines for FF, F+ and ++ ewes were  $-0.06 (-0.03, -0.08)$ ,  $-0.03 (-0.02, -0.05)$  and  $-0.03 (-0.02, -0.06)$  ng ml<sup>-1</sup> h<sup>-1</sup> respectively; the slopes for the FF ewes (n = 12) were significantly steeper than those for F+ ( $P < 0.05$ ; N = 9) or ++ ewes ( $P < 0.05$ ; N = 12). From 36 h after the start of the sampling schedule the differences in the hourly mean FSH concentrations between the genotypes were no longer significantly different from one another.

Asynchronous, FSH peaks around the time of the preovulatory LH surge (see Fig. 1b) were apparent during the final 26 h of continuous blood sampling (i.e. from ram entry). For each animal, the 'maximum FSH value' and the time from cloprostenol injection to that maximum were determined. The median (and 95% confidence limits) of the FSH maximum values for FF, F+ and ++ ewes were  $5.7 (3.7, 8.0)$ ,  $3.4 (2.4, 7.4)$  and  $4.3 (1.3, 10.7)$  ng/ml respectively; these FSH values were not significantly different from one another (Kruskal-Wallis test). The median (and 95% confidence limits) of the times from cloprostenol injection to the FSH maximum values for the FF, F+

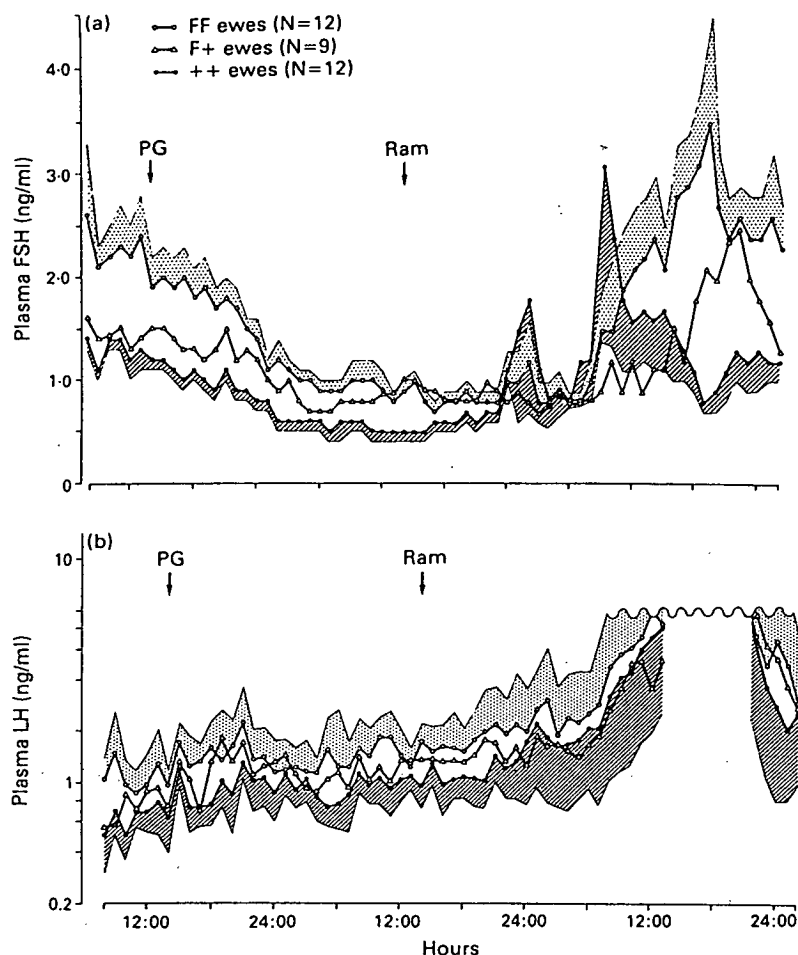


Fig. 1. Changes in the mean plasma concentrations of FSH (a) and LH (b) before and after injection of cloprostenol (PG) with respect to Booroola genotype. All ewes were at Day 10 of the oestrous cycle when injected with PG. For FSH the shaded areas for the F++ and FF ewes represent  $\pm$  s.e.m. For LH, the levels represent geometric means and the shaded areas represent the 95% confidence limits. For both hormones the shaded areas are equidistant each side of their respective means. For the sake of clarity the s.e.m. or 95% confidence limits were not shown for the F+ ewes. The number of ewes for both FSH and LH was 12 for FF, 9 for F+ and 12 ++ ewes.

and ++ ewes were 50 (46, 54), 56 (51, 60) and 44 (34, 52) h respectively; the median time for the F+ ewes was significantly longer ( $P < 0.05$ ) than that for the ++ or FF ewes (Kruskal-Wallis test). The median (and 95% confidence limits) with respect to time of onset of oestrous behaviour for FF, F+ and ++ ewes occurred at 52, (40, 64), 57 (50, 74) and 61 (54, 74) h respectively after cloprostenol injection; these values were not significantly different from one another (Kruskal-Wallis test).

**LH.** The hourly geometric mean concentrations of LH before and after a cloprostenol-induced follicular phase with respect to genotype are summarized in Fig. 1(b). The upper 95% confidence limits for the FF ewes and lower 95% confidence limits for the ++ ewes are also indicated. The 95% confidence limits for the F+ ewes were not included for the sake of clarity. The mean LH

values in the FF ewes were always higher than in the ++ ewes with those in the F+ ewes being in between. However, when the individual mean LH concentrations were compared with respect to genotype throughout the 66-h sampling period, the means were significantly different from one another ( $P < 0.05$ ; ANOVA) on only a few occasions (i.e. 19.4%). For all genotypes the mean LH values increased gradually after cloprostenol injection until the onset of the preovulatory LH surge. From the hourly means there was no obvious convergence of the LH values before the preovulatory LH surge.

The mean  $\pm$  s.e.m. times from cloprostenol injection to the onset of the preovulatory LH surge for the 3 genotypes was  $44 \pm 2$ ,  $48 \pm 2$  and  $46 \pm 3$  h for FF, F+ and ++ ewes respectively; these times were not significantly different from one another. It was not possible to assess either the mean peak heights or the mean durations of the preovulatory LH surges for each of the genotypes. The

**Table 1.** Characteristics of plasma LH concentrations before and after cloprostenol (PG) injection (time 0) in Booroola ewes on Day 10 of the oestrous cycle with respect to Booroola genotype

Time from PG injection (h)	Booroola genotype	Mean LH† conc. (ng/ml)	No. of LH peaks/6 h	Amplitude of LH peaks (ng/ml)	Mean peak length (min)
-6 to 0	++	$0.7 \pm 0.1^a$	$1.9 \pm 0.4^a$	$1.1 \pm 0.1^a$	$18 \pm 2^a$
	F+	$0.8 \pm 0.1^{a,b}$	$1.9 \pm 0.8^a$	$1.3 \pm 0.4^{a,b}$	$31 \pm 7^a$
	FF	$1.0 \pm 0.05^b$	$2.8 \pm 0.6^a$	$2.3 \pm 0.3^b$	$27 \pm 5^a$
6 to 12	++	$0.9 \pm 0.1^d$	$3.0 \pm 0.5^a$	$1.3 \pm 0.1^d$	$20 \pm 2^a$
	F+	$1.2 \pm 0.1^{d,e}$	$3.2 \pm 0.4^a$	$1.6 \pm 0.4^{d,e}$	$24 \pm 4^a$
	FF	$1.3 \pm 0.1^e$	$3.4 \pm 0.6^a$	$2.8 \pm 0.4^e$	$30 \pm 3^a$
24 to 30	++	$1.1 \pm 0.1^a$	$2.8 \pm 0.6^a$	$1.2 \pm 0.1^d$	$23 \pm 5^a$
	F+	$1.4 \pm 0.2^a$	$4.1 \pm 0.7^a$	$2.1 \pm 0.6^{d,e}$	$17 \pm 2^a$
	FF	$1.4 \pm 0.1^a$	$3.4 \pm 0.7^a$	$3.3 \pm 0.6^e$	$20 \pm 3^a$

†Mean level (i.e. the 'smoothed mean') was calculated from the baseline values after all the peak samples were omitted.

Values in each column and within each time frame not sharing a common superscript were significantly different from one another.  $d$  vs  $e = P < 0.05$ ;  $a$  vs  $b = P < 0.01$  (ANOVA, Neuman-Keuls test). Values with common superscripts are not different from one another.

**Table 2.** FSH concentrations (ng/ml) in Booroola ewes with respect to genotype and reproductive status

Reproductive status	Booroola genotype			No. of ewes per genotype
	++	F+	FF	
Days 6-10 oestrous cycle	$1.5 \pm 0.1^a$	$1.8 \pm 0.1^{a,b}$	$2.1 \pm 0.2^b$	35
Anoestrus	$1.3 \pm 0.1^a$	$1.8 \pm 0.2^{a,b}$	$2.5 \pm 0.3^b$	10

Values are means  $\pm$  s.e.m.

++, F+, FF are non-carriers, heterozygous carriers or homozygous carriers respectively of the Booroola F-gene. The FSH value for each ewe was the average from 6 consecutive blood samples collected at hourly intervals. For each row  $a$  vs  $b = P < 0.01$  (ANOVA with Neuman-Keuls test). Numbers sharing the same superscript are not different from one another.

peak LH values exceeded the assay sensitivity and, in some instances, the preovulatory secretory episodes were still elevated at the end of the LH sampling period.

The characteristics of the plasma LH concentrations with respect to mean levels, LH peak, frequency and amplitude and mean LH peak length are summarized in Table 1. At -6 to 0 h and 6 to 12 h from cloprostenol injection (at time 0), the mean concentrations of plasma LH and the mean amplitudes of the LH peaks in FF ewes were significantly higher than in ++ ewes (i.e. both  $P < 0.05$ ; Table 1) with the respective values in F+ ewes being in between the other two genotypes. At 24-30 h, the mean amplitudes of the LH peaks but not the mean LH concentrations in FF ewes were significantly higher ( $P < 0.05$ ) than those in the ++ ewes with those for F+ ewes being in between. In all three time frames, no significant gene-specific differences were noted in the number of LH peaks per 6 h or in the mean peak lengths. For each genotype, the mean LH concentration for the 24-30 h interval after cloprostenol injection was significantly higher than that for the -6 to 0 h interval ( $P < 0.05$  for all genotypes) with that for the 6-12 h interval being intermediate for each of the genotypes.

#### *FSH concentrations with respect to Booroola genotype and time of year (Exp. 2)*

These data are summarized in Table 2. During both the luteal phase of the oestrous cycle and anoestrus, FF ewes contained significantly higher mean plasma FSH concentrations compared to those in ++ ewes with the concentrations in the F+ animals being in between the other two genotypes. Although the overall FSH means were significantly different with respect to genotype, the range of mean FSH values between sheep within each genotype was 0.8-4.3, 0.6-3.0 and 0.6-3.5 ng/ml for the FF, F+ and ++ animals respectively. The likelihood of determining Booroola genotype in an individual ewe from one or more blood samples during anoestrus or the luteal phase is therefore minimal. In the ewes during the luteal phase, the mean  $\pm$  s.e.m. number of corpora lutea in the FF, F+ and ++ genotypes ( $N = 35$  ewes/genotypes) were  $4.7 \pm 0.2$ ,  $3.1 \pm 0.1$  and  $1.2 \pm 0.07$  respectively.

#### *Immunoreactive FSH and LH contents in the pituitary glands of Booroola ewes (Exp. 3)*

The geometric mean (and 95% confidence limits) for the FSH pituitary contents in ++ ( $N = 8$ ), F+ ( $N = 7$ ) and FF ( $N = 7$ ) ewes were 26 (20, 32), 52 (34, 82) and 36 (22, 60)  $\mu$ g/pituitary gland respectively. The FSH content in F+ ewes was significantly higher than in ++ ewes ( $P < 0.05$ ) but not different from that in FF ewes. The geometric mean (and 95% confidence limits) for the LH pituitary contents in the above ++, F+ and FF ewes were 590 (473, 735), 493 (302, 804) and 889 (578, 1366)  $\mu$ g/gland respectively. For LH there were no significant differences between the genotypes.

The mean  $\pm$  s.e.m. number of corpora lutea in the aforementioned ++, F+ and FF ewes were  $1.4 \pm 0.2$ ,  $3.3 \pm 0.3$  and  $4.9 \pm 0.3$  respectively.

#### *Effects of GnRH on the plasma concentrations of LH and FSH with respect to GnRH dose and Booroola genotype (Exp. 4)*

The mean  $\pm$  s.e.m. concentrations for LH in the FF, F+ and ++ ewes before GnRH injection were  $1.7 \pm 0.1$ ,  $1.1 \pm 0.1$  and  $0.9 \pm 0.05$  ng/ml ( $N = 10$ /genotype). The concentrations in FF ewes were significantly higher than those in F+ ( $P < 0.05$ ) and ++ ewes ( $P < 0.01$ ; ANOVA, Neuman-Keuls test); the concentrations in F+ and ++ ewes were not significantly different from one another. At 10 min after GnRH injection, and irrespective of GnRH dose, all LH values were elevated ( $>2.5$ -fold) with respect to the pretreatment values. Thereafter, depending on the GnRH dose, the LH values remained elevated for between 0.5 and 5.0 h; after these times the plasma LH concentrations returned to the pretreatment levels.

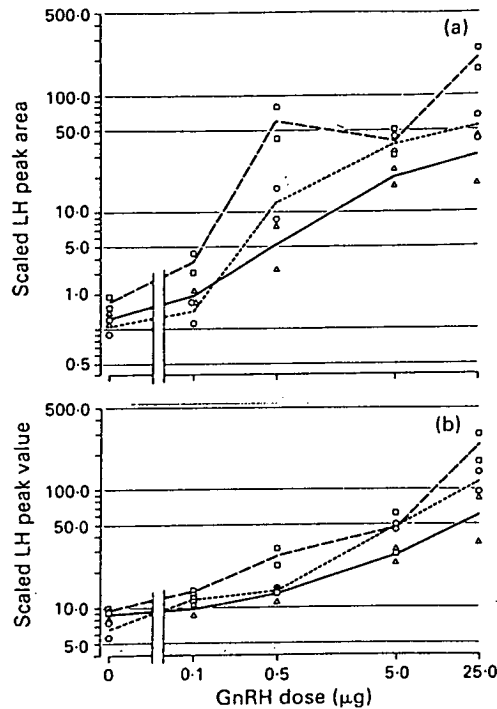


Fig. 2. Changes in the scaled LH peak values (a) and scaled LH peak areas (b) in individual ++ ( $\square$ ), F+ ( $\circ$ ) and FF ( $\triangle$ ) ewes with respect to GnRH dose. The lines (---, ++ ewes; ----, F+ ewes; —, FF ewes) are drawn through the midpoints of the results for the 2 ewes at each GnRH dose. The results were scaled to normalize the results between genotypes (see 'Statistical procedures') since the pretreatment values were related to genotype.

The scaled LH peak values and peak areas following GnRH injection are shown in Fig. 2. The values were scaled as described in 'Materials and Methods' to normalize the results between genotypes because the pretreatment LH values were related to genotype. For the ++ ewes, 7 out of 8 produced higher scaled peak and area values than those for the FF ewes with the results for the F+ ewes being in between. When a two-way ANOVA was performed on these data (i.e. genotype vs GnRH dose; Fig. 2), significant effects of genotype ( $P < 0.01$ ), GnRH dose ( $P < 0.01$ ) and genotype  $\times$  dose interaction ( $P < 0.01$ ) were noted with respect to both the scaled LH peak values and scaled LH peak areas.

The mean  $\pm$  s.e.m. concentrations for FSH in the FF, F+ and ++ ewes before GnRH injection were  $2.5 \pm 0.1$ ,  $1.8 \pm 0.1$ ,  $1.3 \pm 0.1$  (N = 10 sheep/genotype). The concentrations in FF ewes were significantly higher than those in F+ ( $P < 0.05$ ) and ++ ewes ( $P < 0.01$ ; ANOVA, Neuman Keuls test); the mean concentrations for F+ and ++ ewes were not significantly different from one another.

Irrespective of GnRH dose (i.e. 0, 0.1, 0.5, 5.0 and 25.0  $\mu$ g) there was little or no increase (i.e. <2-fold) in plasma FSH concentrations in the FF ewes (N = 10) relative to the pretreatment values. Similarly, in the F+ (N = 10) and ++ ewes (N = 10), the FSH responses were minimal (i.e. <2-fold) for all but the highest GnRH dose (i.e. 25.0  $\mu$ g). After the 25  $\mu$ g injection, the scaled peak FSH values in the ++ and F+ ewes (N = 2 ewes/genotype) increased 2.5- to 4.3-fold; in these animals FSH was elevated for the first 3 h after GnRH treatment. The scaled FSH peak areas followed a pattern similar to those described for the peak values. Thereafter, in these 4 animals and



in all the others, the FSH concentrations either returned to the pretreatment values or to concentrations lower than those before treatment. At 5–8 h after GnRH injection, the FSH values sometimes declined to concentrations which were only 30% of pretreatment concentrations. When the frequency of these low values was compared with respect to genotype and GnRH dose, a significant effect of genotype ( $P < 0.05$ ) but not dose or genotype  $\times$  dose interaction, was observed. The average numbers of low FSH values after GnRH were 3.7, 2.5 and 1.0 (s.e.d. = 0.94;  $N = 10$  ewes/genotype) for FF, F+ and ++ ewes. Therefore, compared to ++ but not F+ ewes, treatment of FF ewes with GnRH eventually resulted in a greater frequency of low FSH concentrations relative to those before treatment.

### Discussion

These studies show that the mean plasma concentrations of FSH and LH are significantly higher in FF Booroola ewes than in ++ Booroola ewes with those of F+ animals being consistently in between. These gene-specific differences in mean values were found during anoestrus, the luteal phase and during a cloprostenol-induced follicular phase. These differences in plasma FSH and LH were noted for the overall means for each genotype but only after frequent blood samplings from each animal. However, as can be noted from the results of Exps 1 and 2, these differences are not always obvious in individual animals. Nevertheless, it seems likely that the ovaries of ewes with the F-gene are more often exposed to high concentrations of FSH and LH than is the case in ++ ewes. These studies have confirmed that peripheral blood and the pituitary together with the ovary are compartments in which F-gene differences can be observed (Bindon, 1984). However, they do not establish whether the F-gene differences in pituitary function are a cause or a consequence of F-gene expression in some other tissue such as the ovary or hypothalamus.

It is possible that some of the ewes were misclassified with respect to genotype. Davis *et al.* (1982) found that 3% of New Zealand Merino ewes with a mean  $\pm$  s.e.m. ovulation rate of  $1.39 \pm 0.02$  may have a triple ovulation at least once in their lifetime and 7% of F+ ewes from the same flock were misclassified as FFs. Ewes in the present study were also generated from the same sources of animals reported in the above study. It therefore seems reasonable to assume that the numbers of misclassified ewes in the present study were small. Moreover, errors of the aforementioned magnitudes are unlikely to affect the overall conclusions of this study since they would tend to reduce the observed differences between the three groups.

In Romney ewes, exogenous FSH is known to increase oestrogen synthetase (aromatase) activity in granulosa cells from 3–4.5 mm diameter follicles to levels similar to those in cells from preovulatory follicles (i.e.  $\geq 5$  mm diam.) of the controls (McNatty *et al.*, 1985a). Perhaps the exposure of ovaries in FF and F+ ewes to higher FSH concentrations compared to that in ++ ewes is the reason why peak levels of aromatase activity as well as LH receptors are observed in the granulosa cells of 3–4.5 mm diameter follicles in F-gene carriers whereas in ++ ewes these characteristics are not observed unless the granulosa cells are recovered from follicles  $\geq 5$  mm diameter (Henderson *et al.*, 1985; McNatty *et al.*, 1986a). If this is so, then the elevated FSH concentrations in F-gene carriers may be partly responsible for the maturation of follicles at smaller diameters (i.e. 3–5 mm) than in ++ ewes (i.e.  $\geq 5$  mm).

In contrast with previous reports (see Bindon, 1984, for review), the present studies have shown a consistent correlation between plasma LH concentrations and Booroola genotype. In the earlier studies, the blood sampling frequencies were at 20-min intervals and so possible gene-specific differences in LH amplitude may have been missed. Moreover, it is difficult to discern from the earlier studies the accuracy of the genotypic classifications. And, as mentioned earlier, errors due to misclassification may mask any putative gene-specific differences in gonadotrophin secretion. In the present study, the gene-specific differences in LH secretion arose because the amplitude of each LH pulse was 2–3 times greater in FF compared to ++ ewes, with those for F+ ewes being in

between. In all three genotypes, the LH pulse frequencies were similar. The amplitudinal differences were recorded during the luteal and cloprostenol-induced follicular phases. It is likely that the differences in LH amplitude also occur during anoestrus as there were gene-specific differences in mean LH values at this time (see 'Results' for Exp. 4). The reasons why the LH amplitude differed between the genotypes are unclear. LH pulse amplitude (but not LH pulse frequency) as well as FSH secretion has been reported to be inhibited after the administration of sheep follicular fluid to ovariectomized ewes (Clarke *et al.*, 1986). It has been suggested that differences in the production of ovarian inhibin, a protein present in high concentrations in follicular fluid, may lead to F gene-specific differences in FSH secretion and thereby the ovulation rate in Booroola ewes (Cummins *et al.*, 1983; Bindon, 1984). Perhaps differences in the production of a follicular protein (i.e. inhibin?) may also be responsible for the gene-specific differences in LH pulse amplitude. However, an alternative explanation might be that the above differences in LH pulse amplitude are indicative of gene-specific differences in the amplitudes and/or the widths of the hypothalamic GnRH pulses (McIntosh & McIntosh, 1983b), independent of inhibin feedback.

Notwithstanding the underlying mechanisms leading to gene-specific differences in LH pulse amplitudes these might be important for stimulating a level of steroid synthesis in F-gene carriers comparable to that in ++ ewes. The ovarian secretion rates of androstenedione, testosterone and oestradiol are not significantly different between the genotypes during the luteal- or cloprostenol-induced follicular phase (McNatty *et al.*, 1985b; unpublished data). Moreover, it is known that granulosa cell oestradiol synthesis is critically dependent on theca interna androgen synthesis (Baird, 1977) and that, under in-vitro conditions, thecal androgen output is directly proportional to the wet weight of tissue (McNatty *et al.*, 1984). The respective mean  $\pm$  s.e.m. wet weights (mg) of theca interna recovered from all follicles  $\geq 1$  mm in diameter in FF, F+ and ++ ewes were  $10.0 \pm 0.6$  (N = 10 sheep),  $12.0 \pm 0.7$  (N = 12) and  $15.5 \pm 1.0$  (N = 15) (K. P. McNatty, unpublished data). On a per unit wet weight basis, the androgen output from theca interna was found to be the same for all genotypes (McNatty *et al.*, 1985b, 1986b). Although the amount of thecal tissue is smaller in the FF or F+ ewes than in ++ ewes, the steroidogenic responses in the former might be compensated by the higher amplitude LH pulses than those delivered to the thecae in ++ ewes. In part, this notion is supported by the finding that, under in-vitro conditions, the output of thecal androstenedione from ovine theca interna is directly correlated with LH pulse amplitude (McNatty *et al.*, 1986d).

An unexpected result in the present study was that the pituitary sensitivity to exogenous GnRH in anoestrous Booroola ewes was influenced by genotype. With regard to the LH responses to GnRH, the FF ewes did not respond as well as the ++ ewes, with the F+ ewes being in between. With regard to FSH, none of the FF ewes produced an appreciable FSH response (i.e. > 2-fold) to any of the GnRH doses. For the other genotypes (F+, ++) only the highest GnRH dose (i.e. 25  $\mu$ g) produced an appreciable FSH response (i.e. > 2-fold). The finding that GnRH caused a decrease in FSH output in the FF ewes, and to a lesser extent in F+ ewes, suggests that their pituitary responses to endogenous GnRH were being down-regulated whereas this was not the case in the ++ ewes. The lower FSH and LH outputs in the FF and F+ ewes are unlikely to have been due to an exhaustion of pituitary reserves since, in luteal-phase ewes at least, the pituitary contents of LH and FSH were either similar in all genotypes or higher in the F-gene carriers compared to those in ++ ewes (see 'Results' for Exp. 3; and also Bindon, 1984; Robertson *et al.*, 1984). Collectively, these findings from the GnRH experiments suggest that the pituitary glands in F-gene carriers may experience a different pattern of endogenous GnRH secretion relative to that in ++ ewes. However, the possibility cannot be discounted that the pituitary glands in the F-gene carriers have a different level of sensitivity to GnRH than is the case in the ++ ewes.

In conclusion, the present studies have shown that significant gene-specific differences exist in the plasma concentrations of FSH and LH and for LH, at least, that these differences are due to the pituitary release of LH at higher amplitudes in F-gene carriers than in ++ ewes. These findings, together with those showing gene-specific differences in pituitary sensitivity to GnRH raise the

possibility that gene-specific differences between Booroola ewes with and without the F-gene may also extend to the hypothalamus and/or other regions of the brain.

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## Differences in gonadotrophin-stimulated cyclic AMP production by granulosa cells from Booroola × Merino ewes which were homozygous, heterozygous or non-carriers of a fecundity gene influencing their ovulation rate

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**Summary.** Granulosa cells from follicles of different sizes from Booroola × Merino ewes which were homozygous (FF), heterozygous (F+) or non-carriers(++) of a fecundity gene were obtained 0–48 h after cloprostenol injection on Day 10 of the oestrous cycle. The highest mean amounts of cAMP produced by the cells did not differ between the genotypes. However, in the ++ ewes it was attained by cells from follicles ≥5 mm in diameter, whereas in F+ and FF ewes it was attained by cells from follicles 3–4.5 mm in diameter. Cells from 1–2.5-mm diameter follicles of FF ewes were more sensitive to FSH and LH than were corresponding cells from F+ or ++ ewes. Granulosa cells from ≥5 mm diameter follicles of ++ ewes 12–24 h after injection of cloprostenol had a lower mean response to FSH and LH than did cells obtained 0–6 or 36–48 h after cloprostenol. No such effect of time was evident for cells from any size of follicles obtained from F+ or FF ewes. In 1–2.5-mm diameter follicles, the mean aromatase activity of granulosa cells from ++ and F+ ewes was similar, but significantly lower than that of cells from FF ewes. In 3–4.5 mm diameter follicles, the mean aromatase activity of cells from F+ and FF ewes was similar, and significantly higher than that of cells from ++ ewes. For all 3 genotypes, there was a significant positive relationship between FSH or LH stimulation of granulosa cell cAMP production and cellular aromatase activity.

### Introduction

The increased prolificacy of Booroola crossbred ewes endowed with the so-called F, or fecundity gene, is a consequence of their increased ovulation rate compared to ewes without the gene (see Bindon, 1984, for review). Sheep that are homozygous (FF), heterozygous (F+) and non-carriers (++) of the fecundity gene have been segregated on the basis of at least one ovulation rate recording of ≥5, 3 or 4, and 1 or 2 respectively (Davis *et al.*, 1982). In a previous study we compared the ability of FSH and LH to stimulate cAMP production by granulosa cells from different sized follicles obtained during the preovulatory period from F+ and ++ Booroola ewes (Henderson *et al.*, 1985). It was found that the highest mean amounts of cAMP produced by cells challenged with FSH or LH did not differ between the genotypes. However, they were achieved using cells from ≥5 mm diameter follicles in ++ ewes but only 3–4 mm diameter follicles in F+ ewes. On the basis of this and other studies (McNatty *et al.*, 1985) it has been suggested that follicles from F+ ewes mature at a smaller diameter than those from ++ ewes. In addition, it was found that in ++ but not F+ ewes the amount of cAMP produced by granulosa cells challenged with FSH was dependent upon the time the cells were collected during a cloprostenol-induced follicular

phase. FF ewes have an even higher ovulation rate than F+ ewes. The responsiveness to gonadotrophins of granulosa cells from FF ewes may therefore differ from that of cells from F+ ewes. The present study was therefore undertaken to compare the ability of FSH and LH to stimulate cAMP production by granulosa cells obtained from various sized follicles of FF, F+ and ++ Booroola ewes during a cloprostenol-induced follicular phase.

### Materials and Methods

**Sheep.** This study was performed using 15 FF, 17 F+ and 18 ++ Booroola × Merino ewes aged 5–8 years. They were grazed on open pasture, and run with a vasectomized ram fitted with a marking harness to detect oestrous activity.

**Recovery of granulosa cells.** On Day 10 of the oestrous cycle (Day 0 = day of oestrus) the ewes were injected intramuscularly with 125 µg cloprostenol (Coopers Animal Health, Upper Hutt, New Zealand) to induce luteolysis. Ovariectomy was performed 0, 6, 12, 24, 36 or 48 h later (3 ewes of each genotype at each time, except at 6 h when only 2 F+ and 0 FF ewes were studied, due to the limited number of ewes available). Immediately after ovariectomy, the corpora lutea and all antral follicles ≥ 1.0 mm in diameter were individually dissected into sterile Medium 199 with Earle's salts (Eagle, 1959), supplemented with HEPES buffer (20 mM), gentamicin (50 µg/ml; Gibco, Grand Island, NY, U.S.A.), sodium heparin (50 i.u./ml; Weddel Pharmaceuticals Ltd, London, U.K.) and 1.0% bovine serum albumin (Fraction V; Sigma Chemical Co., St Louis, MO, U.S.A.) (Medium A). After recording the follicular diameter and external appearance, and removing the follicular fluid, the granulosa cells were recovered from each follicle by washing the internal face of the follicle wall repeatedly with Medium A. After removing the oocyte, released clumps of cells were dispersed by pipetting several times through a finely drawn Pasteur pipette. The total number of granulosa cells recovered from each follicle was determined by counting using a haemocytometer. Each follicle was classified as non-atretic or atretic on the basis of its morphological characteristics and number of granulosa cells as described previously (Henderson *et al.*, 1985). The influence of follicular health on steroid production by these granulosa cells has been reported previously (McNatty *et al.*, 1986b). However, because of the relatively few cells in small and/or atretic follicles, and the large number of cells required to perform replicate dose-response studies, cells were pooled according to follicle size irrespective of health. For each ewe, cells were pooled from follicles of 1–2.5 mm, 3–4.5 mm and, in ++ ewes, ≥ 5 mm diameter.

**Determination of cellular cAMP production in response to LH and FSH, and oestradiol-17β synthetase (aromatase activity).** Each pool of granulosa cells was split into 2 fractions, one to study cAMP production in response to challenge with LH or FSH, the other to study aromatase activity. The cells were centrifuged at 200 g for 10 min and resuspended in Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin (DBS-BSA) for determining the cAMP response to gonadotrophins, or in Medium A devoid of sodium heparin (Medium B) for determination of aromatase activity. Aliquants of each were taken for determination of cell number by haemocytometer counts. To determine responsiveness to gonadotrophins, aliquants of granulosa cells ( $1.5 \times 10^5$  cells) in 0.5 ml DBS-BSA were dispensed into a series of 10 × 75 mm plastic test-tubes. Ovine FSH (NIADDK-oFSH-16) or ovine LH (NIADDK-oLH-24) was added in 0.5 ml DBS-BSA to give a final concentration of gonadotrophin of 0, 0.1, 1, 10, 100 or 1000 ng/ml. In some instances, 3-isobutyl-1-methylxanthine was also included in the incubations at final concentration of 500 µM. Duplicate or triplicate tubes were established for each dose. The tubes were capped and incubated at 37°C for 1 h in a shaking water bath before being transferred to an 80°C water bath for 15 min. Some tubes were transferred directly to the 80°C bath so that the endogenous cAMP content of the cells at zero time could be determined. All the tubes were frozen (–20°C) until assayed for cAMP by radioimmunoassay. Preliminary studies showed that the rate of cAMP production by granulosa cells incubated for 2 h at 37°C was constant.

For determination of aromatase activity, aliquants of cells ( $6-60 \times 10^4$ ) in 0.5 ml Medium B were pipetted into duplicate or triplicate tubes containing 0.5 ml of a solution of testosterone (2 µg/ml) in Medium B. The tubes were gassed with 5% CO<sub>2</sub> in air, capped and incubated for 3 h at 37°C in a shaking water bath. At the end of the incubation, the tubes were snap frozen to –70°C. Subsequently, the contents of the tubes were thawed, centrifuged and the supernatants assayed for oestradiol-17β. Under these conditions, the rate of oestradiol-17β production is constant for 3 h.

**Radioimmunoassays.** Concentrations of cAMP and oestradiol-17β in incubation media were measured by specific, direct radioimmunoassays described previously (McNatty *et al.*, 1984, 1986a). The limit of sensitivity of the assays (per tube) was 5 fmol for cAMP and 5 pg for oestradiol-17β. The intra- and inter-assay coefficients of variation were < 12%. Values for cAMP and oestradiol-17β were normalized with respect to granulosa cell number, and the results were expressed as pmol cAMP or ng oestradiol-17β per 10<sup>6</sup> cells.

**Statistics.** The data were analysed statistically using analysis of variance in conjunction with Newman-Keuls multiple range test, Student's *t* test or  $\chi^2$  as appropriate. When heterogeneity of variance was indicated by Bartlett's test (data for Table 1 and Figs 1 & 2), the variances were equalized by transforming the data to logarithms ( $\ln(x + 1)$ ). In these instances the data have been presented as geometric means together with 95% confidence limits, except in Fig. 1 where for clarity means and the s.e.m. have been presented. The level of significance was set at  $P < 0.05$ .

## Results

### *Influence of genotype on the number of corpora lutea and antral follicles*

The number of corpora lutea recovered from each genotype was  $5.1 \pm 0.4$  (mean  $\pm$  s.e.m.) for FF ewes (N = 15),  $2.8 \pm 0.1$  for F + ewes (N = 17) and  $1.2 \pm 0.1$  for ++ ewes (N = 18) (FF > F+ > ++,  $P < 0.01$ ). There was no influence of genotype on the mean numbers of follicles of 1–2.5 or 3–4.5 mm diameter, or the percentage of these follicles which were considered non-atretic. FF, F+ and ++ ewes had  $37 \pm 4$ ,  $32 \pm 3$  and  $30 \pm 2$  follicles respectively of 1–2.5 mm diameter, of which  $44 \pm 4\%$ ,  $45 \pm 4\%$  and  $53 \pm 3\%$  respectively were non-atretic. There were  $4.1 \pm 0.7$ ,  $3.5 \pm 0.3$  and  $2.5 \pm 0.5$  follicles of 3–4.5 mm diameter in FF, F+ and ++ ewes respectively, of which  $86 \pm 5\%$ ,  $80 \pm 5\%$  and  $72 \pm 7\%$  respectively were non-atretic (all values are mean  $\pm$  s.e.m.). No follicles of  $\geq 5$  mm diameter were recovered from the FF ewes while only 2 (both atretic) were recovered from one F+ ewe. Of the 18 ++ ewes, 15 had one follicle  $\geq 5$  mm in diameter (all non-atretic), one ewe had 2 follicles of this size (1 atretic and 1 non-atretic) and 2 ewes had no follicles  $\geq 5$  mm in diameter. The number of follicles of each diameter recovered was independent of the time after cloprostenol treatment for all genotypes.

### *Influence of genotype and follicular diameter on LH and FSH stimulation of cAMP production by granulosa cells*

As follicular diameter increased there was a shift to the left in the dose–response curves for FSH and LH stimulation of cAMP production for all genotypes (Fig. 1). With increasing follicular diameter, lower doses of gonadotrophin were able to stimulate cAMP production above basal levels, while higher amounts of cAMP were produced in response to the top doses of gonadotrophin. The minimum doses able to stimulate cAMP production were 10 ng FSH/ml and 0.1 ng LH/ml. At each dose of FSH  $\geq 10$  ng/ml and of LH  $\geq 0.1$  ng/ml there was no significant difference between the amounts of cAMP produced by cells from  $\geq 5$  mm diameter follicles of ++ ewes and cells from 3–4.5 mm diameter follicles of FF or F+ ewes. However, cells from 3–4.5 mm diameter follicles of FF and F+ ewes produced significantly more cAMP than did cells from similar sized follicles of ++ ewes at each dose of FSH  $\geq 10$  ng/ml or LH  $\geq 0.1$  ng/ml. There was little difference between FF and F+ ewes except that 100 ng FSH or LH/ml caused a slight, but significant, increase in cAMP production (relative to 0 ng FSH or LH/ml) with cells from 1–2.5 mm diameter follicles in FF, but not F+ ewes. Cells of 1–2.5 mm diameter follicles of FF ewes were therefore on average more sensitive to gonadotrophins than were cells of similar sized follicles of F+ or ++ ewes.

Inclusion of 3-isobutyl-1-methylxanthine (500  $\mu$ M) in the incubation medium to inhibit granulosa cell phosphodiesterase activity increased gonadotrophin stimulation of cAMP production by approximately 1.8- to 3-fold, regardless of ewe genotype or follicular diameter.

### *Influence of time after cloprostenol treatment on FSH and LH stimulation of cAMP production by granulosa cells*

Granulosa cells collected from large follicles ( $\geq 5$  mm diam.) of ++ ewes 12–24 h after cloprostenol treatment produced a significantly lower mean amount of cAMP when challenged with FSH or LH (100 ng/ml) than did cells obtained 0–6 h or 36–48 h after cloprostenol (Fig. 2). In F+ and FF ewes, the response of granulosa cells to FSH and LH 12–24 h after cloprostenol was not consistently low, in contrast to the ++ ewes, but was much more variable, as indicated by the large 95% confidence limits. No significant effect of time on the mean response of granulosa cells to FSH or LH was thus evident for the F+ and FF ewes. Mean cyclic AMP production in response to FSH or LH stimulation by cells from follicles < 5 mm in diameter in ++ ewes or 1–2.5 mm in diameter in F+ or FF ewes was also independent of time of collection of the cells.



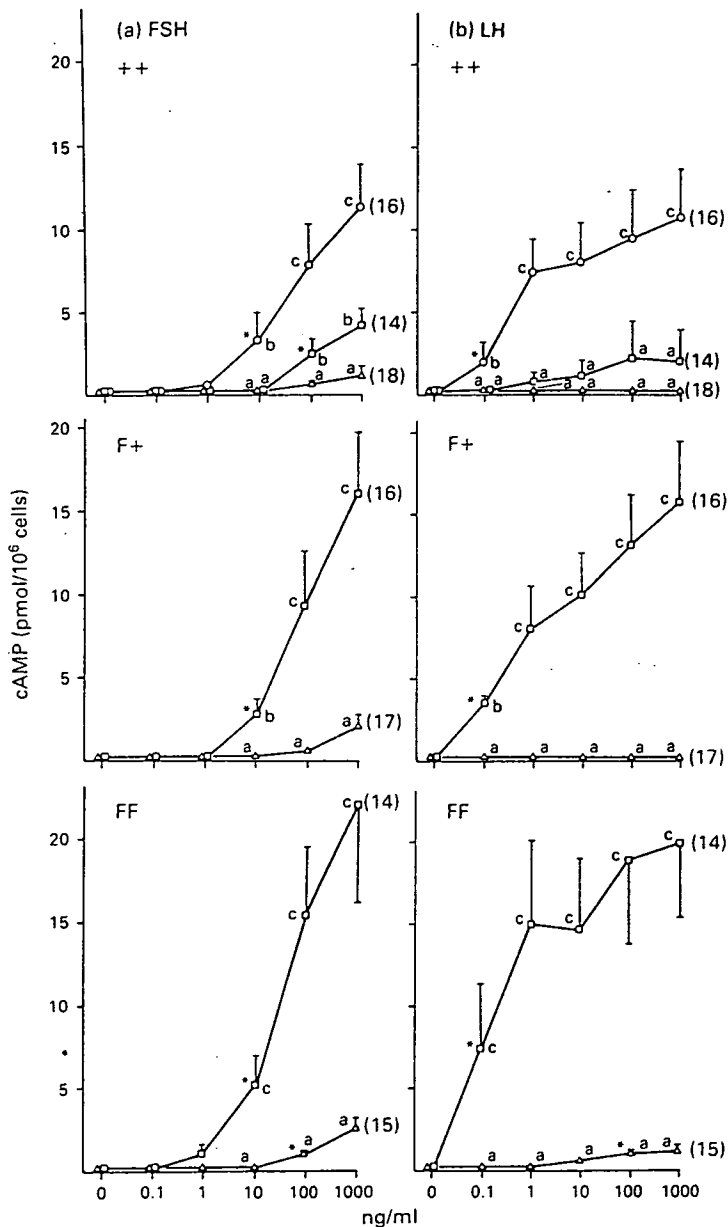


Fig. 1. Effect of increasing concentrations of (a) FSH and (b) LH on in-vitro cAMP production by granulosa cells from 1-2.5 mm ( $\Delta$ - $\Delta$ ), 3-4.5 mm ( $\square$ - $\square$ ) and  $\geq$  5 mm ( $\circ$ - $\circ$ ) diameter follicles of ++, F+ and FF Booroola  $\times$  Merino ewes. Values are means of (N) ewes. Vertical lines show the s.e.m. when larger than the size of the symbol. \*Lowest concentration of gonadotrophin to stimulate cAMP production significantly (relative to 0 ng/ml) for that follicle size range ( $P < 0.05$ ). Mean values with different letter superscripts for the same dose of FSH or LH are significantly different ( $P < 0.05$ ). Groups of mean values without superscripts are not significantly different.

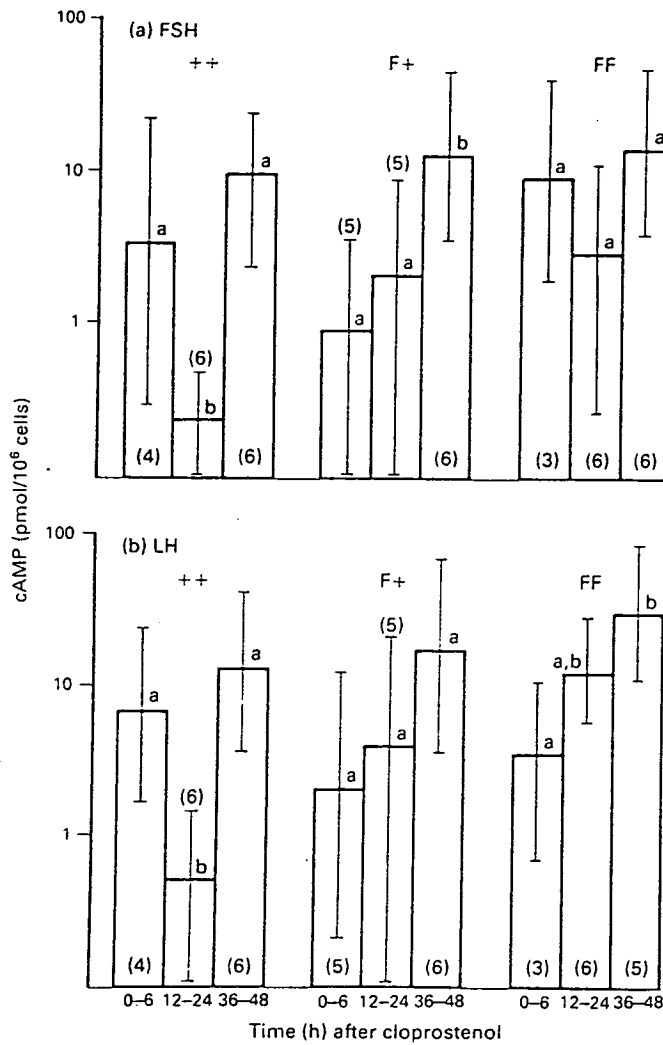


Fig. 2. Effect of time after cloprostenol treatment on (a) FSH and (b) LH (100 ng/ml for each) stimulation of cAMP production by granulosa cells of large follicles from ++, F+ and FF ewes. Large follicles in ++ ewes are those  $\geq 5$  mm in diameter and in FF and F+ ewes those 3-4.5 mm in diameter. Values are geometric means with 95% confidence limits indicated by the vertical lines for (N) ewes. Mean values with different letters within the same genotype are significantly different ( $P < 0.05$ ).

*Influence of follicular diameter and ewe genotype on granulosa cell aromatase activity*

There was a significant increase in granulosa cell aromatase activity as follicular diameter increased in all 3 genotypes (Table 1). The aromatase activity of cells from 1-2.5 mm diameter follicles of F+ and ++ ewes did not differ significantly, but was significantly lower than that of similar sized follicles from FF ewes. The aromatase activity of cells from 3-4.5 mm diameter follicles of F+ and FF ewes was significantly higher ( $P < 0.01$ ) than that of cells from similar sized follicles of ++ ewes, but not significantly different ( $P > 0.05$ ) from the aromatase activity of cells from  $\geq 5$  mm diameter follicles of ++ ewes.

**Table 1.** Influence of follicular diameter and genotype on granulosa cell aromatase activity (ng oestradiol-17 $\beta$ /10<sup>6</sup> cells)

Genotype	Follicular diameter (mm)		
	1-2.5	3-4.5	$\geq 5$
++	0.20 <sup>a</sup> (18) 0.15-0.27	1.36 <sup>c</sup> (14) 0.56- 2.57	3.89 <sup>d</sup> (16) 2.13-6.63
F+	0.40 <sup>a</sup> (17) 0.27-0.54	4.43 <sup>d</sup> (16) 2.71- 6.95	—
FF	0.73 <sup>b</sup> (15) 0.33-1.26	6.67 <sup>d</sup> (14) 3.78-11.54	—

Values are geometric means with 95% confidence limits below for (N) sheep.

Values not sharing a common superscript in the same row or column are significantly different ( $P < 0.05$ ).

**Table 2.** Frequency table showing the relationship between FSH (100 ng/ml) or LH (100 ng/ml) stimulation of cAMP production and cellular aromatase activity

Genotype	Aromatase (ng oestradiol-17 $\beta$ per 10 <sup>6</sup> cells)	cAMP production (pmol/10 <sup>6</sup> cells) in response to stimulation with:					
		FSH			LH		
		<1	1-5	>5	<1	1-5	>5
++	<1	22	8	0	27	10	0
	1-5	5	3	2	0	1	2
	>5	0	2	6	0	2	6
F+	<1	16	1	0	17	3	0
	1-5	4	3	2	2	1	2
	>5	0	2	5	0	1	7
FF	<1	9	4	0	8	4	0
	1-5	3	2	2	3	3	1
	>5	0	2	7	0	2	8

*Relationship between FSH and LH stimulation of granulosa cell cAMP production, and cellular aromatase activity*

For all 3 genotypes, there was a significant positive relationship ( $P < 0.01$ ,  $\chi^2$  analyses) between the ability of granulosa cells to metabolize testosterone to oestradiol-17 $\beta$  (aromatase activity) and the amount of cAMP produced by the cells in response to challenge with FSH or LH (Table 2).

### Discussion

In our previous study, we demonstrated differences in LH and FSH stimulation of cAMP production between granulosa cells obtained from F+ and ++ Booroola  $\times$  Romney ewes (Henderson *et al.*, 1985). The present study of Booroola  $\times$  Merino ewes has confirmed these findings, and in addition shown that the aromatase activity and the responsiveness to LH and FSH

of granulosa cells from FF ewes in turn differs from that of F+ and ++ ewes. In this study, granulosa cells were pooled according to follicular diameter, irrespective of follicle health, whereas in our previous study cells from non-atretic and atretic follicles were studied separately. However, the finding that cells from ++ and F+ ewes responded to LH and FSH in a similar fashion in both studies indicates that, provided follicles are of the same diameter, it makes little difference to the final results whether cells from non-atretic and atretic follicles are pooled or not. This is further supported in that the effects of follicular diameter on granulosa cell aromatase activity, for all 3 genotypes, are similar to findings of the previous study (McNatty *et al.*, 1986b), in which cells from non-atretic and atretic follicles were studied separately.

Follicles of FF, F+ and ++ Booroola  $\times$  Merino ewes reach preovulatory maturity when 2–4.5 mm, 3–4.5 mm and  $\geq 5$  mm in diameter, respectively (McNatty *et al.*, 1986b). This is reflected in the follicular diameters at which granulosa cells show their highest mean response (in terms of cAMP production) to FSH and LH (Fig. 1), and highest mean aromatase activity (Table 1). Although cells from 3–4.5 mm diameter follicles of FF and F+ ewes responded in a similar fashion to both FSH and LH, and had similar aromatase activities, cells from 1–2.5 mm diameter follicles of FF ewes were slightly more sensitive to FSH and LH than were corresponding cells of F+ ewes (Fig. 1), and had a higher mean aromatase activity (Table 1). This is consistent with some follicles of FF ewes attaining preovulatory maturity earlier (i.e. at a smaller diameter) than follicles of F+ ewes. It cannot be ascertained from the present study whether it is an increase in the rate of follicular maturation and/or a decrease in the rate of follicular atresia which allows follicles of F gene-bearing ewes to attain preovulatory maturity at smaller diameters than in ++ ewes.

In all 3 genotypes, granulosa cells became more sensitive to gonadotrophins as follicular diameter increased, as indicated by the shifts in the dose–response curves to FSH and LH (Fig. 1). Although FSH is essential for antral follicle development, the later stages of follicular development in sheep occur in the face of declining plasma concentrations of FSH, brought about by the negative feedback effects of steroids and inhibin produced by the developing follicle(s) (Baird *et al.*, 1981; Miller *et al.*, 1981; Henderson *et al.*, 1986a). Falling plasma FSH concentrations may therefore be compensated for to some extent by granulosa cells becoming more sensitive to FSH, as follicle size increases.

At 12–24 h after cloprostenol treatment, the mean response to FSH and LH of granulosa cells from  $\geq 5$  mm diameter follicles of ++ ewes was significantly less than at other times (Fig. 2). Therefore, despite the increased sensitivity of large follicles to FSH, the low plasma FSH concentrations occurring at this time may result in the gonadotrophic environment being inadequate to support fully proper functioning of such large follicles. Previous studies with low fecundity Romney ewes have also shown that when plasma FSH concentrations were depressed, after treatment with follicular fluid, granulosa cells from large ( $\geq 5$  mm diam.) but not medium or small follicles had a reduced ability to produce cAMP when challenged *in vitro* with FSH or LH (Henderson *et al.*, 1986b). None of the large follicles from ++ ewes examined 12–24 h after cloprostenol treatment were judged atretic by our morphological criteria, despite the reduced responsiveness of the granulosa cells to FSH and LH. However, a reduction in the ability of gonadotrophins to stimulate cAMP production by granulosa cells may represent a very early, possibly reversible, stage of atresia. Morphological signs of atresia first appear relatively late in the atretic process, some considerable time after the initial biochemical lesions have occurred. In F+ and FF ewes there was no significant effect of time on FSH or LH stimulation of cAMP production by granulosa cells from large follicles (Fig. 2). Cells from a lower proportion of the follicles examined from F+ and FF ewes, than from ++ ewes, showed a low responsiveness to FSH and LH 12–24 h after cloprostenol treatment. Perhaps in F gene-bearing ewes large follicles may be more resistant to the deleterious effects of low plasma FSH concentrations, and/or plasma FSH concentrations are higher than those of ++ ewes (Bindon *et al.*, 1984; McNatty *et al.*, 1987).

There was a significant positive relationship between granulosa cell aromatase activity and FSH or LH stimulation of cAMP production in all genotypes (Table 2). There were no instances of the

cells having a high responsiveness to FSH or LH but a low aromatase activity, or *vice versa*. In sheep, as in the rat (Wang *et al.*, 1982), FSH stimulation of cAMP production may be an essential early event necessary for full functioning of the aromatase enzyme complex. Whether production of large amounts of cAMP in response to LH is a cause or consequence of high cellular aromatase activity is uncertain.

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## GONADOTROPHINS, FECUNDITY GENES AND OVARIAN FOLLICULAR FUNCTION

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**Summary**—The Booroola Merino is a sheep breed having a major gene(s) (F) influencing its ovulation-rate. Homozygous (FF), heterozygous (F+) and non-carriers (++) of the gene have ovulation-rates of  $\geq 5$ , 3 or 4 and 1 or 2 respectively with the durations of each oestrous cycle and oestrous behaviour being similar in all genotypes. Although the principal site(s) of gene expression are obscure, FF genotypes have mean plasma concentrations of FSH and LH which are higher than in the F+ ewes, which in turn are higher than in the ++ animals. Thus, the FF and F+ animals provide a unique system in which to examine ovarian function under continual exposure to elevated gonadotrophin concentrations.

At the ovarian level, F gene-specific differences in follicular development and function were noted. In small follicles (0.1–1.0 mm dia.), the basal levels of cAMP and the *in vitro* synthesis of cAMP, progesterone, androstenedione and oestradiol-17 $\beta$  in response to LH and FSH were significantly influenced by genotype (FF > F+ > ++;  $P < 0.05$ ). In larger follicles (1–4.5 mm dia.) the granulosa cells from FF and F+ ewes were more responsive to FSH and/or LH than in ++ ewes with respect to cAMP synthesis and they also had higher levels of aromatase activity. *In vivo*, the ovarian secretion-rates of oestradiol from  $\geq 5$  ("oestrogenic") follicles in FF ewes, 3–4 such follicles in F+ ewes, and 1–2 such follicles in ++ animals during the follicular phase were similar. In FF and F+ ewes, the preovulatory follicles ovulated at a smaller diameter (i.e. 3–5 mm) than in ++ ewes (> 5 mm diam.) and also produced smaller corpora lutea. Thus, after continual exposure to elevated levels of gonadotrophins, follicles may synthesize steroid and mature at smaller diameters compared to those exposed to normal levels of FSH and LH.

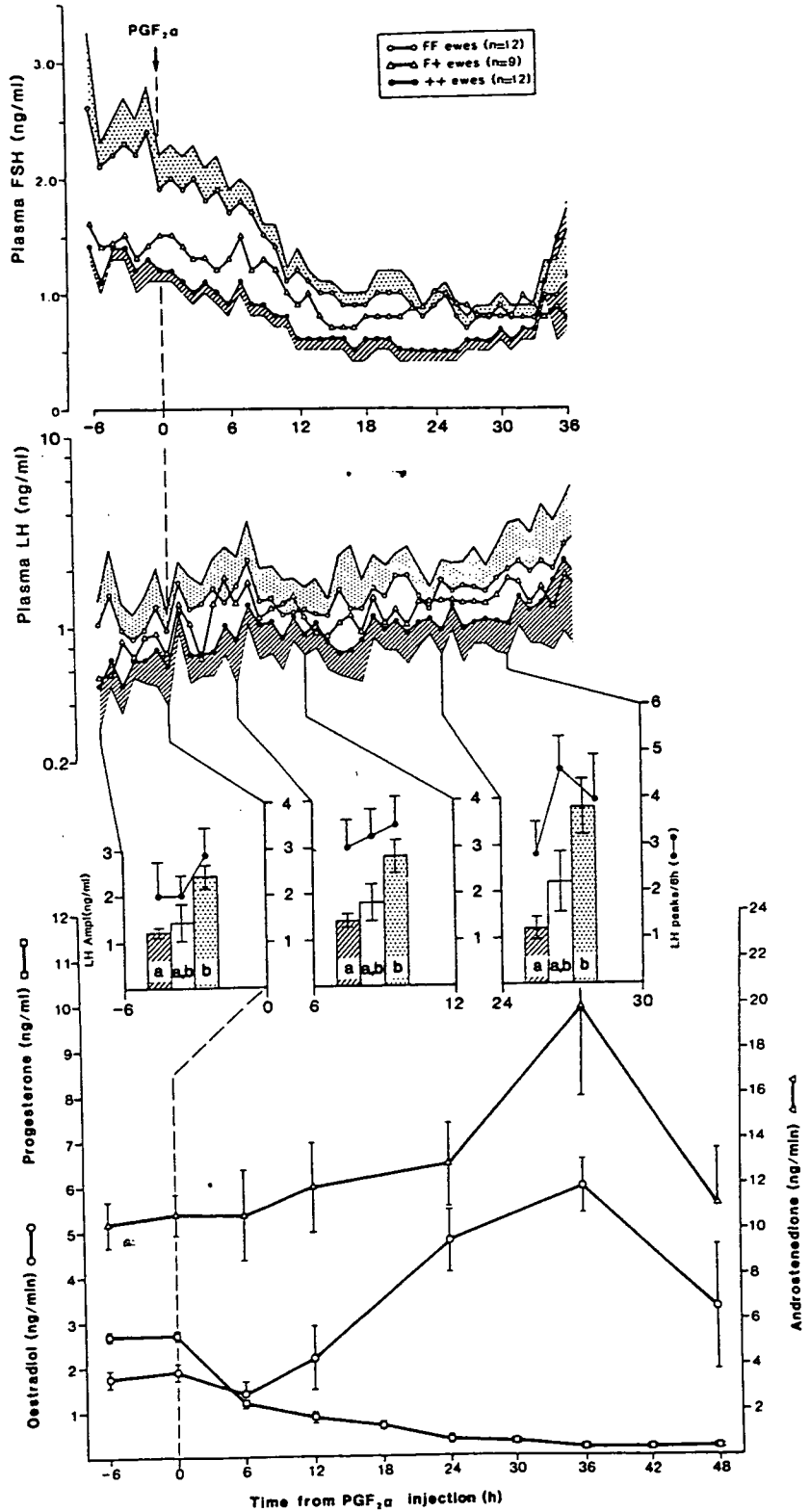
### INTRODUCTION

The Booroola Merino is one of the most prolific sheep breeds in the world [1]. The term Booroola refers to a commercial sheep property in Cooma, New South Wales, Australia, where the prolificacy of this strain of Merino sheep was first noted. The history and origins of the Booroola sheep flock are obscure; all the available data have been reviewed by Turner [2]. The exceptional prolificacy of the Booroola has been attributed to a major gene(s) which influences its ovulation-rate (i.e. as judged by the number of corpora lutea formed each oestrous cycle) [3–5]. The putative fecundity gene(s) is referred to as the F-gene. Homozygous (FF), heterozygous (F+) and non-carriers (++) have been segregated on the basis of at least one ovulation-rate recording of  $\geq 5$ , 3 or 4 and 1 or 2 respectively [4]. Apart from ovulation-rate, no other overt difference between the genotypes has been recorded. For example, the animals are identical in appearance and the lengths of each oestrous cycle (i.e. 16–18 days) and durations of oestrous activity (i.e. ~33 h) are similar. Moreover, no differences have been noted in the time from the onset of oestrus to the preovulatory surge of luteinizing hormone (LH) (i.e. 8–11 h) nor in the duration of the preovulatory LH surge itself (i.e. 11–12 h) [6]. In addition, there is no evidence, as yet, for expression of the F-gene in the male [1]. In the female Booroola, the principal site(s) of F-gene expression remains to be elucidated. However, despite a lack of knowledge as to how or where the

F-gene is expressed, Booroola sheep provide a unique system in which to investigate physiological mechanisms that determine the number of ovarian follicles that ovulate. The aim of this paper is to summarise recent findings on gonadotrophins and ovarian follicular function in FF, F+ and ++ Booroola ewes.

### Gonadotrophins and ovarian steroid secretion

The pituitary gonadotrophins, follicle stimulating hormone (FSH) and LH are key determinants of ovarian follicular maturation and of ovulation-rate in sheep [7–9]. Thus, before examining ovarian function in the different Booroola genotypes, it is of value to have some insight into the plasma concentration of FSH and LH in these animals. In FF ewes, the mean FSH and LH values are consistently higher than in ++ ewes with those for F+ animals being consistently in between [10]. These F gene-specific differences were found during both the luteal and follicular phases of the oestrous cycle. For example, the composite data in Fig. 1 summarize the plasma concentrations (ng/ml) of FSH, LH and progesterone and also the ovarian secretion-rates (ng/min) of androstenedione and oestradiol for FF, F+ and ++ ewes before and after a prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> )-induced follicular phase. For the gonadotrophin data in Fig. 1, the ovulation rates during the cycle under study were not known. However, in the subsequent cycle they were  $4.8 \pm 0.3$  ( $n = 12$  FF ewes),  $2.8 \pm 0.1$  ( $n = 9$  F+ ewes) and  $1.3 \pm 0.1$  ( $n =$





12 ++ ewes). The mean FSH concentrations in the FF ewes before luteolysis were significantly higher than those for the F+ and ++ ewes (both  $P < 0.01$ , Kruskal-Wallis test) whereas the values in the F+ and ++ ewes were not significantly different from one another. Although the rate of decline of FSH in the FF ewes from PGF<sub>2α</sub> injection until 20 h later was significantly steeper than that for either the F+ and ++ ewes (both  $P < 0.05$ , regression analysis), the FSH values were consistently higher in FF ewes than those in either the F+ and ++ ewes.

For LH, the mean values in the FF ewes were always higher than in the ++ ewes with those for the F+ ewes being in between. For all genotypes, the mean LH values increased gradually after PGF<sub>2α</sub> injection until the end of the sampling period. In these animals, the preovulatory LH surge for the 3 genotypes began some 44–48 h after PGF<sub>2α</sub>. The characteristics of the LH concentrations with respect to LH peak frequency and amplitude as assessed from 10-min blood sampling regimens for 6 hourly "windows" at -6 to 0, +6 to +12 and +24 to +30 h from PGF<sub>2α</sub> injection are shown in the inserts to Fig. 1. At each of these 6 hourly "windows", there were no gene-specific differences in LH peak frequency ( $P > 0.1$ ). However, during all of the above time frames, the LH peak amplitudes were 2–3-fold higher in FF ewes compared to ++ ewes ( $P < 0.05$  at all times tested; analysis of variance, Neuman-Keuls test) with those for the F+ ewes being in between. Thus, the gene-specific differences in mean LH concentration were, at least in part, due to differences in the amplitudes of pituitary LH secretion. It is not known whether similar secretory characteristics existed for pituitary FSH release.

For the steroid data in Fig. 1, the respective ovulation-rates in the FF, F+ and ++ ewes for the cycle under study were  $5.1 \pm 0.4$  ( $n = 15$  FF ewes),  $2.8 \pm 0.1$  ( $n = 30$  F+ ewes) and  $1.2 \pm 0.1$  ( $n = 30$  ++ ewes). For progesterone, androstenedione, testosterone (data not shown) and oestradiol, there were no F gene-specific differences in concentration or secretion-rates at each of the time points ( $n = 2$ –6 animals/point), consequently, the data for all the

genotypes were pooled. At 12 h after PGF<sub>2α</sub> injection, the progesterone concentrations were only 65% of those beforehand and at 36 h afterwards the values were at the lowest limits of assay detection indicating that progesterone secretion from the corpora lutea had ceased. After PGF<sub>2α</sub> injection there was a progressive increase in the overall ovarian secretion-rates of androstenedione, testosterone and oestradiol which, at 36 h after injection, were 2–5-fold higher than beforehand.

Thus, although gene-specific differences were found in the plasma concentrations of FSH and LH (FF > F+ > ++), the ovarian secretion-rates of progesterone, androstenedione, testosterone and oestradiol did not differ between the genotypes.

#### *Follicle numbers, follicle cell populations and diameter of preovulatory follicles*

The relationships (if any) between the plasma concentrations of FSH and/or LH and follicle numbers are obscure. However, it is known that the plasma levels of FSH may influence the numbers of granulosa cells in ovarian follicles and also the sizes at which follicles may secrete oestradiol [8, 9]. Thus, as F gene-specific differences were found to exist with respect to the plasma concentrations of FSH and LH, it is possible that these may influence the numbers of follicles or the numbers of cells within follicles and/or the sizes of the follicles at ovulation.

There is no information available on whether there are F gene-specific differences in the numbers of primordial follicles between FF, F+ and ++ ewes. However, no significant gene-specific differences were noted in the total numbers of small (0.1–1 mm dia.) or larger-sized follicles ( $\geq 1$  mm dia.) or in the numbers of non-atretic follicles ( $\geq 1$  mm dia.; Table 1) [11–13]. No data are available on the numbers of non-atretic small follicles. The diameters of the largest follicles (atretic or non-atretic) in FF and F+ ewes were between 2 and 5 mm whereas in ++ ewes the largest were  $\geq 5$  mm. In contrast to follicle numbers, there were F gene-specific differences in the numbers of granulosa cells in non-atretic follicles of similar diameter (Fig. 2). Over all the ranges of

Fig. 1. A composite figure derived from two separate studies. The first study shows the changes in the hourly mean plasma concentrations of FSH and LH before and after an injection of a PGF<sub>2α</sub> derivative (cloprostenol, 125 µg subcutaneously) with respect to Booroola genotype. All ewes were at Day 10 of the oestrous cycle when injected with PGF<sub>2α</sub>. For FSH, the shaded areas for the ++ and FF ewes represent  $\pm$  SEM. For LH the levels represent geometric means and the shaded areas represent the 95% confidence limits. For both hormones, the shaded areas are equidistant each side of their respective means. For the sake of clarity the respective SEM and 95% confidence limits were not shown for the F+ ewes. The inserts refer to the LH amplitudes and LH peak frequencies determined from 10-min blood samplings over 6 hourly intervals from -6 to 0, +6 to +12 and +24 to +30 h from PGF<sub>2α</sub> injection. The diagonal striped, white and stippled histograms represent the LH amplitudes in ++, F+ and FF ewes respectively. The number of ewes for both FSH and LH was 12 for FF, 9 for F+ and 12 for ++ ewes. The second study shows the mean plasma concentrations of progesterone (□—□) and total (left and right) ovarian venous androstenedione (△—△) and oestradiol (○—○) secretion rates at various times before and after PGF<sub>2α</sub> injection in FF ( $n = 15$ ), F+ ( $n = 30$ ) and ++ ( $n = 30$ ) ewes. For the ovarian steroid secretion rates the number of observations per genotype per data point was between 2 and 6 except at +6 h when no FF ewes were studied. For each of the above ewes the ovarian steroid secretion rates were determined at one time only. The vertical bars represent  $\pm$  SEM. Data obtained from Refs [10, 12, 13], and unpublished results.

Table 1. Follicle numbers with respect to diameter, follicular health and genotype. Values are geometric means (and 95% confidence limits)

Follicular diam. (mm)	Total number (T) or number of non-atretic follicles (NA)	Genotype		
		++	F+	FF
0.1-1.0	T	26 (20, 34)	26 (16, 46)	27 (17, 43)
≥1.0	T	32 (28, 36)	31 (24, 39)	38 (31, 47)
≥1.0	NA	17 (15, 19)	15 (11, 19)	18 (14, 22)

Data from [11, 13].

follicle sizes which could be compared, the numbers of granulosa cells in the FF animals were significantly lower (all  $P < 0.05$ ) than in the ++ ewes with those for the F+ animals being in between. No data are available with respect to the populations of theca interna cells in each of the genotypes.

At the time of the preovulatory LH surge (i.e. at 48 h after a PGF<sub>2α</sub>-induced follicular phase), the respective mean ( $\pm$ SEM) diameters of the presumptive preovulatory follicles in FF, F+ and ++ ewes were  $3.4 \pm 0.3$  mm,  $4.1 \pm 0.2$  and  $6.8 \pm 0.3$  mm ( $n = 3$  ewes/genotype; all values are significantly different from one another,  $P < 0.05$ ). Moreover, the respective mean ( $\pm$ SEM) numbers of granulosa cells in these follicles were  $1.8 (\pm 0.3) \times 10^6$ ,  $2.2 (\pm 0.3) \times 10^6$  and  $6.6 (\pm 0.3) \times 10^6$  (all values are significantly different from one another,  $P < 0.05$ ).

#### Adenosine cyclic 3',5-monophosphate (cAMP) production

Specific plasma membrane receptors for LH are located in the theca interna and granulosa cells whereas specific plasma membrane receptors for FSH are located exclusively in granulosa cells [14, 15]. The LH receptors in granulosa cells are only present in large antral follicles [14]. Both

FSH and LH, on interacting with their receptors, stimulate the production of cAMP. Thus the levels of cAMP produced by intact follicles or isolated granulosa or theca cells provide a convenient index of the responsiveness of the cells to FSH and/or LH.

The tissue contents of cAMP in freshly dissected small follicles (0.1-0.5 mm dia.) were significantly higher in F-gene carriers than in ++ ewes (Table 2). After a 1-h incubation with LH (1  $\mu$ g/ml, NIH-LH-S23) and FSH (1  $\mu$ g/ml, NIH-FSH-S11) there was also a significant cAMP response in the small follicles (0.1-1 mm dia.) from ewes with the F-gene but not in those from ++ ewes (Table 2).

In large-sized follicles ( $\geq 1$  mm dia.) the gonadotrophin-induced cAMP response has been evaluated in isolated granulosa cells and theca interna but not in whole follicles [15, 16]. In dispersed granulosa cells from follicles ( $\geq 1$  mm dia.) there was a significant influence of follicular size on FSH and LH stimulation of cAMP production *in vitro* (Table 3). For example, in 1-2.5 and 3-4.5 mm dia. follicles, the granulosa cells from FF ewes produced, on average, between 1.8 and 10.2 times more cAMP than those from ++ ewes after exposure to 100 ng/ml FSH (NIH-FSH-S16). The maximum levels of cAMP production in FF ewes were reached in 3-

Table 2. Cyclic AMP content (pmol) in follicular tissue at zero time and after incubation with LH and FSH (both 1.0  $\mu$ g/ml) for 1 h with respect to Booroola genotype and follicle diameter

Follicular diameter (mm)	Time (h)	Genotype		
		++	F+	FF
0.13-0.52 (n)	0	0.13 (0.09, 0.16) <sup>a</sup> (50)	0.17 (0.15, 0.20) <sup>b</sup> (50)	0.20 (0.16, 0.24) <sup>b</sup> (34)
	1	0.14 (0.12, 0.16) <sup>a</sup> (114)	0.26 (0.21, 0.31) <sup>c</sup> (112)	0.35 (0.23, 0.48) <sup>c</sup> (62)
0.53-1.00 (n)	0	0.40 (0.31, 0.51) <sup>b</sup> (30)	0.48 (0.39, 0.57) <sup>b</sup> (42)	0.40 (0.32, 0.49) <sup>b</sup> (32)
	1	0.32 (0.26, 0.39) <sup>b</sup> (86)	0.80 (0.67, 0.95) <sup>c</sup> (112)	1.14 (0.90, 1.41) <sup>d</sup> (73)

Values are geometric means (and 95% confidence limits);  $n$  is the number of follicles. For each follicular diameter, the values in rows or columns having a different superscript are significantly different from one another: a vs b,  $P < 0.025$ ; b vs c,  $P < 0.01$ ; c vs d,  $P < 0.01$ . Data from [11].

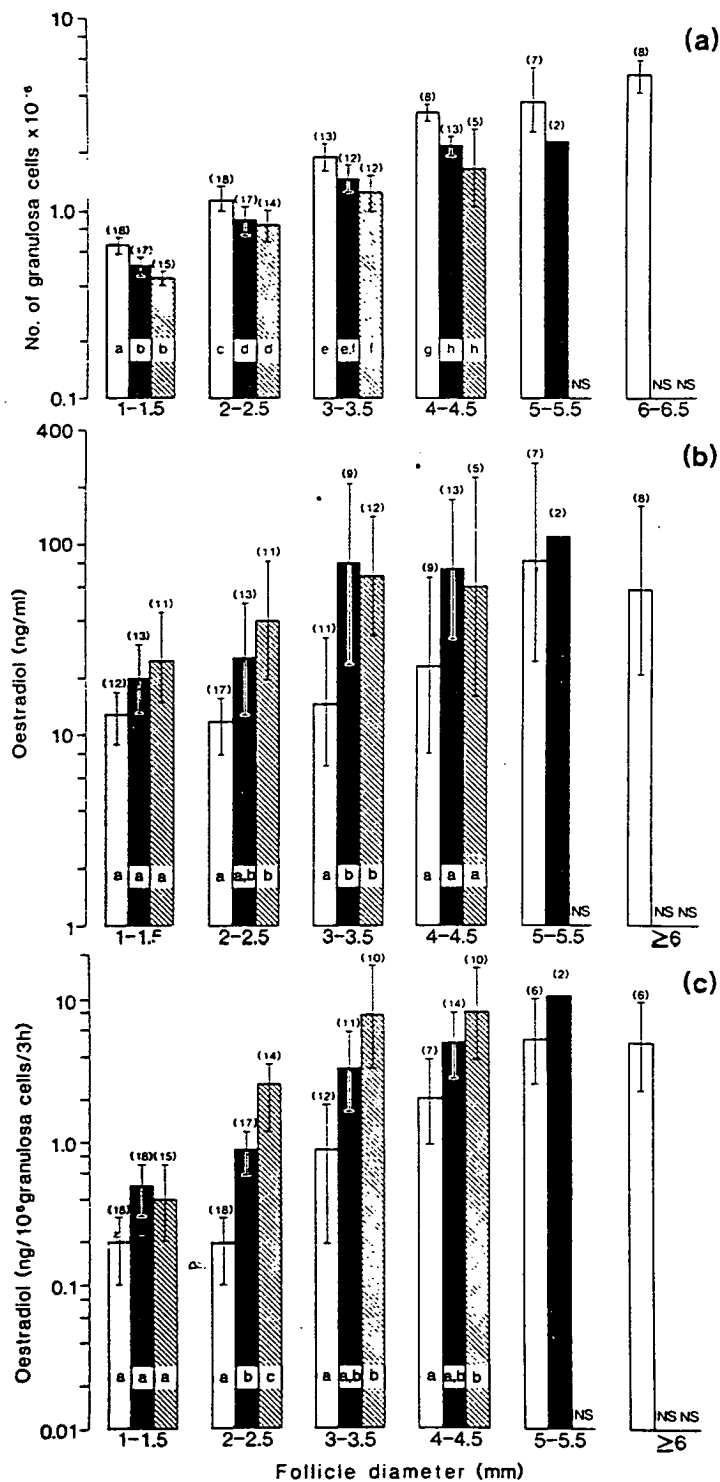


Fig. 2. The number of granulosa cells (a); the concentrations of oestradiol- $17\beta$  in follicular fluid (b); and aromatase activity in granulosa cells (c) from different sized non-atretic follicles of Booroola Merino ewes previously segregated as homozygous (FF, striped histograms), heterozygous (F+; black histograms) and non-carriers (++; white histograms) of a major gene influencing their ovulation rates. Results are geometric means with vertical bars representing 95% confidence limits. Numbers in parenthesis refer to number of ewes studied. For each range of diameters in (a), (b) or (c) the histograms not sharing a common alphabetical superscript were significantly different from one another,  $P < 0.05$ . Data obtained from [13]. NS = no samples.

Table 3. FSH or LH stimulation of cAMP production (pmol/10<sup>6</sup> cells/per h) by granulosa cells with respect to Booroola genotype and follicle diameter\*

Hormone (100 ng/ml)	Booroola genotype	Follicle diameter (mm)		
		1-2.5	3-4.5	≥5
FSH	++	0.4 <sup>a</sup> [18] (0.1-0.8)	0.7 <sup>a</sup> [14] (0.1-1.5)	2.9 <sup>b</sup> [16] (1.0-6.5)
	F+	0.3 <sup>a</sup> [17] (0.1-0.6)	3.7 <sup>b</sup> [16] (1.4-8.5)	—
	FF	0.7 <sup>a</sup> [15] (0.1-1.2)	7.2 <sup>b</sup> [14] (2.9-16.4)	—
LH	++	0.2 <sup>a</sup> [18] (0.1-0.3)	0.5 <sup>a</sup> [14] (0.02-1.3)	3.4 <sup>b</sup> [16] (1.1-8.0)
	F+	0.2 <sup>a</sup> [17] (0.1-0.3)	5.5 <sup>b</sup> [16] (2.2-12.4)	—
	FF	0.7 <sup>c</sup> [15] (0.3-1.1)	9.5 <sup>b</sup> [14] (4.1-20.9)	—

Number of ewes indicated by numbers in square brackets.

Different superscripts in rows or columns are significantly different  $P < 0.05$ .

\*Values are geometric means (and 95% confidence limits).

4.5 mm follicles whereas an equivalent response from cells in ++ ewes was not reached until follicles were ≥5 mm dia.

F gene-specific differences were also noted in the size of follicle in which granulosa cells acquire functional receptors for LH (Table 3) [15, 16]. Moreover, the maximum cAMP response to LH in FF and F+ ewes was observed in 3-4.5 mm dia. follicles whereas in ++ ewes, it was observed in ≥5 mm dia. follicles. The characteristics of the LH/hCG receptor in the granulosa cells (i.e.  $B_{max}$ ,  $K_d$ ) were identical in both the F-gene and non-carriers [14].

In isolated theca interna tissue from follicles ≥1 mm dia. no F gene-specific differences were noted with respect to LH-induced cAMP synthesis *in vitro*. Moreover, the characteristics of the thecal LH/hCG receptor (i.e.  $B_{max}$ ,  $K_d$ ) were identical in F-gene and ++ ewes [14].

#### Steroid synthesis

FSH and/or LH stimulation of cAMP may, in turn, lead to the synthesis of certain steroids. A key steroid associated with the final phases of ovarian follicle maturation is oestradiol. However, in sheep, as in other species, follicles first acquire the ability to synthesize progestins and androgens before some eventually acquire the ability to synthesize oestradiol.

**Small follicles (0.1-1.0 mm dia.).** The steroid contents in the tissues of freshly isolated small ovine follicles were undetectable (i.e. <0.6 ng/follicle) [11]. However, under *in vitro* conditions, follicles between 0.1 and 0.2 mm dia. (i.e. during antrum formation) were capable of synthesizing progesterone and androstenedione and those between 0.2 and 0.5 mm dia. were also capable of synthesizing

testosterone [11]. Some follicles ≥0.3 mm dia. with the potential to synthesize the above steroids were also capable of synthesizing oestradiol. Thus, when follicles were ≥0.5 mm dia. some synthesized all the above-mentioned steroids. In Booroola ewes there were significant F gene-specific effects in the proportions of follicles (0.5-1.0 mm dia.) which produced progesterone ( $P < 0.01$ ), androstenedione ( $P < 0.05$ ) and oestradiol ( $P < 0.025$ ) when exposed to LH+FSH (both 1 µg/ml) for 48 h *in vitro*; no gene-specific differences were noted for testosterone [11]. For progesterone, the respective proportions of FF, F+ and ++ follicles which produced ≥4 ng/48 h were 65% ( $n = 82$  follicles), 47% ( $n = 58$ ) and 38% ( $n = 55$ ). For androstenedione, the respective proportions of FF, F+ and ++ follicles which produced ≥3 ng/48 h were 40% ( $n = 82$ ), 26% ( $n = 58$ ) and 22% ( $n = 55$ ). For oestradiol, the respective proportions of FF, F+ and ++ follicles which produced ≥0.8 ng oestradiol/48 h were 39% ( $n = 82$ ), 26% ( $n = 58$ ) and 25% ( $n = 55$ ).

In summary, the evidence suggests that, irrespective of genotype, some small follicles (0.5-1.0 mm dia.) were capable of secreting peak quantities of all the above-mentioned steroids. However, a greater proportion of those from FF ewes produced peak quantities relative to the proportions from either F+ or ++ ewes.

**Large follicles (≥1 mm dia.).** There are technical difficulties in devising an optimal environment in which to culture whole follicles >1 mm dia. *in vitro*. However, an indication of the steroidogenic competence of these follicles can be obtained from the measurement of steroids in follicular fluid. In most follicles between 1 and 1.5 mm dia. the progesterone concentrations are relatively high (i.e. ≥20 ng/ml) whereas in larger-sized follicles (e.g. ≥3 mm dia.)

the concentrations are usually low (i.e.  $\leq 10$  ng/ml) [12]. In most follicles, irrespective of size, the intrafollicular concentrations of androstenedione are low (i.e.  $\leq 10$  ng/ml), probably because most of this steroid which is of thecal origin, is metabolized to testosterone and/or oestradiol by the granulosa cells. Testosterone is usually present in 1–1.5 mm dia. follicles in relatively high concentrations (i.e.  $\geq 40$  ng/ml) [12, 13]. However, as was the case for progesterone, the testosterone concentrations in the largest non-atretic follicles were usually low (i.e.  $\leq 15$  ng/ml) [12, 13]. No gene specific effects have been noted for progesterone, androstenedione or testosterone concentrations in follicular fluid over any range of follicle sizes (Refs [12], [13] and unpublished data).

In contrast to progesterone, androstenedione and testosterone, F gene-specific differences were found for oestradiol (Fig. 2). In 2–2.5- and 3–3.5-mm follicles, the oestradiol concentrations were significantly higher in both the FF and F+ genotypes compared to those in ++ ewes. Moreover, in the FF and F+ ewes, peak oestradiol concentrations were reached in 3–3.5 mm dia. follicles whereas in ++ ewes peak concentrations were not reached until follicles were  $\geq 5$  mm dia.

**Granulosa cells.** In the sheep ovary, >95% of the oestradiol originates from the granulosa cells; the output of oestradiol from the theca interna under optimal *in vitro* perfusion conditions is negligible. There was a significant F gene-specific influence on follicular diameter with respect to aromatase activity in granulosa cells from non-atretic follicles (Fig. 2). Peak aromatase activity in granulosa cells from FF, F+ and ++ ewes was reached when the non-atretic follicles were between 3 and 4.5 mm, 3 and 5.5 mm and  $\geq 5$  mm diameter respectively. Of interest is the finding that peak aromatase activities in granulosa cells from each of the genotypes were similar on a per cell basis.

**Theca interna.** In the sheep as in other species, androstenedione is the major steroid secreted by theca interna *in vitro* [12, 13, 17]. Other steroids to be secreted in substantially smaller amounts are testosterone and progesterone. Irrespective of follicular diameter and Booroola genotype, the geometric mean androstenedione content (and 95% confidence limits) in the theca interna of freshly isolated tissue was 2 (1,3) ng/10 mg wet weight theca interna. However, when theca interna was perfused *in vitro* with oxygenated media at a flow rate of 1.4 ml/min and exposed to a "saturating pulse" of LH (i.e. 8 ng/ml for 20 min), the cumulative androstenedione outputs [geometric mean (and 95% confidence limits)] during and after the LH pulse were 55 (40, 74), 47 (33, 66) and 48 (37, 63) ng/10 mg theca interna/3 h for theca from FF ( $n =$

14), F+ ( $n = 116$ ) and ++ ( $n = 18$ ) ewes respectively.

In summary, the lack of any F gene-specific differences in the LH-receptor characteristics, LH-induced cAMP synthesis, LH-induced androstenedione synthesis, the concentrations of androstenedione or testosterone in follicular fluid suggest that the theca interna is functionally similar in all Booroola genotypes. However, it is noteworthy that there was a significantly greater proportion of small follicles (0.1–1.0 mm dia.) in the FF genotype which produced peak levels of androstenedione relative to the proportions from the other genotypes. This finding suggests the theca in the FF genotype is more likely to synthesize steroid earlier in follicular development than is the case for theca from the other genotypes.

*Interrelationships among the number of "oestrogenic" follicles, follicular diameter, granulosa-cell number, oestradiol secretion-rate and Booroola genotype*

At 12–36 h after the initiation of a PGF<sub>2 $\alpha$</sub> -induced follicular phase, most Booroola ewes have an elevated ovarian output of oestradiol (Fig. 1) and contain at least one ovary with an "oestrogenic" follicle (i.e.  $\geq 50$  ng oestradiol/ml follicular fluid [12, 13]). At other times both before and after the above time frame, some ewes have no "oestrogenic" follicles [12, 13]. The data summarizing the aforementioned interrelationships are shown in Table 4. On average, the FF and F+ ewes contained 3.2- and 2.1-fold respectively more "oestrogenic" follicles than did the ++ ewes. However, the respective mean follicular diameters and numbers of granulosa cells in each of these follicles were smaller in the FF and F+ ewes than in the ++ ewes (Table 4). If the number of "oestrogenic" follicles was multiplied by the number of these follicles for each genotype, then the total number of cells in the "oestrogenic" follicles from each genotype was identical. Moreover, the oestradiol secretion-rates of either the ovaries containing the "oestrogenic" follicles or from both ovaries from each ewe with respect to genotype were not significantly different.

*Corpora lutea (CL)*

Detailed information on the formation and function of CL in FF, F+ and ++ ewes is not available. However, during the mid-luteal phase, there were no gene-specific differences in plasma progesterone concentrations (Fig. 1), even though there were differences in the mean numbers ( $\pm$ SEM) of CL in the genotypes; in the FF ( $n = 15$  ewes), F+ ( $n = 30$  ewes) and ++ ewes ( $n = 30$  ewes) the respective CL numbers were 5.1 ( $\pm 0.4$ ), 2.8 ( $\pm 0.1$ ) and 1.2 ( $\pm 0.1$ ). During the mid-luteal phase, the respective mean ( $\pm$ SEM) weights of each CL in the above FF, F+ and ++ ewes were 0.16  $\pm$  0.01 ( $n = 6$  ewes), 0.25  $\pm$  0.02 ( $n = 9$ ) and 0.53  $\pm$  0.03 g ( $n = 9$ ). Irrespective of genotype there was an inverse relationship

Table 4. Number and diameter of 'oestrogenic' follicles\* and the number of granulosa cells in FF, F+ and ++ ewes together with the oestradiol secretion rate† at 12–36 h after a PGF<sub>2α</sub> injection‡

Ewes	'Oestrogenic' follicles				Oestradiol secretion rate (ng/min) (geometric means (95% confidence limits))	
	No./ewe (mean ± SEM)	Dia. (mean ± SEM, mm)	Granulosa cell no./follicle (mean ± SEM × 10 <sup>-6</sup> )	Foll. no. × granulosa cell no. (mean ± SEM × 10 <sup>-6</sup> )		
Genotype	P	No.	Dia.	Granulosa cell no./follicle	Foll. no. × granulosa cell no.	Oestradiol secretion rate
++		8	5.1 ± 0.3 <sup>b</sup>	3.8 ± 0.4 <sup>a</sup>	5.4 ± 0.4	3.2 (1.6, 5.8)
F+		9	3.3 ± 0.2 <sup>b</sup>	1.7 ± 0.1 <sup>b</sup>	5.4 ± 0.4	4.5 (3.0, 6.5)
FF		9	2.9 ± 0.1 <sup>b</sup>	1.1 ± 0.05 <sup>c</sup>	5.4 ± 0.5	3.0 (1.9, 4.5)

\*Follicle containing ≥ 50 ng oestradiol/ml follicular fluid.

†Oestradiol secretion rate: total output from left and right ovaries.

‡A PGF<sub>2α</sub> derivative (cloprostenol, 125 µg subcutaneously) was injected into all ewes on the 10th day of the oestrous cycle. For each column

a vs b,  $P < 0.01$ ; a vs c,  $P < 0.01$ ; b vs c,  $P < 0.025$  (ANOVA). Data from [13].

between ovulation-rate and CL weight which could be expressed by the equation  $Y = 0.01 \pm 1.73X$  where  $X$  is the CL weight in g, and  $Y$  is the ovulation rate ( $R = 0.91$ ,  $P < 0.001$ ) [13]. In summary, as the ovulation rate increased, the weight of the CL decreased and the overall levels of plasma progesterone in three genotypes were similar.

#### CONCLUSIONS

The ovaries of animals homozygous for the F-gene are more frequently exposed to elevated FSH and LH than are the ovaries of animals without the F-gene. In the smallest follicles studied (i.e. 0.1–1.0 mm dia.), the basal levels of cAMP and the *in vitro* synthesis of cAMP, progesterone, androstenedione and oestradiol-17β, in response to LH + FSH, were significantly influenced by genotype (FF > F+ > ++,  $P < 0.05$ ). In the larger-sized follicles (3–4.5 mm dia.), granulosa cells from FF and F+ but not ++ ewes had peak responses to FSH and LH in terms of cAMP synthesis and they also had peak aromatase activity. In the ++ ewes, the peak cAMP responses to FSH and LH and peak aromatase activity in granulosa cells were only observed in follicles ≥ 5 mm dia. *In vivo*, the total number of granulosa cells within the ≥ 5 'oestrogenic' follicles in FF ewes, 3–4 such follicles in F+ ewes and 1–2 such follicles in ++ animals during a PGF<sub>2α</sub>-induced follicular phase were similar as were the overall ovarian secretion-rates of oestradiol. Collectively, these findings support the notion that as a consequence of chronic exposure to elevated plasma FSH and LH, follicles in FF and F+ ewes mature at smaller diameters (i.e. ~ 3 mm in FF ewes; ~ 4 mm in F+ ewes) than in ++ ewes (i.e. ≥ 5 mm). In turn, the maturation of ≥ 5 follicles in FF ewes and 3–4 follicles in F+ ewes may each be necessary to provide a follicular cell mass capable of producing the same quantity of oestradiol as that from 1–2 preovulatory follicles in ++ ewes.

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## Differences in the plasma concentrations of FSH and LH in ovariectomized Booroola FF and ++ ewes

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**Summary.** During 12 sampling days before ovariectomy the mean plasma FSH but not LH concentrations in FF ewes were higher ( $P < 0.01$ ) than those in ++ ewes (16 ewes/genotype). After ovariectomy increases in the concentrations of FSH and LH were noted for ewes of both genotypes within 3-4 h and the rates of increase of FSH and LH were  $0.18 \text{ ng ml}^{-1} \text{ h}^{-1}$  and  $0.09 \text{ ng ml}^{-1} \text{ h}^{-1}$  respectively for the first 15 h. From Days 1 to 12 after ovariectomy, the overall mean  $\pm$  s.e.m. concentrations for FSH in the FF and ++ ewes were  $8.1 \pm 0.6$  and  $7.1 \pm 0.4 \text{ ng/ml}$  respectively and for LH they were  $2.7 \pm 0.3$  and  $2.1 \pm 0.2 \text{ ng/ml}$ ; these differences were not statistically significant ( $P = 0.09$  for both FSH and LH; Student's *t* test). However, when the frequencies of high FSH or LH values after ovariectomy were compared with respect to genotype over time, significant F gene-specific differences were noted ( $P < 0.01$  for both FSH and LH; median test).

In Exp. 2 another 21 ewes/genotype were blood sampled every 2nd day from Days 2 to 60 after ovariectomy and the plasma concentrations of FSH and LH were more frequently higher in FF than in ++ ewes ( $P < 0.01$  for FSH and LH). The F gene-specific differences in LH concentration, observed at 21-36 days after ovariectomy were due to higher mean LH amplitudes ( $P < 0.025$ ) but not LH peak frequency in FF than in ++ ewes.

Collectively the evidence shows that the greater frequency of high plasma gonadotrophin concentrations in FF compared to ++ Booroola ewes occurs independently of ovarian hormones and that the principal site(s) of F gene expression may not be in the gonad.

**Keywords:** Booroola ewes; ovariectomy; plasma FSH; plasma LH

### Introduction

High fecundity Booroola ewes contain a major gene(s) (F) which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative gene(s) are segregated on the basis of ovulation rate recordings of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). Previous studies have demonstrated differences both in ovarian activity and plasma gonadotrophin concentrations between ++ and FF ewes (see McNatty & Henderson, 1987 for review). With regard to the gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the mean plasma concentrations were shown to be significantly higher in FF than in ++ ewes, with those of F+ animals being consistently in between. Despite these differences, it is not known whether the principal sites of F gene expression are in the pituitary, ovary or elsewhere. The aim of this study was to examine whether the F gene-specific differences in plasma gonadotrophin concentrations persisted after ovariectomy.

## Materials and Methods

### Animals and experiments

Booroola Merino ewes born in 1978–80 were classified as being of the ++ (N = 37) or FF (N = 37) genotypes based on pedigree analysis and were confirmed by laparoscopy according to the criteria of Davis *et al.* (1982). All the FF ewes, aged 6–7 years at the time of the study, were recorded as having at least one annual ovulation rate of  $\geq 5$  whereas the ++ ewes were never recorded as having an ovulation rate of  $> 2$ .

Experiment 1 was designed to examine the daily plasma concentrations of FSH and LH from 12 days before until 12 days after ovariectomy (day of ovariectomy = Day 0) in 16 FF and 16 ++ ewes. In addition to the daily blood samplings, hourly samples were collected from 26 h before to 36 h after ovariectomy to examine in more detail the changes in hormone concentrations shortly before and after removal of the ovaries. The mean  $\pm$  s.e.m. day of the oestrous cycle when ovariectomy was performed was  $7.8 \pm 0.5$  and  $7.6 \pm 0.5$  for sheep of the FF and ++ genotypes respectively. The ovaries were removed surgically, after the animals had been anaesthetized. Thiopentone was used to induce anaesthesia which was maintained using halothane (Fluothane; Coopers Animal Health, Upper Hutt, New Zealand). The whole surgical procedure from induction of anaesthesia to cessation of halothane administration was 20 min. At ovariectomy, the number of corpora lutea (CL) in each ovary was recorded. On the day before the hourly blood sampling was due to be started the animals were penned indoors and each was fitted with an intrajugular cannula. For the once hourly collections 2.5 ml blood were collected via the jugular cannulae. The blood sample at 0 h was taken during thiopentone treatment and that at 1 h was taken while the animals were recovering from the effect of anaesthesia. At 2 h after induction of anaesthesia the animals were standing and some were eating. For the once daily collections 5 ml blood were collected from the jugular vein into a heparinized vacutainer.

Experiment 2 was designed to examine the plasma concentrations of FSH and LH every 2nd day from 2 to 60 days after ovariectomy (Day 0) in a second set of 21 FF and 21 ++ ewes. The numbers of CL in each ovary were recorded at ovariectomy which was performed as described in Exp. 1. On each sampling day, a blood sample (5 ml) from each ewe was collected from the jugular vein into heparinized vacutainers. At 21–36 days after ovariectomy, the animals were brought indoors and each was fitted with an intrajugular cannula. The following day, each animal was bled (2.5 ml) via the jugular cannula once every 10 min for 12 h to determine the pulsatile characteristics of the plasma LH concentrations.

All blood samples were centrifuged at 4000 g at room temperature for 8 min within 30 min of collection and the plasma samples were stored at  $-20^{\circ}\text{C}$  until assayed.

### Hormone assays

**FSH.** The radioimmunoassay kit was that supplied by The National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, MD, USA. The ovine (o) FSH for iodination was NIAMDD-oFSH-I-1; the oFSH reference preparation was NIAMDD-oFSH-RP-1 (biopotency  $75 \times$  NIH-FSH-3) and the oFSH antiserum was NIAMDD-anti oFSH-1 (AFP-C5288113). At a final FSH antiserum dilution of 1:80 000 this homologous assay had a working range of 0.01 to 5 ng per assay tube. The volume of plasma which was assayed was 0.1 ml and each sample was assayed in duplicate. The reference standards and standard curve samples were prepared in hypophysectomized ewe plasma which contained undetectable concentrations of FSH. The intra- and interassay coefficients of variation were  $< 4$  and  $< 5\%$  respectively.

**LH.** The radioimmunoassay was similar to that described by McNatty *et al.* (1987). The iodination standard was NIDDK-oLH-I-3 (AFP-9598B), the oLH reference preparation was NIAMDD-oLH-S23 (biopotency  $2.3 \times$  NIH-LH-S1) and the LH antiserum was raised at Wallaceville (characteristics were as described by McNatty *et al.*, 1987). The volume of plasma which was assayed was 0.1 ml and each sample was assayed in duplicate. The minimum detectable concentration of LH was 0.03 ng/tube. The reference standards and standard curve samples were prepared in hypophysectomized ewe plasma which contained undetectable amounts of LH. The intra- and interassay coefficients of variation were  $< 5$  and  $< 8\%$  respectively.

### Statistical analysis

For each genotype separately, the plasma LH or FSH concentrations in each daily or hourly period showed no major departures from normality when assessed by the Shapiro–Wilk test (Zar, 1974). The variance in the FF genotype was significantly higher than that in the ++ genotype using Bartlett's test and so the Student's *t* test with the Satterthwaite approximation (Snedecor & Cochran, 1980) was used for genotypic comparisons within each time. This *t* test was also used for overall genotype differences. Box and whisker plots (Velleman & Hoaglin, 1981) were used to show the distribution of these overall means within each genotype. For each genotype sample correlations were performed at various times to test whether the FSH or LH concentrations in the ewes remained in the same order over time.

Partial autocorrelations for each sheep over time were calculated so that the relationship between times could be investigated. These were not found to be significant for most sheep and so the median test was used to see whether there were genotypic differences in the proportions of high values after adjusting for time differences (Conover, 1971).

Within each genotype, although there was some heterogeneity of variance between times, the effects of time were examined by analysis of variance.

The episodic LH data were examined using the method of Van Look (1976). A pulse was defined as occurring when the hormone concentrations of 2 consecutive samples were greater than that of the mean of the 2 previous samples (basal samples) and the value of at least one of the peak samples exceeded the mean basal value by more than twice the coefficient of variation of the assay which was set at 8%. The amplitude of each pulse was measured by subtracting the basal value from the peak value. The 'smoothed' mean value was defined as the overall mean basal value after all peak values were removed. Genotype differences in episodic LH data were compared by Student's *t* test.

## Results

### *Plasma FSH and LH concentrations before and after ovariectomy (Exp. 1)*

The daily mean  $\pm$  s.e.m. plasma concentrations of FSH and LH for each genotype from 12 days before until 12 days after ovariectomy are summarized in Fig. 1.

The mean  $\pm$  s.e.m. ovulation rates as judged by the number of CL in the ovaries at ovariectomy (time 0 h; Fig. 1) were  $5.8 \pm 0.3$  ( $N = 16$ ) for the FF ewes and  $1.8 \pm 0.1$  ( $N = 16$ ) for the ++ ewes.

Before ovariectomy, the daily mean concentrations of FSH but not LH were consistently higher in FF than in ++ ewes. Significant differences ( $P < 0.05$ ) for FSH were observed on 9 of the 13 sampling days (i.e. Days -10, -9, -6, -5, -4, -3, -2, -1 and 0) whereas none was observed for LH. The mean  $\pm$  s.e.m. FSH concentrations of ewes of the FF genotype were significantly higher than those of the ++ genotype when the values for each sheep were averaged over time ( $P < 0.001$ ; FF,  $1.7 \pm 0.1$  ng/ml; ++,  $1.1 \pm 0.1$  ng/ml). The corresponding mean  $\pm$  s.e.m. LH concentrations in sheep of the FF and ++ genotypes were  $0.32 \pm 0.05$  and  $0.33 \pm 0.05$  ng/ml respectively. After ovariectomy, the concentrations of FSH and LH in both genotypes began to increase some 3 h later (Fig. 1). Thereafter there was a steady increase in the daily FSH and LH concentrations for 5-6 days and 2 days respectively after which the concentrations for both hormones remained relatively constant until 12 days after ovariectomy (the last day of blood sampling). Regression analysis of the mean FSH (1) and LH (2) concentrations during the first 15 h after ovariectomy for ewes of each genotype gave the following equations: (1) for FF ewes,  $y = 0.18x + 1.84$  ( $r = 0.974$ ; 15 df), and for ++ ewes,  $y = 0.18x + 1.11$  ( $r = 0.960$ ; 15 df) where  $y$  = plasma FSH concentrations in ng/ml and  $x$  = time in hours; (2) for FF ewes  $y = 0.09x + 0.18$  ( $r = 0.930$ ; 15 df) and for ++ ewes,  $y = 0.09x + 0.18$  ( $r = 0.898$ ; 15 df) where  $y$  = plasma LH concentrations in ng/ml and  $x$  = time in hours. Moreover, over this time frame the mean FSH values in FF ewes were consistently (i.e.  $\sim 0.7$  ng/ml) higher than in the ++ ewes whereas the mean LH values in ewes of the two genotypes were essentially the same.

After ovariectomy no F gene-specific differences in mean concentrations were observed on any individual sampling day for either FSH or LH. For FSH from Days +1 to +12, the overall mean  $\pm$  s.e.m. concentrations in the FF and ++ ewes ( $N = 16$ /genotype) were  $8.1 \pm 0.6$  and  $7.1 \pm 0.4$  ng/ml respectively and for LH they were  $2.7 \pm 0.3$  and  $2.1 \pm 0.2$  ng/ml respectively. The differences between the overall means were not significant (FF v ++: FSH and LH;  $P = 0.09$ ) but (as shown in the box and whisker plots; Fig. 2) about 3/4 of the FF ewes had mean FSH or LH values above the median of the ++ sheep. The correlation coefficients for the FSH and LH concentrations averaged over Days -12 and -11, +1 and +2 or +11 and +12 were not significantly correlated with one another, indicating that the animals with the highest values at any of the above times were not necessarily those with the highest at the other times. When the proportions of high FSH or LH values were compared with respect to genotype over time (by the Median test), significant F gene-specific differences were noted for both FSH ( $P < 0.01$ ) and LH ( $P < 0.01$ ); 60% of the FSH and 55% of the LH values in FF ewes were above the daily overall median values for ewes of both genotypes (192 samples per genotype).

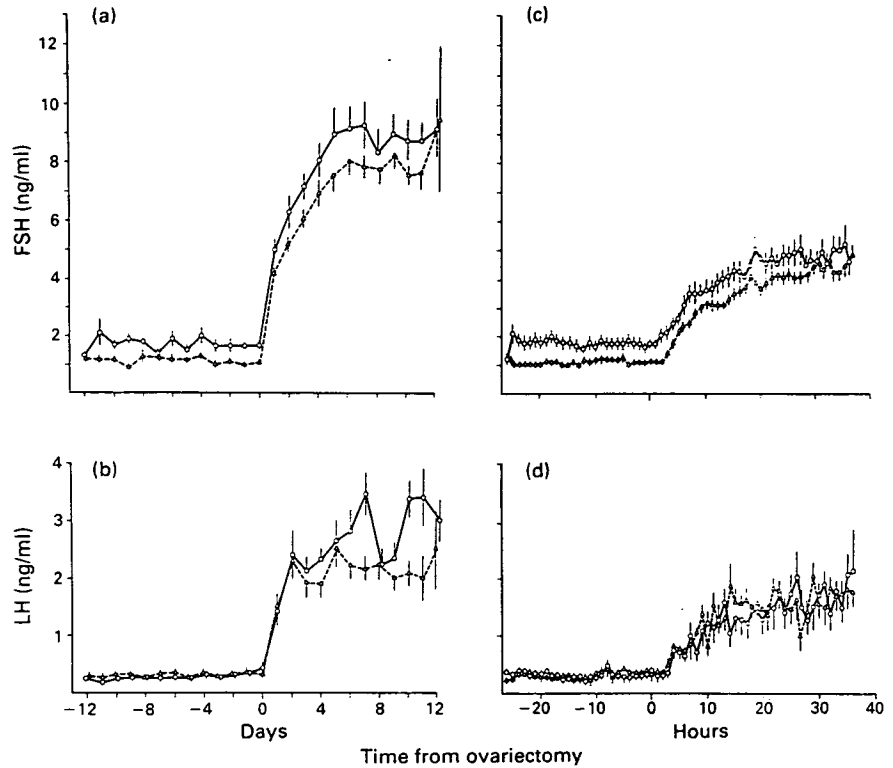


Fig. 1. Changes in the plasma concentrations of FSH and LH measured once daily for 12 days before and after ovariectomy (FSH, 1a; LH, 1b) and from 26 h before until 36 h after ovariectomy (FSH, 1c; LH, 1d) with respect to Booroola genotype (FF,  $\circ$ — $\circ$ ; ++,  $\triangle$ --- $\triangle$ ). Ovariectomy was performed within minutes of the blood sample collected at 0 h. Values are means  $\pm$  s.e.m. for 16 ewes/genotype.

#### Plasma concentrations of FSH and LH after ovariectomy (Exp. 2)

The mean  $\pm$  s.e.m. ovulation rates in the FF and ++ ewes as judged by the number of CL at ovariectomy were  $4.7 \pm 0.2$  and  $1.3 \pm 0.1$  respectively.

The mean  $\pm$  s.e.m. plasma concentrations of FSH and LH for each genotype from Day +2 to Day +60 after ovariectomy are summarized in Fig. 3 as are the box and whisker plots of the overall mean FSH and LH concentrations over time for each genotype. The mean concentrations of FSH and LH in both genotypes were variable with time after ovariectomy but overall they increased from 10 ng/ml and 1 ng/ml respectively at Day +2 to 16–20 ng/ml and 2 ng/ml respectively at Day +60: for each genotype, the increases in the mean concentrations of FSH and LH were significantly different with respect to time (one-way ANOVA for each genotype separately;  $P < 0.01$  for both FSH and LH). Overall, the variances of the FSH and LH concentrations for ewes of the FF genotype were  $\sim 2$ – $2.5$  times those for the ++ genotype.

When the mean FSH and LH concentrations within each time were compared, significant genotype-specific differences ( $P < 0.05$ ) were noted for only 3 of the 30 sampling days for FSH (i.e. Days 14, 38 and 50) and for only 1 of the 30 sampling days for LH (i.e. Day 30). The mean  $\pm$  s.e.m. FSH concentrations over time for ewes of the FF and ++ genotypes (21 ewes/genotype) were  $16.0 \pm 1.2$  and  $12.6 \pm 0.8$  ng/ml respectively. Likewise, the mean  $\pm$  s.e.m. LH concentrations for the FF and ++ genotypes were  $2.2 \pm 0.3$  and  $1.7 \pm 0.2$  ng/ml. For FSH but not LH, the overall

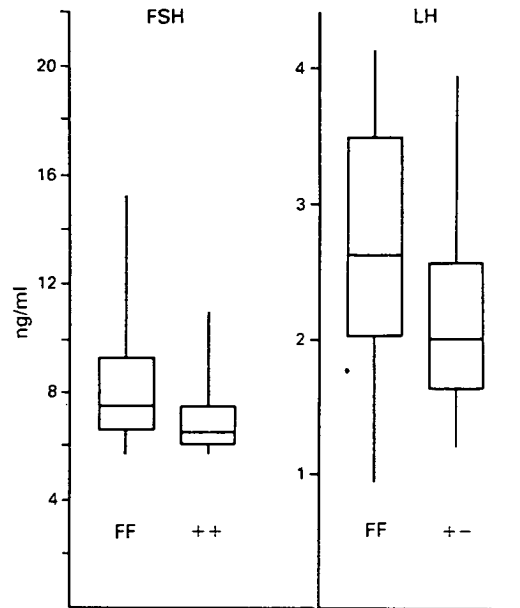
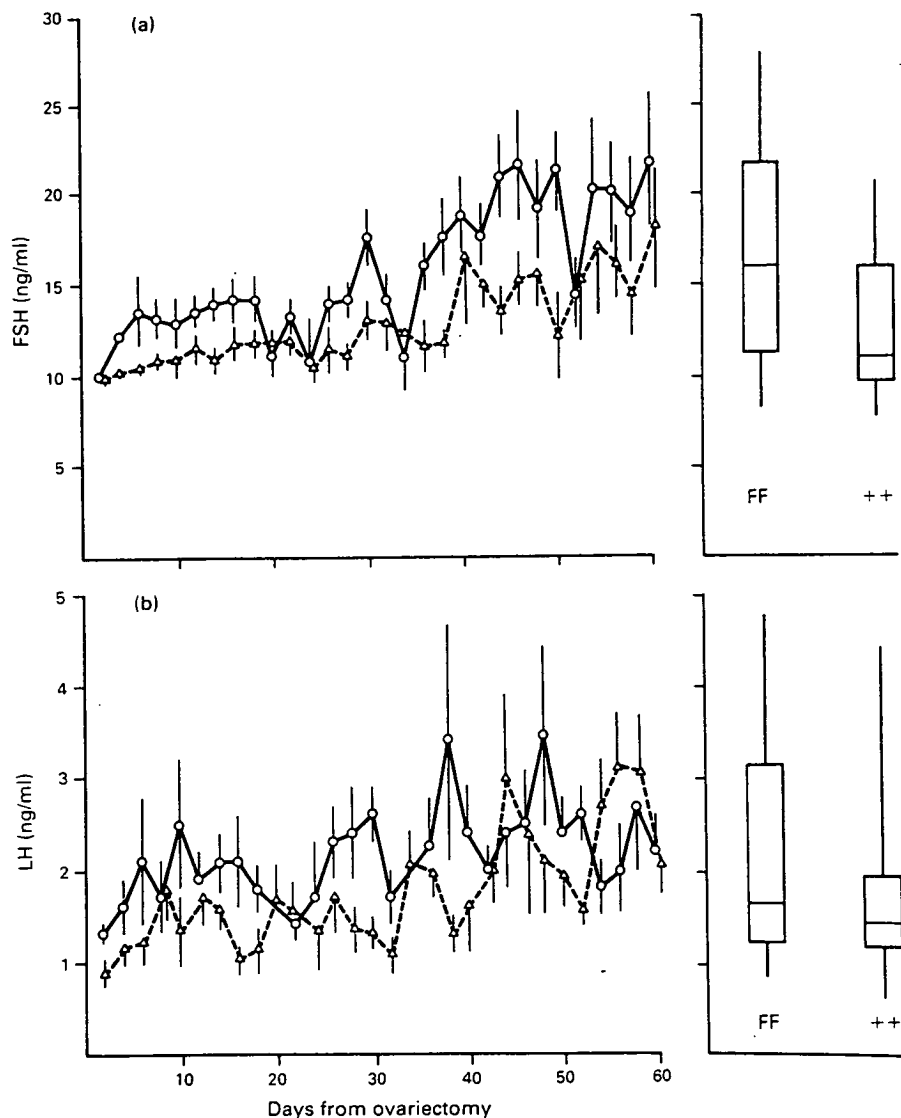


Fig. 2. Box and whisker plots of the mean FSH and LH data for each ewe averaged over 12 days from ovariectomy with respect to genotype. The extreme ends of the vertical (whisker) lines represent the maximum and minimum values. The upper and lower horizontal lines of the box refer to the upper and lower quartiles and the lines through the box represent the median values.

means for the FF ewes were significantly different from those for the ++ ewes ( $P < 0.02$ ). For FSH this difference between the overall means was not due to only 1 or 2 sheep as assessed by sample correlation analysis. From examination of the box and whisker plots, half of the FF ewes had mean FSH values higher than 75% of the ++ ewes (Fig. 3). For LH, the box and whisker plots showed a similar tendency for more FF ewes to have higher mean concentrations than was the case for the ++ animals. The correlation coefficients for the FSH and LH concentrations averaged over Days 2 and 4 and Days 58 and 60 respectively were not significant, indicating that ewes with the highest FSH or LH values 2–4 days after ovariectomy were not necessarily the animals with the highest values at Days 58–60 after ovariectomy. When the proportions of high FSH or LH values were compared with respect to genotype over time (by the median test), significant F gene-specific differences were noted for both FSH and LH (FSH: FF > ++;  $P < 0.01$ ; LH: FF > ++;  $P < 0.01$ ); 60% of the FSH and 58% of the LH values in ewes of the FF genotype were above the daily overall median values for all sheep (630 samples/genotype).

#### *Pulsatile characteristics of plasma LH concentrations in ovariectomized Booroola ewes with respect to genotype*

As summarized in Table 1, there was about 1 LH peak every 75–90 min. No differences were noted in LH peak frequency between the genotypes. However, the LH peak amplitudes were significantly higher in FF compared to ++ ewes ( $P < 0.025$ ) and the smoothed mean LH concentrations were 1.3-fold higher in FF than in ++ ewes ( $P < 0.05$ ).



**Fig. 3.** Changes in the plasma concentrations of FSH (a) and LH (b) once every second day from Day 2 until Day 60 after ovariectomy in FF, (○—○) and ++ (△----△) Booroola ewes. Values are means  $\pm$  s.e.m. for 21 ewes/genotype. Also shown are the box and whisker plots of the mean FSH (a) and LH (b) data for each ewe averaged over all times with respect to genotype. The extreme ends of the vertical (whisker) lines represent the maximum and minimum values. The upper and lower horizontal lines of the box refer to the upper and lower quartiles and the lines through the box represent the median values.

### Discussion

The important finding from this study is that the plasma concentrations of FSH and LH in Booroola ewes are more often higher in those of the FF genotype than in the ++ genotype after ovariectomy. However, statistically significant differences between the genotypes after ovariectomy were rarely observed when the daily mean values were compared (i.e. Exp. 1 or 2) or even

**Table 1.** Characteristics of plasma LH concentrations in ovariectomized Booroola ewes which were homozygous (FF) or non-carriers (++) with respect to the F-gene

Genotype (no. of ewes)	Mean (ng/ml)	Smoothed mean (ng/ml)	No. of peaks per 12 h	LH peak amplitude (ng/ml)	No. of days after ovariectomy
FF (21)	3.0 <sup>a</sup> ±0.3	2.8 <sup>a</sup> ±0.3	9.6 <sup>a</sup> ±0.7	2.6 <sup>a</sup> ±0.3	32 <sup>a</sup> ±1
++ (21)	2.2 <sup>a</sup> ±0.3	2.1 <sup>b</sup> ±0.2	8.0 <sup>a</sup> ±0.4	1.5 <sup>c</sup> ±0.2	33 <sup>a</sup> ±1

Values are means ± s.e.m

Values in columns sharing a common superscript are not different from one another: a vs b,  $P < 0.05$ ; a vs c  $P < 0.025$  (Students's *t* test).

when the mean values over all times (e.g. LH and FSH in Exp. 1 or LH in Exp. 2) were compared. The differences were most obvious in the proportions (i.e. frequency) of higher FSH and LH values in FF compared to ++ ewes. Moreover it was evident that most, if not all, of the ewes contributed to the frequency of higher FSH and LH values over time after ovariectomy since the frequency of high values could not be attributed to 1 or 2 outlier animals (Figs 2 and 3) and since the FSH or LH concentrations in any one ewe on any one day were not necessarily correlated with the concentrations on any previous day. These small and subtle differences in the plasma concentrations of FSH and LH between the genotypes in the ovariectomized ewes are similar in magnitude to those reported in a previous study with intact Booroola ewes (McNatty *et al.*, 1987).

The variances in the FSH and LH values in FF ewes were approximately 2–2.5 times those in ++ ewes. The pattern of pituitary secretion of FSH is not understood in sheep. In large part this has been due to the long half-life of clearance ( $t_1$ ) of FSH in intact (i.e. ~110 min) and ovariectomized (i.e. ~1100 min) ewes (Akbar *et al.*, 1973; Fry *et al.*, 1987). However, as the  $t_1$  of FSH does not differ between the Booroola genotypes and since the various forms of FSH in Booroola are similar to those in non-Booroola Merinos (Robertson *et al.*, 1984), the higher variance associated with the plasma FSH concentrations in FF ewes could indicate a more variable pattern of secretion than in ++ ewes. This is clearly the case for LH. For example, in the ovariectomized Booroola ewes, the LH peak amplitudes but not peak frequencies were significantly higher in FF than in ++ ewes which is similar to that found for intact Booroola ewes (McNatty *et al.*, 1987). The finding that the F gene-specific effect on plasma LH concentration is associated with LH amplitude but not frequency may explain, at least in part, why gene-specific differences in plasma LH concentration are not always observed. For example, LH peak frequency is influenced by steroids (e.g. progesterone) as well as photoperiod (Goodman & Karsch, 1981) and LH peak amplitude is also influenced (i.e. independently of genotype) by steroids (e.g. oestradiol; Goodman & Karsch, 1981) and also by stress (Rasmussen & Malven, 1983). Therefore, if the F gene is influencing only one component of the hypothalamic–pituitary system that controls gonadotrophin secretion then often its influence may be masked by these other factors. In turn, this may explain why the ovulation rate in FF and F+ ewes is so often variable (i.e. between 2 and 12).

The effect of ovariectomy on the plasma concentrations of FSH and LH was biphasic. There was a rapid and relatively linear increase in FSH (~10-fold) and LH (~3-fold) concentration, with both beginning to rise consistently some 3–4 h after ovariectomy. The effects of anaesthesia or surgical stress on the timing of the increase in gonadotrophin concentration are unknown but some effect cannot be ruled out. However, the initial rapid increase in FSH and LH concentrations continued for some 6 and 12 days respectively. Thereafter, for the next 50+ days, the concentrations of both gonadotrophins increased only 2-fold with this increase being gradual and fluctuating. The initial rapid rise in gonadotrophin concentration is most likely to be due to the release

from the negative feedback effects of ovarian hormones with perhaps some effects from the surgical procedure, whereas the second-phase gradual increase in concentration is more likely to be due to alterations in the chemical compositions of FSH and LH (Peckham & Knobil, 1976a,b; Weick, 1977; Montgomery *et al.*, 1984; Fry *et al.*, 1987).

Collectively, the evidence from the present study that ovariectomized FF ewes more frequently have higher plasma concentrations of FSH and LH than do ++ ewes suggests that the principal site(s) of F gene expression is independent of ovarian hormones and may not be in the gonad. Nevertheless, Fry *et al.* (1988) have shown that the ovaries of hypophysectomized Booroola F+ ewes are more responsive to PMSG than those of ++ ewes some 6 weeks after hypophysectomy, thereby suggesting that the ovary cannot be ruled out as a major site of F gene expression. However, it is possible that these ovarian differences are a legacy of previous differences in gonadotrophin concentration. For example, we have previously shown significant F gene-specific differences in the morphological and functional status of preantral and early antral follicles in Booroola ewes (McNatty *et al.*, 1986; Smith & McNatty, 1988). It remains to be determined whether a hypothalamic-pituitary-mediated difference in gonadotrophin secretion can influence follicle maturation very early in development and it is still possible that F gene expression occurs both within and outside the ovary.

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## Binding characteristics of $^{125}\text{I}$ -labelled human FSH to granulosa cells from Booroola ewes which were homozygous, heterozygous or non-carriers of a major gene(s) influencing their ovulation rate

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**Summary.** At 37°C  $^{125}\text{I}$ -labelled human (h) FSH (NIAMDD-hFSH-1-3) bound rapidly to granulosa cells from Booroola and Romney ewes with 50% maximum binding achieved after 3 min and equilibrium being reached within 45 min, irrespective of whether the cells were obtained from the FF, F+ or ++ Booroola genotypes or from Romney ewes. Binding of  $^{125}\text{I}$ -labelled FSH followed second order kinetics and there was no effect of follicle diameter (1-2.5 mm vs  $\geq 3$  mm). Irrespective of breed, genotype or follicle size, the mean ( $\pm$  s.e.m.) calculated association rate constant, ( $k_a$ ) was  $7.3 (\pm 0.8) \times 10^5$  litres mol $^{-1}$  sec $^{-1}$  ( $n = 12$ ). Dissociation of receptor bound  $^{125}\text{I}$ -labelled hFSH was < 5% after 30 min and low but variable (i.e. between 0 and 30%) after 2-6 h irrespective of breed, genotype or follicle size. No gene-specific differences were noted in binding specificity between F+ and ++ genotypes: studies were not performed with cells from FF ewes because of insufficient cells. The binding of  $^{125}\text{I}$ -labelled hFSH could be displaced with sheep FSH (NIH-FSH-S16: 10% cross-reaction) and FSH-P (2.5% cross-reaction) but other sheep pituitary hormones and hCG showed little or no cross-reaction ( $\leq 0.1\%$ ).

The calculated binding capacities ( $B_{\text{max}}$ ) and equilibrium dissociation constants ( $K_d$ ) for  $^{125}\text{I}$ -labelled hFSH binding to granulosa cells did not differ between the Booroola genotypes or between Booroola or Romney follicles of different diameter (i.e. 1-2.5 mm; or  $\geq 3$  mm). The overall mean  $\pm$  s.e.m. ( $n = 24$ )  $B_{\text{max}}$  and  $K_d$  values were  $16.7 \pm 0.8$  fm/mg protein (i.e.  $\sim 800$  available receptor binding sites/cell) and  $1.1 \pm 0.1$  nM respectively.

Collectively, these findings suggest that the earlier maturation of follicles in FF or F+ ewes compared to ++ ewes is unlikely to be due to gene-specific differences in the FSH binding characteristics of the granulosa cells.

**Keywords:** Booroola ewes; FSH binding; granulosa cells

### Introduction

High fecundity Booroola ewes contain a major gene(s) which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the F gene(s) have been segregated by pedigree and by ovulation rate recordings of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). The endocrine basis for the high ovulation rate remains unknown but it has been the subject of considerable interest. A characteristic feature of animals with the F gene is that their ovarian follicles ovulate at a significantly smaller diameter than do those in ++

ewes (McNatty *et al.*, 1986a): This seems to be a consequence of a greater proportion of small follicles (0.1–1.0 mm diam.) in F gene carriers responding to follicle stimulating hormone (FSH) and luteinizing hormone (LH) to synthesize high levels of adenosine cyclic 3',5'-monophosphate (cAMP) and of granulosa cells in F gene carriers synthesizing oestradiol and acquiring functional LH receptors at a smaller follicle diameter relative to those in ++ animals (Henderson *et al.*, 1985, 1987; McNatty *et al.*, 1985a, b, 1986a, b). This so-called earlier maturation of antral follicles might be due to the ovaries of ewes with the F gene being more often exposed to high concentrations of FSH and LH than are those of ++ ewes (McNatty *et al.*, 1987) and/or to genotypic differences in gonadal sensitivity to gonadotrophins (Fry *et al.*, 1988).

The aim of this study was to examine whether F gene-specific differences in Booroola ewes were detectable at the level of the FSH receptor in granulosa cells. Some binding studies were also done with granulosa cells from slaughterhouse Romney ewes for comparative purposes.

### Materials and Methods

**Animals.** The parous Booroola Merino ewes that were used were between 4 and 8 years of age. All the animals (66 ++ ewes, 66 F+ ewes and 31 FF ewes) were offspring of progeny tested FF rams mated with FF ewes, ++ rams mated with ++ ewes and F+ rams mated with ++ or F+ ewes (i.e. to generate the F+ ewes). Ovaries of all Booroola ewes were recovered between Days 6 and 14 of the oestrous cycle. The Romney ewes were of mixed age and of unknown reproductive status and the ovaries were recovered from the slaughterhouse. To test the effects of increased or decreased plasma FSH concentrations on FSH binding characteristics in granulosa cells, ovaries were recovered from: (I) anoestrous Romney ewes (N = 8) which had been pretreated with two injections of charcoal-treated bovine follicular fluid (bFF; 5 ml s.c. per injection) at 12-h intervals before ovariectomy; or (II) anoestrous Romney ewes (N = 8) which had been pretreated for 48 h (i.e. 4 × 12 h injections s.c.) with FSH-P (Burns-Biotech, Omaha, NE, USA; batch 518G85; 3 mg per i.m. injection). Treatment of Romney ewes with bFF led to a 70% reduction in plasma FSH concentration and a marked increase in atresia in all sizes of antral follicles ≥ 1 mm diameter (McNatty *et al.*, 1985b; Henderson *et al.*, 1986). Injections of FSH-P to anoestrous Romney ewes increase the number of non-atretic follicles ≥ 3 mm diameter from 3–8 to 5–40 (unpublished observation).

**Recovery and preparation of granulosa cells for binding studies.** Ovaries were collected into chilled (i.e. 4–6°C) saline (0.9% NaCl) containing 20 mM-Hepes buffer (pH 7.4) which was also used as the dissection medium. The numbers of corpora lutea (CL) in the ovaries of the Booroola but not Romney ewes were recorded. As many follicles as possible (≥ 1 mm diam.) were dissected from the ovaries. Preliminary studies with granulosa cells from Romney ewes revealed that to measure appreciable FSH binding (i.e. 1–2%) a minimum of  $5 \times 10^6$  granulosa cells (i.e. ~400 µg protein) was required per assay tube. To achieve this it was necessary to pool granulosa cells from 4–10 ewes. In several instances, cells from small (1–2.5 mm diam.) and large (≥ 3 mm diam.) follicles were pooled separately from ++ and F+ ewes and from Romney ewes. In FF ewes there were fewer follicles ≥ 3 mm diameter compared to ++ and F+ ewes ( $P < 0.01$ ) and follicles > 4.5 mm diameter were never observed. Therefore, for all FF ewes, granulosa cells from all follicles ≥ 1 mm diameter were pooled. At the level of the dissecting microscope (× 64), there was no evidence that the granulosa cells were contaminated with thecal tissue. We have previously reported that theca but not granulosa cells from 1–2.5 mm diameter follicles in ++ Booroola ewes or from Romney ewes bind  $^{125}\text{I}$ -labelled human chorionic gonadotrophin (McNatty *et al.*, 1986b). Moreover, we have repeatedly shown that granulosa cells from the above-mentioned 1–2.5 mm diameter follicles do not synthesize cAMP after exposure to LH (Henderson *et al.*, 1985, 1986, 1987), suggesting that our technique for recovering granulosa cells is unlikely to introduce significant thecal contamination. Once pools of granulosa cells were prepared they were centrifuged at 450 g at 4–6°C for 20 min and the cellular pellets were then resuspended in Tris buffer (i.e. 0.05 M-Tris-HCl buffer containing 0.02 M-sucrose and 5 mM-MgCl<sub>2</sub> (pH 7.5)) to a final concentration of  $25 \times 10^6$  cells/ml. The cell suspensions were added in 0.2 ml aliquants to assay tubes, capped and stored at –70°C until the binding studies were performed. The only exception to the above was with respect to a study of the relationship between the number of granulosa cells and the amount of  $^{125}\text{I}$ -labelled hFSH specifically bound to the cells. In this instance granulosa cells were stored in the Tris buffer at doubling concentrations from  $3.125 \times 10^6$  cells/0.2 ml to  $200 \times 10^6$  cells/0.2 ml. Aliquants of each pool of granulosa cells were assayed for protein by the method of Lowry *et al.* (1951).

**Recovery and preparation of granulosa cells for cAMP studies.** Ovaries from Booroola Merino FF or ++ ewes were collected into chilled (4–6°C) minimum essential medium (MEM) containing 20 mM-Hepes buffer (pH 7.4) and 1% (w/v) bovine serum albumin (BSA; 98% purity; ICP Ltd, Auckland, NZ). Granulosa cells were isolated, pooled and washed as described for the binding studies. After washing the cellular pellets were resuspended in Dulbecco's phosphate-buffered saline with 1-methyl-3-isobutyl xanthine (0.2 mM, MIX) and 1% (w/v) BSA (i.e. DPBS; pH 7.4) at a concentration of 250 000 cells/ml. The cell suspensions were then added in 0.5 ml aliquants to 12 × 75 mm screw

topped plastic tubes semi-immersed in ice-water and containing 0.5 ml DPBS or 0.5 ml DPBS + 500 ng hFSH (NIAMDD-hFSH-1-3). The tubes were incubated at 37°C for 0 or 45 min and then heated to 80°C for 15 min before being stored at -20°C until assayed for cAMP by radioimmunoassay as described by McNatty *et al.* (1985b).

The cAMP antiserum was kindly provided by Dr A. R. La Barbera (Northwestern University, Chicago, IL, USA). The lyophilized material was made up in assay buffer and 100 µl aliquants were added to the acetylated samples to a final dilution of 1:30 000. The cross-reactions of AMP, ADP, ATP and cGMP with the antiserum in our assays were all <0.00004% while that for dibutyryl cAMP was 0.0049%. About 10 000 c.p.m. iodinated cAMP derivative in sodium acetate buffer (0.05 M, pH 6.5) containing 3% (w/v) BSA was added to each assay tube in 100 µl. The radioactive label was prepared by iodinating 2',O-monosuccinyl adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (Sigma Chemicals, St Louis, MO, USA) to a specific activity of 120-150 µCi/µg.

The assay tubes were incubated overnight at 4°C and the free and bound fractions were separated using 0.2% (w/v) Norit A charcoal in 0.1 M-potassium phosphate buffer (pH 6.3) with 0.25% BSA (w/v). The cAMP results were expressed as pmol cAMP/10<sup>6</sup> cells. The intra- and inter-assay coefficients of variation were 8 and 14% respectively. The limit of detection was ≤0.1 pmol/10<sup>6</sup> cells.

*FSH iodination and binding studies with <sup>125</sup>I-labelled hFSH.* Human FSH (hFSH; 25 µg; NIAMDD-hFSH-1-3; 6887 i.u./mg by RIA; contamination with hLH, hTSH, hGH and human prolactin was 420, 0.12, ≤0.01 and <0.01 i.u./mg respectively as determined by RIA) was iodinated using 1.0 mCi Na<sup>125</sup>I (Amersham, Bucks, UK) and lactoperoxidase (Myachi *et al.*, 1972) and the products were purified on a column of Sephadex (G-75, 30 × 1 cm). The specific activities of the <sup>125</sup>I-labelled hFSH preparations were calculated to be 30-42 µCi/µg as described by Bolton (1977) and confirmed using the self-displacement method (Diekman *et al.*, 1978). The mass of label added to each binding assay was standardized for each iodination. Each <sup>125</sup>I-labelled hFSH preparation was stored at 4°C and used within 14 days of iodination. The bindability of each preparation of <sup>125</sup>I-labelled hFSH was not checked. However, the coefficient of variation between different preparations of <sup>125</sup>I-hFSH binding to granulosa cells from Romney ewes was 12.6% (*n* = 4).

For the binding studies, aliquants of granulosa cells were incubated in polystyrene tubes (12 × 75 mm) with <sup>125</sup>I-labelled hFSH (0.14-120 ng) in a final volume of 350 µl Tris buffer with 5 mM-MgCl<sub>2</sub>, 20 mM-sucrose and 0.5% egg albumin (Sigma). Incubations were performed at 37°C for up to 16 h; specific incubation times for the various studies are indicated in the 'Results' section. The receptor-bound <sup>125</sup>I-labelled hFSH was separated from free <sup>125</sup>I-labelled hFSH by centrifugation at 3000 g for 20 min at 4°C after the addition of 1 ml ice-cold incubation buffer. The pellets containing the bound <sup>125</sup>I-labelled hFSH were washed with 1 ml ice-cold incubation buffer and the radioactivity in the pellets was determined after a second centrifugation at 3000 g. Non-specific binding was determined by co-incubating the cells or homogenate with excess unlabelled gonadotrophin (i.e. Gonadotrophon F.S.H.; Paines & Byrne Ltd, Greenford, UK). For every 1 ng of <sup>125</sup>I-labelled hFSH per assay tube 10 i.u. Gonadotrophon was added for non-specific binding; dose-response studies indicated that this ratio of Gonadotrophon to <sup>125</sup>I-labelled hFSH was 1-2 times the minimum amount necessary to displace the maximum amount of <sup>125</sup>I-labelled hFSH. Gonadotrophon is a serum extract from pregnant mares and 10 i.u. were equivalent to 100 ng hFSH-1-3 in their ability to displace <sup>125</sup>I-labelled hFSH from a testicular receptor homogenate prepared as described by Abou-Issa & Reichert (1977). The amount of specifically bound <sup>125</sup>I-labelled hFSH in c.p.m. was calculated by subtracting non-specific binding from the total amount of <sup>125</sup>I-labelled hFSH bound. The counting efficiency of the gamma counter (Crystal Multidector, Packard, IL, USA) was 75%. Woolf plots (McNatty *et al.*, 1985b) were generated by using a standard amount of <sup>125</sup>I-labelled hFSH (3-5 ng) and increasing amounts of unlabelled hFSH (NIADDK-hFSH-1-3). In one instance the results from these studies were compared to that from a Woolf plot obtained from progressively increasing the amounts of <sup>125</sup>I-labelled hFSH (3.75-120 ng).

Triplicate or in most instances, duplicate measurements were made at each binding point with the coefficient of variation between replicate values being <8%.

*The Woolf plots and analysis of results.* From the equilibrium binding studies, the specific binding data were fitted to the Woolf equation, i.e.

$$\frac{F}{B} = \frac{K_d}{B_{max}} + \frac{1}{B_{max}} F,$$

where *F* = the concentration of free hormone and *B* = the concentration of bound hormone (Keightley & Cressie, 1980). The binding characteristics (*B*<sub>max</sub>, *K*<sub>d</sub>) with respect to follicle size or genotype were examined by Student's *t* test or analysis of variance in conjunction with the Newman-Keuls test.

## Results

The mean ± s.e.m. number of CL recovered from FF, F+ and ++ ewes were 5.32 ± 0.27 (range 4-11; *N* = 31 animals), 2.83 ± 0.09 (range 2-4; *N* = 66) and 1.09 ± 0.07 (range 0-2; *N* = 66) respectively.

*Effect of number of granulosa cells on 'bindability' of  $^{125}\text{I}$ -labelled hFSH*

Specific  $^{125}\text{I}$ -labelled hFSH ( $\sim 0.14$  ng or 9600 c.p.m.:  $41.4 \mu\text{Ci}/\mu\text{g}$ ) binding to ovine granulosa cells varied with the number of cells (Fig. 1). Maximum 'bindability' was achieved with 13.5% of the  $^{125}\text{I}$ -labelled hFSH being specifically bound to the granulosa cell preparations.

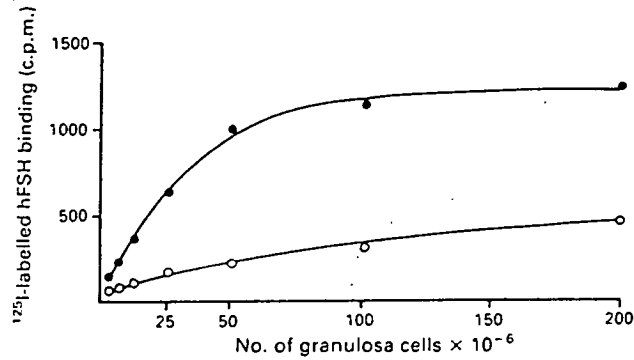


Fig. 1. Specific (●—●) and non-specific (○—○) binding of  $^{125}\text{I}$ -labelled hFSH (0.14 ng/tube: 9600 c.p.m.) to granulosa cells from Romney ewes. The granulosa cells were incubated at  $37^\circ\text{C}$  for 75 min. All values are means of duplicates.

*Effect of incubation time on binding of  $^{125}\text{I}$ -labelled hFSH to granulosa cells*

The specific binding of  $^{125}\text{I}$ -labelled hFSH was a time dependent process (Fig. 2). At  $37^\circ\text{C}$ , specific binding increased rapidly for the first 10 min and thereafter proceeded more slowly to reach a maximum after 20–45 min. Approximately 50% maximum  $^{125}\text{I}$ -labelled hFSH binding was achieved for cells from ewes of FF, F+ and ++ genotypes and for cells from Romney ewes at 3 min.

The rate of association of  $^{125}\text{I}$ -labelled hFSH to granulosa cells at  $37^\circ\text{C}$  as shown in Fig. 2 followed second-order kinetics using the equation:

$$k_a t = \frac{1}{a - b} \ln \left[ \frac{b(a - x)}{a(b - x)} \right]$$

where the initial hormone concentration, receptor concentration, hormone-receptor complex concentration and time are represented by the terms  $a$ ,  $b$ ,  $x$  and  $t$  respectively. The association rates (i.e.  $k_a$ ) were obtained from regression lines fitted through 5–6 datum points over the first 5 min (Fig. 3). In these studies  $a = 1 \times 10^{-9}$  M,  $b = 0.016 \times 10^{-9}$  M and the initial total counts added were 900 000 c.p.m. These data were obtained from pools of cells different from those described in Fig. 2. The  $k_a$  values were calculated for the FF, F+ and ++ Booroola ewes to be  $9.6 \times 10^5$  (1 pool tested; Fig. 3),  $8.2 \times 10^5$  [2 pools:  $5.8 \times 10^5$  (data not shown) and  $10.6 \times 10^5$  (see Fig. 3)] and  $8.9 \times 10^5$  (1 pool; Fig. 3) litres  $\text{mol}^{-1} \text{sec}^{-1}$ , respectively. The calculated mean  $k_a$  values ( $\pm$  s.e.m.) for  $^{125}\text{I}$ -labelled hFSH binding to granulosa cells from small (1–2.5 mm diam.: 4 pools tested) and large ( $\geq 3$  mm diam.: 4 pools) follicles from Romney ewes were  $5.2 (\pm 0.7) \times 10^5$  and  $8.1 (\pm 1.5) \times 10^5$  litres  $\text{mol}^{-1} \text{sec}^{-1}$  respectively; these  $k_a$  values were not significantly different from one another. Figure 3 shows an example of binding with respect to time for a single pool of cells from small or large follicles. The overall  $k_a$  value ( $\pm$  s.e.m.) irrespective of breed (genotype) and follicular diameter was  $7.3 (\pm 0.8) \times 10^5$  litres  $\text{mol}^{-1} \text{sec}^{-1}$  (12 pools).

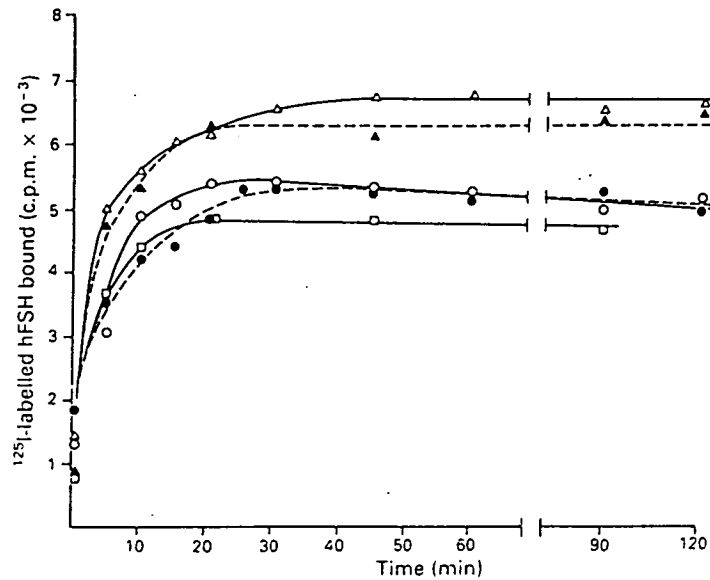


Fig. 2. Time dependence of specific  $^{125}\text{I}$ -labelled hFSH (12–20 ng/tube; sp. act. = 36–41  $\mu\text{Ci}/\mu\text{g}$ ) binding to granulosa cells ( $5 \times 10^6$  cells/tube) from Booroola FF ( $\triangle$ — $\triangle$ ), F+ ( $\circ$ — $\circ$ ), ++ ( $\bullet$ — $\bullet$ ) and Romney ( $\square$ — $\square$ , 1–2.5 mm diam. follicles;  $\blacktriangle$ — $\blacktriangle$ :  $\geq 3$  mm diam. follicles) ewes. Cells from the Booroola ewes were pooled from all follicles  $\geq 1$  mm diam.). The incubations were performed at  $37^\circ\text{C}$  and the values are means of duplicates.

To test for dissociation,  $5 \times 10^6$  granulosa cells/tube were preincubated with  $^{125}\text{I}$ -labelled hFSH (12–20 ng; 36–42  $\mu\text{Ci}/\mu\text{g}$ ) for 75 min at  $37^\circ\text{C}$ . The bound  $^{125}\text{I}$ -labelled hFSH was pelleted by centrifugation and resuspended in 350  $\mu\text{l}$  Tris-HCl buffer at  $37^\circ\text{C}$  for up to 6 h before the final centrifugation. Pools of cells from Booroola F+ (2 pools) and ++ (N = 1 pool) ewes and from Romney ewes (6 pools from large follicles, 6 pools from small follicles) were tested. Overall, the dissociation of specifically bound  $^{125}\text{I}$ -labelled hFSH appeared to be low: after 30 min, < 5% of the label had dissociated whereas after 2–6 h, the amount dissociated varied between 0 and 30%. The variability was unrelated to either sheep breed (genotype) or follicular diameter.

#### Specificity studies

Since hFSH (NIAMDD-hFSH-1–3) contained an appreciable amount of hLH contamination (i.e. 420 i.u./mg), the ability of human chorionic gonadotrophin (hCG; CR121; 13 450 i.u./mg) to displace  $^{125}\text{I}$ -labelled hFSH (3 ng, 40  $\mu\text{Ci}/\text{mg}$ ) binding was tested using a pool of granulosa cells from 1–6 mm diameter follicles from Romney ewes ( $5 \times 10^6$  cells/tube). The degree of cross-reaction with hCG was measured at 0.03%.

The specificity data for the ++ and F+ ewes are summarized in Table 1. Insufficient cells were available to test binding specificity for FF ewes. The % binding figures were obtained by dividing the amount of unlabelled hFSH ( $1.8 \times 10^{-9}$  M) required to displace 50% of the  $^{125}\text{I}$ -labelled hFSH by the amount of the other cross-reacting hormones required to displace 50% of the  $^{125}\text{I}$ -labelled hFSH. No obvious gene-specific differences in binding specificity were noted between granulosa cells from F+ and ++ ewes. The binding of  $^{125}\text{I}$ -labelled hFSH ( $\sim 3$  ng) to granulosa cells from F+ or ++ ewes was displaced to some extent with oFSH and to lesser extent with FSH-P but other ovine pituitary hormones (supplied by the National Pituitary Agency, Maryland, USA) displayed little or no cross reaction.

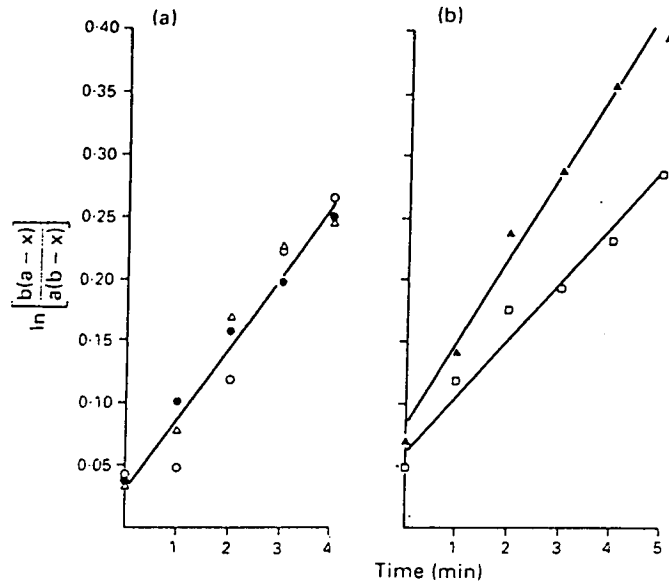


Fig. 3. Rates of  $^{125}\text{I}$ -labelled hFSH binding to granulosa cells ( $5 \times 10^6$  cells/tube from (a) FF ( $\Delta$ : 1.4 mm diam. follicles), F+ ( $\circ$ : 1.4-5 mm diam. follicles) and ++ ( $\bullet$ : 1.6 mm diam. follicles) ewes and (b) small ( $\square$ — $\square$ : 1.2-5 mm diam.) and large ( $\blacktriangle$ — $\blacktriangle$ :  $\geq 3$  mm diam.) follicles from Romney ewes as a function of time at  $37^\circ\text{C}$ . Each value is a mean of duplicate measurements. For clarity a single line is drawn through all points for Booroola ewes although the fitted lines for each genotype differ slightly. The terms a, b and x refer to the initial hormone concentration, receptor concentration and hormone-receptor complex concentration respectively.

Table 1. Specificity of  $^{125}\text{I}$ -labelled hFSH ( $\sim 3$  ng) to granulosa cells from Booroola ewes

Hormone	% Binding with respect to genotype	
	++	F+
hFSH (NIAMDD-hFSH-I-3)	100	100
oFSH (NIH-FSH-S16)	10.00	10.00
oLH (NIADDK-oLH-S25)	0.12	0.13
oTSH (NIH-TSH-S8)	0.08	0.06
oGH (NIH-GH-S11)	<0.01	<0.01
oProlactin (NIH-P-S13)	<0.01	<0.01
FSH-P (Lot 518G85)	2.50	2.50

Granulosa cells ( $5 \times 10^6$ ) were incubated at  $37^\circ\text{C}$  for 75 min with  $^{125}\text{I}$ -labelled hFSH (NIAMDD-hFSH-I-3:  $\sim 3$  ng;  $40 \mu\text{Ci}/\mu\text{g}$ ) and with increasing amounts of one of the above-mentioned unlabelled hormones. Binding in the absence of unlabelled hFSH was regarded as 100%.



## Equilibrium binding studies

The incubations of  $^{125}\text{I}$ -labelled hFSH with granulosa cells were performed at  $37^\circ\text{C}$  for 75 min to achieve equilibrium. The specific binding of  $^{125}\text{I}$ -labelled hFSH to granulosa cells from Booroola FF or ++ ewes with respect to increasing concentrations of unlabelled hFSH is shown in Fig. 4(a, b). Typically, linear plots were obtained when the specific binding data were fitted to the Woolf equation (Keightley & Cressie, 1980; Fig. 4c, d). The calculated binding capacities ( $B_{\text{max}}$ ) and equilibrium dissociation constants ( $K_d$ ) from the Woolf plots were 21.5 fmol/mg protein and 1.2 nM for the cells from FF ewes and 17.2 fmol/mg protein and 1.2 nM for the cells from ++ ewes. Binding of  $^{125}\text{I}$ -labelled hFSH to granulosa cells from Romney ewes as a function of increasing  $^{125}\text{I}$ -labelled hFSH concentration is shown in Fig. 5(a). A linear plot was obtained when the specific binding data were fitted to the Woolf equation. The calculated  $B_{\text{max}}$  and  $K_d$  values were 19.9 fmol/mg protein and 1.2 nM respectively.

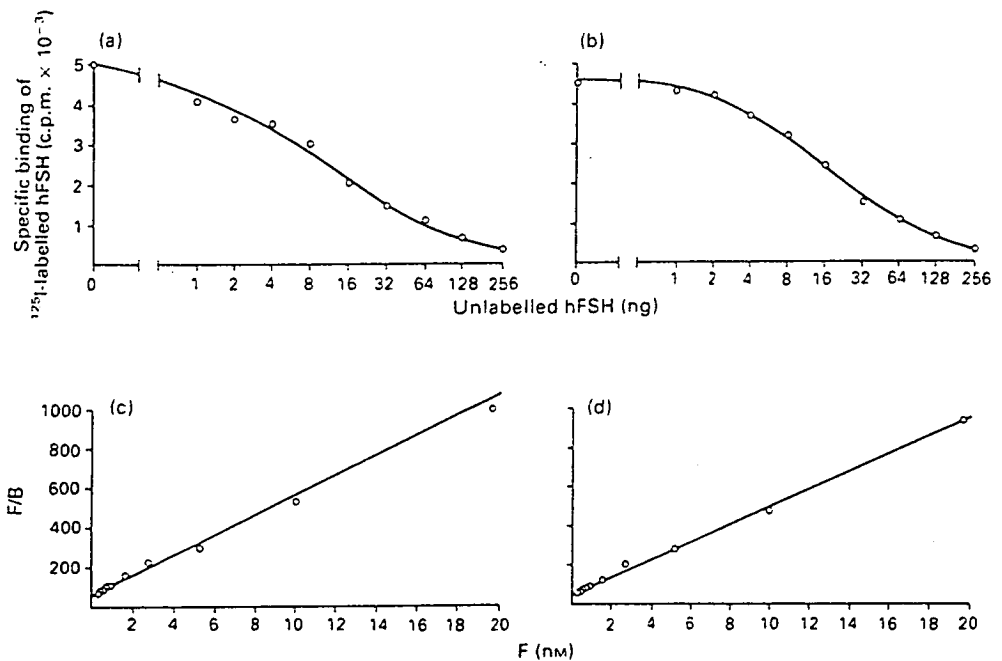


Fig. 4. Specific binding of  $^{125}\text{I}$ -labelled hFSH ( $\sim 3$  ng,  $36 \mu\text{Ci}/\mu\text{g}$ ) to granulosa cells from ++ (a) and FF (b) ewes with respect to increasing concentrations of unlabelled hFSH. For the ++ and FF ewes, the granulosa cells were recovered from 1–2.5 mm diameter and 1–4.5 mm diameter follicles respectively. Binding tubes ( $5 \times 10^6$  granulosa cells/tube) were incubated at  $37^\circ\text{C}$  for 75 min. Woolf plots of the data which included the mass of  $^{125}\text{I}$ -labelled hFSH for the ++ and FF specific binding data are shown in (c) and (d) respectively. The binding characteristic  $B_{\text{max}}$  was derived from the slope of the fitted line (i.e. slope =  $1/B_{\text{max}}$ ) and the  $K_d$  from the  $B_{\text{max}} \times$  intercept.

The characteristics of the FSH receptor in granulosa cells with respect to breed, genotype, and follicle size are summarized in Table 2. No F gene-specific effects, breed or follicle size effects were noted with respect to the FSH binding characteristics in granulosa cells. When all  $B_{\text{max}}$  and  $K_d$  values (i.e. from the self-displacement assays) were pooled irrespective of follicle size, breed or genotype, the respective mean  $\pm$  s.e.m. ( $n = 24$ ) values were  $16.7 \pm 0.8$  fmol/mg protein and  $1.1 \pm 0.1$  nM. Assuming that  $400 \mu\text{g}$  protein is equivalent to  $5 \times 10^6$  cells, the number of available

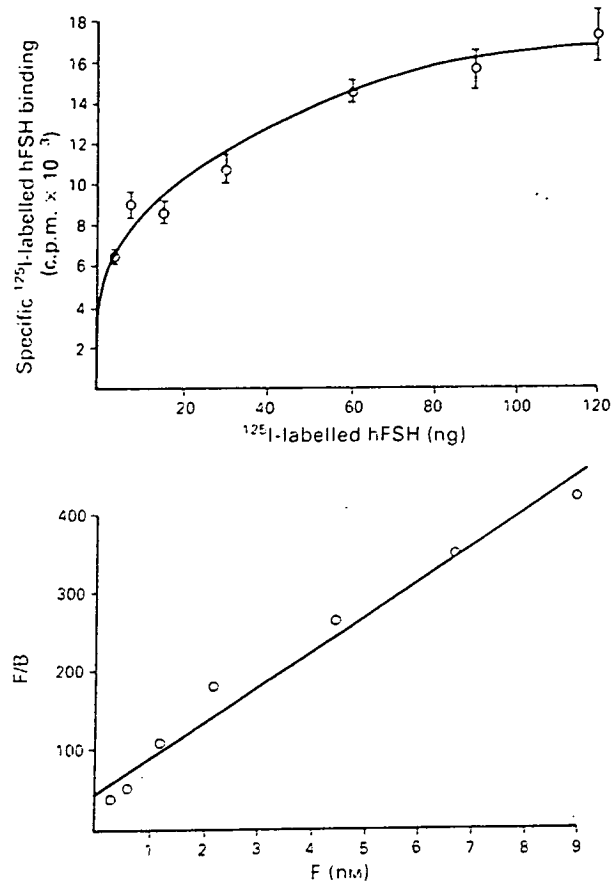


Fig. 5. Effect of addition of increasing  $^{125}\text{I}$ -labelled hFSH (ng) on specific binding of  $^{125}\text{I}$ -labelled hFSH to granulosa cells ( $5 \times 10^6$  cells/tube) from Romney ewes with respect to  $^{125}\text{I}$ -labelled hFSH concentration (a: vertical bars are s.e.m. of triplicate measurements at each point, and granulosa cells were recovered from follicles of 1–6 mm in diameter) and Woolf plot of the above data (b: see Fig. 4 for further details).

FSH receptors per sheep granulosa cell was estimated to be 800. This number of receptors per cell was essentially unchanged even after Romney ewes had been pretreated with bFF or FSH-P. The respective  $B_{\text{max}}$  and  $K_d$  values for cells pooled from the 1–6 mm diameter follicles from all ewes after bFF treatment were 14.1 fmol/mg protein and 1.1 nM. The respective  $B_{\text{max}}$  and  $K_d$  values after FSH-P pretreatment were 13.6 fmol/mg protein and 1.1 nM.

#### *Effect of human FSH on cAMP synthesis by granulosa cells*

These results with respect to follicle diameter and Booroola genotype are summarized in Table 3. Human FSH significantly stimulated more cAMP compared to that produced in control cultures ( $P < 0.01$  for both genotypes). In turn the control cultures produced no further cAMP above that present in the cells at the start of the incubation period (i.e. the  $T_0$  value). In cells from FF but not ++ follicles FSH stimulated more cAMP in large follicles ( $\geq 3$  mm diam.) than in small follicles (1–2.5 mm diam.) ( $P < 0.01$ ). In addition, FSH stimulated significantly more cAMP in cells from both small and large follicles from FF compared to those from ++ ewes ( $P < 0.05$  for small and large follicles).

Table 2. Characteristics of the FSH receptor in granulosa cells of Booroola and Romney ewes with reference to Booroola genotype, sheep breed and follicle size

Breed (genotype)	Follicle diameter (mm)	$B_{max}$ (fmol/mg protein)	$K_d$ (nM)	<i>n</i>
Booroola Merino (++)	1-2.5	15.7 ± 1.7	1.2 ± 0.3	3
	3-6	17.7 ± 1.7	1.2 ± 0.1	5
Booroola Merino (F+)	1-2.5	17.8 ± 2.2	1.0 ± 0.1	3
	3-4.5	15.8 ± 2.0	1.3 ± 0.3	3
Booroola Merino (FF)	1-4.0	17.1 ± 3.4	1.1 ± 0.2	3
Romney	1-2.5	16.3 ± 1.4	0.9 ± 0.1	3
	3-7	16.9 ± 1.0	1.1 ± 0.1	3
	1-7	19.1	1.3	1

Values are means ± s.e.m; *n* = number of pools of granulosa cells tested.

Each pool of granulosa cells was derived from the ovaries of 5-10 sheep.

Table 3. Effect of human FSH (NIAMDD-hFSH-I-3, 250 ng/ml) on cAMP synthesis (pmol/10<sup>6</sup> cells) by granulosa cells with respect to follicle diameter and Booroola genotype

Treatment	Follicle diameter (mm)	Booroola genotype	
		++ ( <i>n</i> = 9)	FF ( <i>n</i> = 8)
$T_0$ †	1-2.5	0.4 (0.3, 0.6)	0.5 (0.3, 0.6)
Control		0.4 (0.3, 0.6)	0.5 (0.3, 0.7)
FSH		2.3 (1.5, 3.3)	4.3* (2.9, 6.0)
$T_0$ †	≥ 3	0.6 (0.4, 0.8)	0.4 (0.3, 0.7)
Control		0.6 (0.4, 0.8)	0.4 (0.2, 0.7)
FSH		3.9 (2.1, 6.7)	8.9* (6.2, 14.8)

Values are geometric means (and 95% confidence limits);

*n* = number of pools of granulosa cells tested.

†cAMP values in cells at time of harvest.

\**P* < 0.05 compared to values for FSH treated cells from ++ ewes.

## Discussion

The principal aim of the present study was to examine the FSH binding characteristics in granulosa cells from the FF, F+ and ++ Booroola genotypes. The ovulation rates as assessed from the number of corpora lutea indicated that the FSH-binding studies were being performed in ovaries which were being influenced by F gene expression. The major findings were that no gene-specific differences were noted with respect to the specificity, capacity ( $B_{max}$ ) or dissociation constant ( $K_d$ ) of  $^{125}I$ -labelled hFSH binding to granulosa cells and, moreover, no obvious differences were noted with respect to the rate of hFSH binding ( $k_a$ ). It could be argued that the lack of a gene-specific difference was masked by the necessity to pool cells from different-sized healthy and atretic follicles. Nevertheless, significant F gene-specific differences are observed in the level of FSH-induced cAMP production after pooling cells as described above (Henderson *et al.*, 1987; McNatty *et al.*, 1988). Also, it could be argued that the lack of a gene-specific difference in FSH binding was due to the use of a human FSH preparation whereas the FSH-induced cAMP differences reported by Henderson *et al.* (1985, 1987; McNatty *et al.*, 1988) were found using an ovine FSH preparation. However, the present study reports that a gene-specific difference in cAMP production also occurs in granulosa cells when the same human FSH preparation as that used in the  $^{125}I$ -labelled FSH binding studies was employed. Collectively these findings suggest that the F gene difference in FSH-induced cAMP synthesis is the result of an event(s) distal to the FSH receptor.

In the present study, the FSH binding characteristics of low total binding (<2%) and high specificity (i.e. >60%) with a  $^{125}I$ -labelled FSH probe of 13.5% 'bindability' are similar to those reported in several previous studies with rat testis (Bhalla & Reichert, 1974), rat granulosa cells (Louvet & Vaitukaitis, 1976; Nimrod *et al.*, 1976), bovine granulosa cells (Darga & Reichert, 1978), human corpora lutea (McNeilly *et al.*, 1980; Bramley *et al.*, 1986) and hamster ovaries (Kin & Greenwald, 1986).

This study confirms that specific receptors for  $^{125}I$ -labelled hFSH are present in sheep granulosa cells (Carson *et al.*, 1979). In the earlier study of Carson *et al.* (1979), intact follicles were preincubated at 37°C for 27–36 h with  $^{125}I$ -labelled hFSH before isolation of granulosa cells and measurement of FSH binding activity. This technique precluded any accurate assessment of the FSH binding characteristics of granulosa cells (i.e.  $k_a$ ,  $K_d$ ,  $B_{max}$ ) because the studies were made in the presence of follicular fluid which is known to inhibit FSH binding (Darga & Reichert, 1978). For example, any of the differences in the amount of  $^{125}I$ -labelled hFSH bound to granulosa cells in small/large, healthy/atretic follicles could have been related to the relative amounts of inhibitor(s) in follicular fluid (Sluss *et al.*, 1983).

In the present study with isolated granulosa cells, ~50% of the  $^{125}I$ -labelled hFSH was bound to the receptor after 3 min and maximum binding was achieved after 45 min. Similarly, in bovine granulosa cells, the binding of  $^{125}I$ -labelled hFSH was rapid with maximum uptake being recorded within 2 h (Darga & Reichert, 1978). The rapid binding of FSH to its receptor is consistent with the finding of a  $\geq 3$ -fold increase in cAMP accumulation within 10 min of exposing ovine or bovine granulosa cells to FSH *in vitro* (K. P. McNatty, S. Lun, D. Heath & N. Hudson, unpublished observations). There was no significant effect of follicle diameter or sheep breed on the rate of uptake of  $^{125}I$ -labelled hFSH to granulosa cells. These findings suggest that the timing of the cAMP response to FSH stimulation *in vitro* is unlikely to be influenced by follicular diameter or sheep breed. *In vivo*, however, the granulosa cell cAMP response to FSH is likely to be affected by other factors such as the extent of the blood supply.

We were unable to measure the rates of dissociation of  $^{125}I$ -labelled hFSH from granulosa cells at 37°C. The dissociation of hFSH appeared to be minimal as has been shown for the rat, rabbit and hamster (Nimrod *et al.*, 1976; Kim & Greenwald, 1986). The variability of dissociation (i.e. 0–30%) of ligand within the first 6 h was a problem which remained unresolved. In bovine granulosa cells, the specific binding of  $^{125}I$ -labelled hFSH at 37°C declined steadily after 4 h (Darga & Reichert, 1978); this decline was thought to be due to the thermal lability of the granulosa

cell receptor as no such decline in binding was noted at 4°C. Perhaps in the present study the variable loss in ligand was due to the long-term (i.e. hours) stability/lability of the receptor at 37°C.

The calculated  $K_d$  value of 1.1 nM for sheep granulosa cells was similar to that recorded for the cow (Darga & Reichert, 1978). Likewise, with bovine granulosa cells no significant change in the  $B_{max}$  was noted with follicle diameter. We estimate that, for the sheep, the number of available FSH binding sites per cell is about 800 and that this remains relatively unchanged between sheep breeds (Merino and Romney), follicle diameter or following modification of ovarian follicular development *in vivo* by pretreating sheep with bovine follicular fluid or supplementary FSH. Louvet & Vaitukaitis (1976) showed that oestrogen pretreatment of rat granulosa cells did not alter the specific number of available FSH receptors per cell. Therefore, the findings from the sheep, cow and rat suggest that as follicles mature any increased FSH uptake would occur solely as a consequence of an increased number of granulosa cells as has been demonstrated in the present study (see Fig. 1). Despite no change in the FSH receptor binding characteristics (i.e.  $B_{max}$ ,  $K_d$ ,  $k_2$ ) with increasing follicular diameter, there is an increase in the mean level of FSH-induced cyclic AMP synthesis with respect to follicular diameter (Henderson *et al.*, 1985, 1987; McNatty *et al.*, 1985c; Table 3). This difference in FSH-stimulatable cAMP synthesis with respect to follicular diameter is not due to any change in cAMP-phosphodiesterase enzyme activity (McNatty *et al.*, 1988) but might be due, in part, to increased activity or altered composition of the regulatory or catalytic components of the adenylate cyclase system (Richards & Hedin, 1988). In addition, part of the increase in cAMP synthesis could be due to LH contamination in the various FSH preparations (e.g. 6% LH contamination of NIAMDD-hFSH-1-3 by RIA). In large follicles (i.e.  $\geq 5$  mm diam. in Romney or Booroola ++ ewes or  $> 2.5$  mm diam. in FF ewes) the level of LH contamination is sufficient to stimulate at least a proportion of the increase in cAMP via the LH receptor (see Henderson *et al.*, 1985, 1987).

In summary, the greater FSH-induced cAMP responses in granulosa cells in antral follicles, the earlier induction of granulosa cell aromatase and also the earlier maturation of follicles at a small diameter in FF ewes compared to ++ ewes (see McNatty & Henderson, 1988, for review) are all unlikely to be due to gene-specific differences in the  $k_2$ ,  $K_d$  or  $B_{max}$  FSH binding characteristics of the granulosa cells.

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**Title:** Effect of oestradiol-17 $\beta$ , progesterone or bovine follicular fluid on the plasma concentrations of FSH and LH in ovariectomized Booroola ewes which were homozygous carriers or non-carriers of a fecundity gene.

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## Effects of oestradiol-17 $\beta$ , progesterone or bovine follicular fluid on the plasma concentrations of FSH and LH in ovariectomized Booroola ewes which were homozygous carriers or non-carriers of a fecundity gene

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**Summary.** The plasma concentrations of FSH and LH were measured in ovariectomized Booroola FF and ++ ewes before and after treatment with subcutaneous implants of oestradiol-17 $\beta$  (0, 2 or 8 cm Silastic capsules; 5 ewes/genotype per dose) or progesterone (0, 1 or 3 Silastic envelopes; 5 ewes/genotype per dose) or subcutaneous injections of steroid-free bovine follicular fluid (bFF; 0, 0.5, 1.0, 2.5 or 5 ml; 4 ewes/genotype per dose). During the first 50 h after implantation of oestradiol or progesterone, or the first 24 h after bFF treatment, the FSH and LH concentrations in plasma were not different between the genotypes although there were significant effects of the steroids and bFF with respect to dose ( $P < 0.05$ ). At 6 days after steroid implantation, no gene-specific effects were noted for the plasma concentrations of FSH although significant effects of dose of oestradiol ( $P < 0.01$ ) but not progesterone were noted. Also at 6 days after steroid implantation, no gene-specific differences in the pulsatile patterns (i.e. peak frequency or amplitude) of plasma LH concentrations were noted although there were significant effects of steroid dose ( $P < 0.05$ ) on frequency and/or amplitude.

It is concluded that the higher ovulation-rate in FF than ++ Booroola ewes is unlikely to be due to gene-specific differences in the sensitivity of the hypothalamic-pituitary axis to ovarian hormones.

**Keywords:** Booroola ewes; oestradiol-17 $\beta$ ; progesterone; bovine follicular fluid; FSH; LH; ovariectomy

### Introduction

High fecundity Booroola ewes contain a major gene(s) (F) which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative gene(s) are segregated on the basis of ovulation-rate recordings of  $\geq 5$ , 3 or 4 and 1 or 2 respectively (Davis *et al.*, 1982). Previous studies have demonstrated differences both in ovarian activity and plasma gonadotrophin concentrations between ++ and FF ewes (McNatty & Henderson, 1987; Fry *et al.*, 1988; McNatty *et al.*, 1987, 1989). However, it is not known whether F gene-specific differences exist in the sensitivity of the hypothalamic-pituitary axis to oestradiol, progesterone or other ovarian hormones. The highly fecund Finnish Landrace ewe is known to be less sensitive to oestradiol negative feedback than the less fecund Scottish Blackface ewe as assessed by the plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Webb *et al.*, 1985a). Ovarian hormones other than oestradiol or progesterone also contribute to



the feedback control of FSH secretion (Goodman *et al.*, 1981). In this context, steroid-free bovine follicular fluid (bFF) has been used experimentally to lower FSH, and to a lesser extent, LH concentrations in plasma of sheep (Martin *et al.*, 1986) by inhibiting, at least in part, the mRNA for the  $\beta$ -subunit of FSH (Mercer *et al.*, 1987).

The aim of this study was to examine the effects of different doses of oestradiol-17 $\beta$ , progesterone or bFF on the plasma concentrations of FSH and LH with respect to Booroola genotype.

## Materials and Methods

### *Animals and experiments*

Booroola Merino ewes born in 1978–80 were classified as ++ (N = 35) or FF (N = 35) genotypes based on pedigree analysis: these genotypic classifications were confirmed by laparoscopy according to the criteria of Davis *et al.* (1982). All the FF ewes, aged 6–7 years at the time of the study, were recorded as having at least one annual ovulation rate of  $\geq 5$  whereas the ++ ewes, aged 6–7 years, were never recorded with an ovulation of  $> 2$ . All the ewes were ovariectomized at least 40 days before the first experiment at the start of the breeding season. At the time of ovariectomy the mean  $\pm$  s.e.m. liveweights of the ewes were 47.2  $\pm$  1.2 kg for the FF genotype and 49.1  $\pm$  1.5 kg for the ++ genotype; there was no significant difference in liveweight between the genotypes. On the day before the experiments, the animals were penned indoors and each was fitted with an intrajugular cannula under local anaesthesia (Xylocaine 2%: Astra Pharmaceuticals Pty Ltd, NSW, Australia). When blood sampling began, the animals were each bled (2.5 ml) *via* the jugular cannula.

Experiments 1 and 2 were designed to examine the plasma concentrations of FSH and LH with respect to genotype before and after treating ewes with oestradiol-17 $\beta$  (Exp. 1) or progesterone (Exp. 2). For Exp. 1, 0, 2 or 8 cm Silastic capsules (3.35 mm i.d., 4.65 mm o.d.; Cat. No. 601-335; Dow Corning Corp., Midland, MI, USA), were packed with oestradiol-17 $\beta$  as described by Karsch *et al.* (1973) and 5 ewes/genotype at each dose of oestradiol were studied. For Exp. 2, 0, 1 or 3 Silastic envelopes (55  $\times$  75 mm; Cat. No. 500-1; Dow Corning), were packed with progesterone as described by Karsch *et al.* (1977) and 5 ewes/genotype at each dose of progesterone were studied. All steroid capsules or envelopes were presoaked in water for 12–18 h to avoid any potential transient 'peak release' of steroid. The implants were inserted in the subcutaneous fascia of the hind leg(s) while the animals were under local anaesthesia; the time taken to implant each capsule or envelope was approximately 10 min. The ewes in Exp. 1 were the same as those used in Exp. 2 but all were from a different flock from those used in Exp. 3. The period between Exps 1 and 2 was 55–59 days.

The blood sampling regimens were identical for Exps 1 and 2. On the 1st day of the experiment, all animals were blood sampled hourly for 24 h before the implanting of steroids and thereafter, each hour for a further 50 h. On the 6th day after steroid implantation, the animals were blood sampled every 10 min for 12 h. The plasmas from all hourly samples were assayed for FSH and LH whereas the samples taken at 10-min intervals were assayed for LH only.

Experiment 3 was designed to examine the plasma concentrations of FSH and LH with respect to genotype after injecting FF and ++ ewes with 0, 0.5, 1.0, 2.5 and 5.0 ml bFF (s.c.; N = 4 ewes/genotype per dose of bFF). All ewes in this experiment had been ovariectomized 60–65 days previously and had not been pretreated with any exogenous steroid or follicular fluid. On the day of the experiment, hourly blood sampling began at 09:00 h for 29 h with the bFF being administered immediately after the 13:00 h blood sample, and again just after the subsequent 01:00 h blood sample. All ewes were treated with the same pooled batch of bFF (Batch V), which was prepared free of steroids as previously described (Henderson *et al.*, 1986).

At the end of each experiment, all steroid implants and cannulae were removed and the animals were returned to pasture.

### *Blood samples*

All blood samples were centrifuged at 4000 g at room temperature for 8 min within 30 min of collection and the plasma samples were stored at  $-20^{\circ}\text{C}$  until assayed.

### *Hormone assays*

**FSH.** The radioimmunoassay kit was that supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, MD, USA. The ovine (o) FSH for iodination was NIAMDD-oFSH-I-1; the oFSH reference preparation was NIAMDD-oFSH-RP-1 (biopotency 75  $\times$  NIH-FSH-3) and the oFSH antiserum was NIAMDD-anti oFSH-1 (AFP-C5288113). At a final FSH antiserum dilution of 1:80 000 this homologous assay had a working range of 0.01 to 5 ng per assay tube. The volume of plasma which was assayed was 0.1 ml and each sample was assayed in duplicate. The reference standards and standard curve samples were prepared in hypophysectomized ewe plasma which contained undetectable concentrations of FSH. The intra- and interassay coefficients of variation were  $< 4$  and  $< 5\%$  respectively.

**LH.** The radioimmunoassay was similar to that described by McNatty *et al.* (1987). The iodination standard was NIDDK-oLH-I-3 (AFP-9598B), the LH antiserum was raised at Wallaceville and its characteristics are described by McNatty *et al.* (1987). The oLH reference preparation was NIAMDD-oLH-S23 (biopotency  $2.3 \times$  NIH-LH-S1). The volume of plasma which was assayed was 0.1 ml and each sample was assayed in duplicate. The minimum detectable level of LH was 0.03 ng/tube. The reference standards and standard curve samples were prepared in hypophysectomized ewe plasma which contained undetectable amounts of LH. The intra and interassay coefficients of variation were  $<5$  and  $<8\%$  respectively.

**Progesterone.** The progesterone radioimmunoassay together with the dilution and specificity of the progesterone antiserum (WA26) were as described by McNatty *et al.* (1981). The volume of plasma assayed was 200  $\mu$ l and the detection limit was 20 pg progesterone/tube. All the samples were measured in one assay and the intra-assay coefficient of variation was  $<8\%$ .

**Oestradiol-17 $\beta$ .** The extraction of oestradiol-17 $\beta$  from plasma was similar to that described by Webb *et al.* (1985b). In brief, 3 ml plasma plus [2,4,6,7- $^3$ H]oestradiol (1000 c.p.m.; sp. act. 94 Ci/mmol) in 100  $\mu$ l assay buffer, namely 0.1 M-phosphate buffer + 0.1% gelatin (w/v; pH 7.4), was incubated at room temperature for 30 min. Thereafter 10 ml double glass-distilled (DG-D) water plus 2 ml oestradiol antibody conjugated to Sepharose 4B were added to the mixture and mixed for 2 h at room temperature. The oestradiol antibody (WA-27) used was identical to that described elsewhere (McNatty *et al.*, 1984); 150  $\mu$ l of the antibody was conjugated to 9 g Sepharose 4B as described by Webb *et al.* (1985b). At the end of the 2-h incubation the plasma, water, [ $^3$ H]oestradiol, oestradiol antibody-Sepharose mixture was poured directly onto a prewashed sintered glass column (1  $\times$  20 cm; Econo-column, Biorad Labs., Richmond, CA, USA). Before use the column was washed with 3 ml 90% (v/v) methanol and twice with 20 ml DG-D water. The tube from which the incubation contents were transferred to the column was rinsed with 7 ml DG-D water and again with 20 ml DG-D water with each of these rinses being transferred to the column. The residual water in the antibody-Sepharose mixture was then removed by applying slight positive pressure. The bound hormone was then eluted with 3 ml 90% methanol which was evaporated to dryness. Thereafter the assay was performed as previously described by McNatty *et al.* (1984). All aqueous solutions (e.g. buffer, dextran-charcoal solutions) were prepared in DG-D water. The limit of detection for oestradiol was  $\leq 3$  pg per tube and the intra- and inter-assay coefficients of variation over the range of 3–30 pg were 15 and 22% respectively.

### Statistical procedures

Because of between-sheep and genotype variation in the pretreatment concentrations of plasma FSH and LH, both the hourly pre- and post-treatment values for each ewe were normalized by expressing the data as a percentage of the overall pretreatment mean value. Homogeneity of variance was checked using the Bartlett test. The genotype and dose effects as specific time intervals were then analysed by a 1- or 2-way Analysis of Variance (ANOVA) and multiple comparisons were made using Scheffe's test. Because of extensive overlap of the mean percentage data of each time between the genotypes, the data in Figs 1–4 are presented as hourly means without errors or ranges for clarity. In these instances, the variability of the individual means for each genotype are presented as an overall coefficient of variation (CV) which was determined by averaging the CVs over each hour of the study.

The episodic LH data were examined using the method of Van Look (1976) and Backstrom *et al.* (1982). An LH peak was defined as occurring when the hormone concentrations of two consecutive samples was greater than that of the mean of the two previous samples (basal samples) and the value of at least one of the peak samples exceeded the mean basal value by more than twice the coefficient of variation of the assay which was set at 8%. The amplitude of each peak was measured by subtracting the basal from the peak value. The 'smoothed' mean value was defined as the overall mean basal value after all peak values were removed. Genotype and dose differences in LH data were compared by ANOVA.

## Results

### *Effect of different doses of oestradiol on the plasma concentrations of oestradiol, FSH and LH before and after implantation of oestradiol (Exp. 1)*

Before implantation, oestradiol was not detectable in the plasma of any individual animal irrespective of genotype. Moreover, in the control animals (i.e. with no oestradiol), oestradiol was not detectable at any time during the experimental period. In the animals given the 2-cm implants, the mean  $\pm$  s.e.m. oestradiol concentrations at  $-24$  h,  $+5$  h,  $+24$  h and  $+49$  h from implantation were  $<1.0$ ,  $2.2 \pm 0.8$ ,  $1.9 \pm 0.5$  and  $2.5 \pm 0.3$  pg/ml respectively for the FF genotype and  $<1.0$ ,  $1.4 \pm 0.2$ ,  $2.6 \pm 0.7$  and  $2.4 \pm 0.2$  pg/ml respectively for the ++ genotype. For those given the 8-cm implants, the mean  $\pm$  s.e.m. concentrations at  $-24$  h,  $+5$  h,  $+24$  h and  $+49$  h from implantation were  $<1.0$ ,  $17.8 \pm 4.2$ ,  $15.8 \pm 2.6$  and  $16.2 \pm 1.5$  pg/ml respectively for the

FF genotype and  $<1.0$ ,  $17.2 \pm 2.3$ ,  $14.3 \pm 1.2$  and  $14.4 \pm 3.0$  pg/ml respectively for the ++ genotype. No gene-specific differences in oestradiol concentrations were noted for the 2- or 8-cm implants at any of the above mentioned times.

The mean FSH and LH data expressed as a percentage of the pretreatment mean value with respect to Booroola genotype are summarized in Fig. 1 (FSH) and Fig. 2 (LH). The pretreatment mean  $\pm$  s.e.m. FSH concentrations for the FF (N = 15) and ++ (N = 15) genotypes were  $7.8 \pm 0.8$  ng/ml and  $7.5 \pm 0.7$  ng/ml respectively and the corresponding LH concentrations were  $5.1 \pm 0.4$  and  $5.4 \pm 0.5$  ng/ml respectively; for each hormone the pretreatment mean values were not significantly different. In the ewes not receiving oestradiol, there were no appreciable changes after 0 h (i.e. the time corresponding to implantation of oestradiol in the treated groups) in the plasma concentrations of FSH or LH in either genotype compared to the pretreatment values. Moreover, when the hourly values after 0 h for each of the untreated genotypes were compared, no gene-specific differences were noted for either FSH or LH.

After receiving 2- or 8-cm oestradiol capsules, there was a progressive decline in the mean FSH and LH concentrations in ewes of both genotypes to a nadir around 4–10 h after implantation followed by a rise to peak values at 15–16 h. From 17 h onwards, the mean FSH and LH values declined once more to reach basal values between 30 and 50 h after oestradiol implantation. No gene-specific differences were observed in the time to the onset of the FSH 'peak' values or in the FSH peak areas for either dose (Fig. 1). For 2-cm implants the mean  $\pm$  s.e.m. times were  $15.4 \pm 0.4$  and  $14.8 \pm 0.4$  h for the ++ and FF genotypes respectively, whereas for 8-cm implants the mean  $\pm$  s.e.m. times were  $15.0 \pm 0.5$  h for both genotypes. Moreover, no difference was noted with respect to peak areas or amplitudes with respect to genotype or dose of oestradiol. Likewise, no gene-specific differences were observed in the time of steroid implantations to the onset of the LH 'surge': 2-cm implants; mean  $\pm$  s.e.m. =  $11.8 \pm 0.8$  h for ++ ewes and  $10.6 \pm 0.4$  h for the FF ewes; 8-cm implants; mean  $\pm$  s.e.m. =  $10.0 \pm 0.6$  h for the ++ ewes and  $9.4 \pm 0.5$  h for the FF ewes. Moreover, no differences were noted with respect to peak areas or amplitude either with respect to genotype or dose of oestradiol. When the FSH or LH means (one mean value for each ewe) from 30 to 50 h for each oestradiol dose for each genotype were examined by 2-way ANOVA, no gene effects were observed but for both the 2-cm and 8-cm oestradiol treatment groups values were significantly lower than in the controls ( $P < 0.01$ ) but not significantly different from one another.

*Effect of different doses of progesterone implants on the plasma concentrations of progesterone, LH and FSH before and after implantation of progesterone (Exp. 2)*

The mean LH data together with the changes in the mean plasma concentrations of progesterone with respect to Booroola genotype are summarized in Fig. 3. Before progesterone implantation, progesterone was not detectable in the plasma of any individual animal irrespective of genotype. Moreover, in the control animals, progesterone was not detectable at any time during the experimental period. In the animals given 1 or 3 implants, the progesterone concentrations were above the detection limits in all animals after 2 h and the concentrations in both treatment groups continued to increase rapidly over the first 11 h and then more gradually over the next 38–39 h. There were no significant differences between the genotypes in the concentrations of progesterone at any of the sampling times.

For LH, the pretreatment mean  $\pm$  s.e.m. values for the FF (N = 15 animals) and ++ (N = 15 animals) genotypes were  $5.4 \pm 0.4$  and  $5.1 \pm 0.5$  ng/ml respectively. Concomitant with the rapid rise in progesterone over the first 11 h after implantation of progesterone there was a precipitous fall in the plasma concentrations of LH with no gene-specific differences noted. In the animals treated with 1 or 3 implants, the LH concentrations fell to the lowest recorded values at 5 h afterwards, remained at that level for the next 5 h and then partly recovered. This pattern of partial recovery occurred in a similar manner in ewes of both genotypes. When the overall means of the

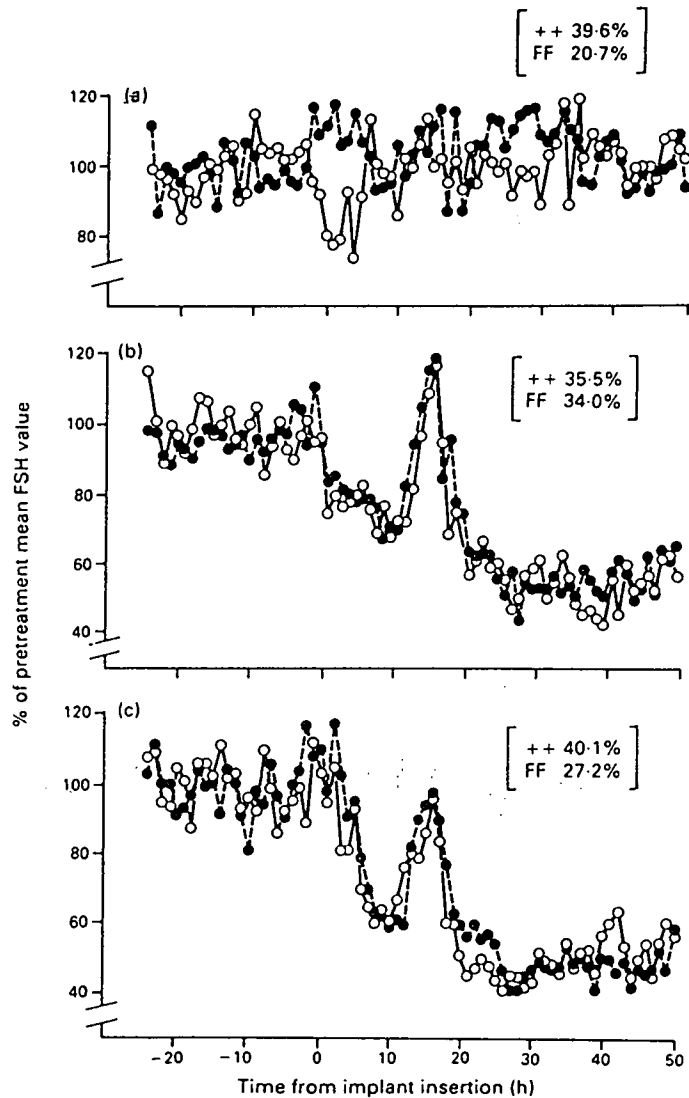


Fig. 1. Hourly changes in the mean plasma concentrations of FSH before and after implantation of 0 (a), 2-cm (b) and 8-cm (c) Silastic capsules of oestradiol-17 $\beta$  in ovariectomized FF (O—O) and ++ (●---●) ewes (5 animals/genotype per dose of oestradiol). Implants were inserted within 30 min of 0 h. For clarity the hourly s.e.m. values for each genotype are omitted. The overall coefficients of variation of the means are shown within the square brackets for each treatment group.

individual mean values for each sheep between 20 and 50 h were examined by 2-way ANOVA with respect to genotype and number of progesterone implants, a significant effect of dose ( $P < 0.01$ ) but not genotype or genotype  $\times$  dose interactions was observed, with the values from each of the progesterone dose levels being significantly different from one another ( $P < 0.05$ ).

In contrast to the effects of progesterone on plasma LH, there were no effects of dose of progesterone or genotype on plasma FSH concentrations during the first 50 h after implantation.

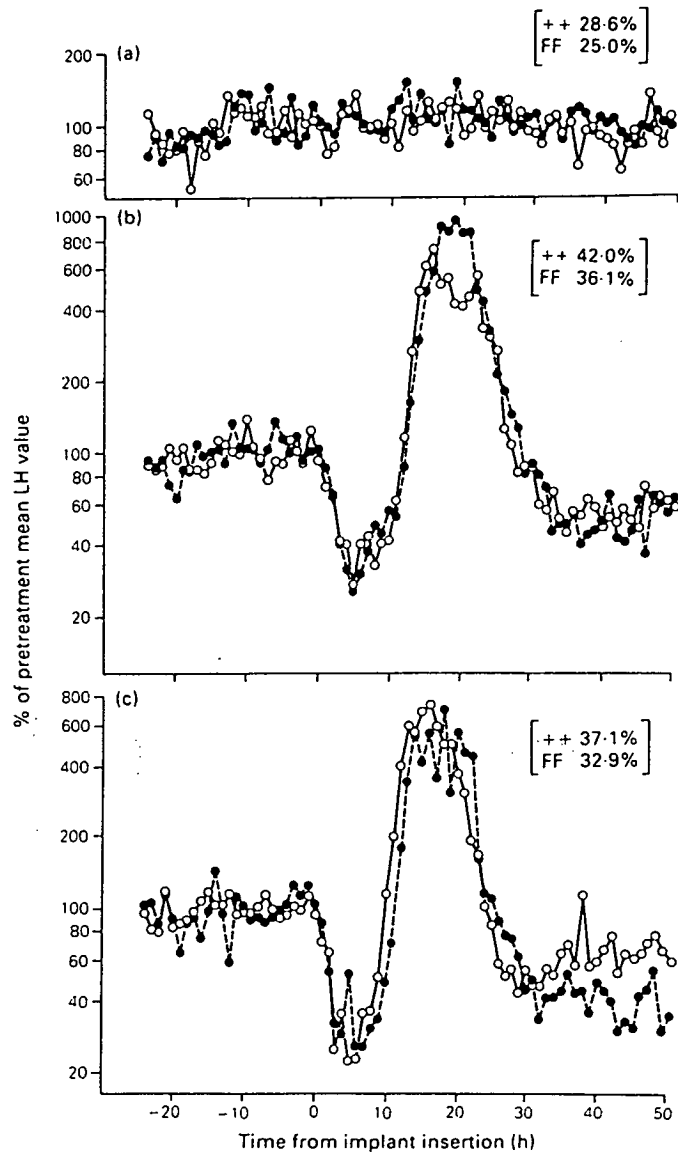
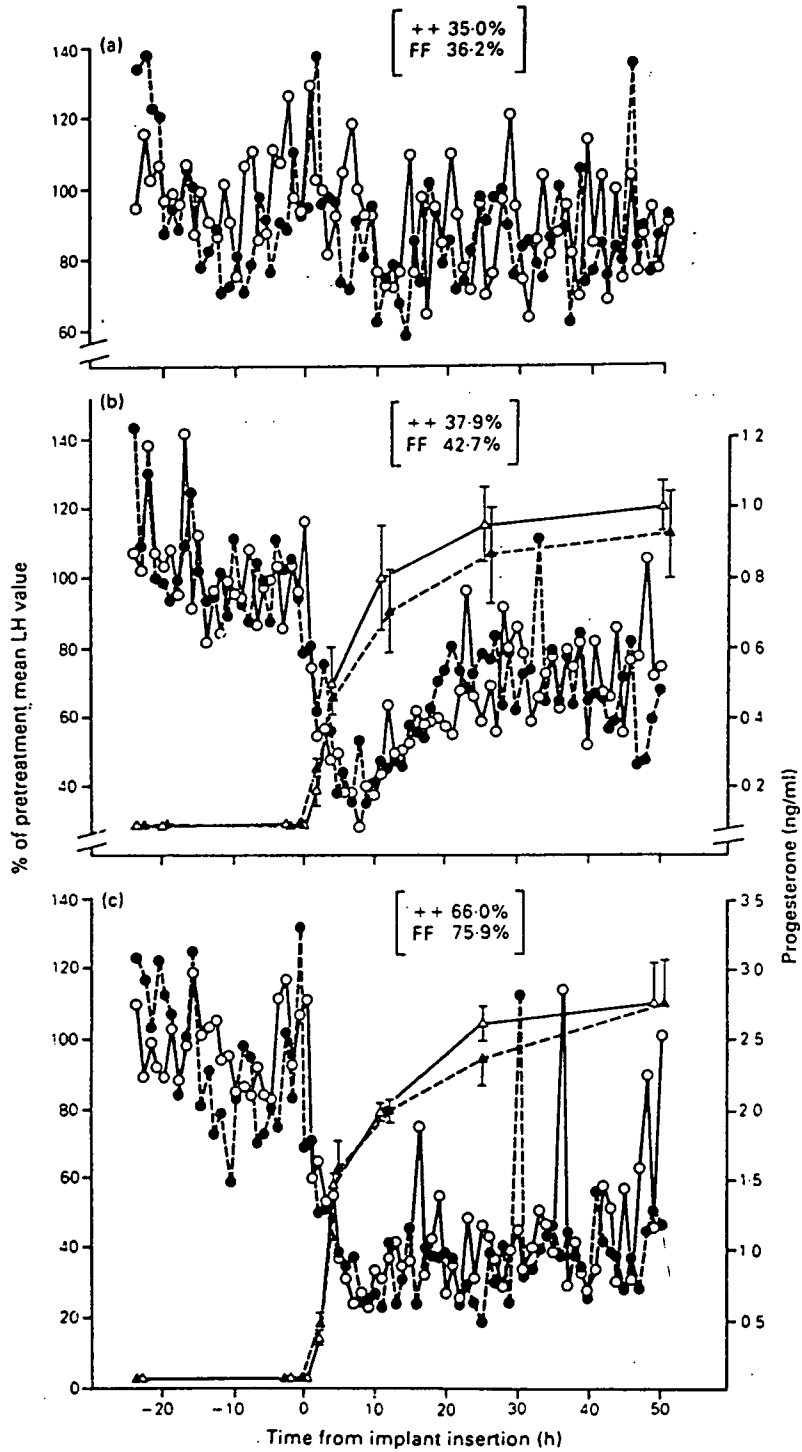


Fig. 2. Hourly changes in the mean plasma concentrations of LH before and after implantation of 0 (a), 2-cm (b) and 8-cm (c) Silastic capsules of oestradiol-17 $\beta$  in ovariectomized FF (○—○) and ++ (●---●) ewes (5 animals/genotype per dose of oestradiol). Implants were inserted within 30 min of 0 h. For clarity the hourly s.e.m. values for each genotype are omitted. The overall coefficients of variation of the means for each genotype are shown within the square brackets for each treatment group.

*FSH and LH concentrations 6 days after implantation of oestradiol or progesterone (Exps 1 and 2)*

The mean  $\pm$  s.e.m. plasma concentrations of oestradiol in the ovariectomized ewes with 0, 2-cm or 8-cm implants of oestradiol-17 $\beta$  were  $<1.0$ ,  $2.4 \pm 0.4$  and  $16.1 \pm 3.1$  pg/ml respectively for the FF genotype (5 ewes/dose) and  $<1.0$ ,  $2.3 \pm 0.4$  and  $16.3 \pm 4.1$  pg/ml respectively for the ++ genotypes (5 ewes/dose). The mean  $\pm$  s.e.m. plasma concentrations of progesterone in the ovariectomized ewes with 0, 1 or 3 Silastic envelopes of progesterone were  $<0.1$ ,  $1.1 \pm 0.1$  and



**Fig. 3.** Hourly changes in the mean plasma concentrations of LH before and after implantation of 0 (a), 1 (b) or 3 (c) Silastic envelopes of progesterone in ovariectomized FF (○—○) and ++ (●—●) ewes (5 ewes/genotype per progesterone treatment). Implants were inserted within 30 min of 0 h. The overall coefficients of variation of the means for each genotype are shown within square brackets for each treatment group. Changes in the mean  $\pm$  s.e.m. (vertical bars) plasma concentrations of progesterone before and after insertion of the Silastic envelopes of progesterone are also shown for the FF ( $\Delta$ — $\Delta$ ) and ++ ( $\blacktriangle$ — $\blacktriangle$ ) genotypes in (b) and (c). In (a), the progesterone concentrations were undetectable.

3.0 ± 0.4 ng/ml respectively for the FF genotype (5 ewes/dose) and <0.1, 0.9 ± 0.1 and 2.9 ± 0.4 ng/ml respectively for the ++ genotype (5 ewes/dose).

The mean ± s.e.m. (N = 5 sheep/steroid dose) concentrations of FSH and LH with respect to Booroola genotype (the gonadotrophin value for each ewe was averaged over time) and dose of oestradiol or progesterone 6 days after implantation are summarized in Fig. 4. When the oestradiol implant data were analysed by a 2-way ANOVA (genotype and dose of oestradiol), there were significant effects of dose of oestradiol for both FSH ( $P < 0.01$ ) and LH ( $P < 0.01$ ) but no significant effects of genotype for either hormone and no dose × genotype interactions were noted. The effect of the 2-cm oestradiol capsule was to lower the FSH ( $P < 0.01$ ) and LH ( $P < 0.05$ ) concentrations with respect to the untreated controls while the 8-cm capsules lowered the LH ( $P < 0.05$ ) but not the FSH concentrations beyond those in the ewes with the 2-cm implants. When the progesterone implant data were examined by a 2-way ANOVA (genotype and number of progesterone envelopes), there was a significant effect of dose of progesterone for LH ( $P < 0.01$ ) but not for FSH, and there were no dose × genotype effects for either LH or FSH. The effect of 1 envelope of progesterone significantly lowered the mean LH concentration ( $P < 0.05$ ) with respect to the untreated control while 3 envelopes lowered the mean LH concentrations significantly below those found in the ewes with one implant ( $P < 0.01$ ).

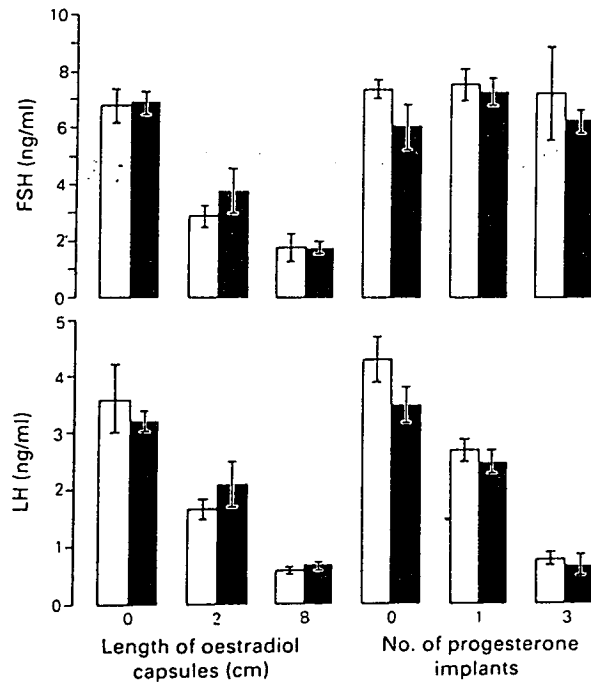


Fig. 4. Effects of length of Silastic capsules of oestradiol-17 $\beta$  (0, 2- or 8-cm) or number of Silastic progesterone envelopes on the mean plasma concentrations of FSH and LH in ovariectomized ewes with respect to genotype. Values are mean ± s.e.m. (5 ewes/genotype per steroid implant).

*Pulsatile plasma LH characteristics 6 days after implantation of oestradiol or progesterone (Exps 1 & 2)*

These data are summarized in Table 1. Significant effects were noted on the overall mean, the smoothed mean or LH peak frequency with respect to the length of the oestradiol capsule

( $P < 0.01$ ) or number of progesterone envelopes ( $P < 0.01$ ) but not for genotype and, moreover, no interactions were noted. Significant effects were also noted with respect to oestradiol ( $P < 0.01$ ) but not progesterone or genotype on LH peak amplitude. With respect to the length of the oestradiol capsule the overall mean LH concentration and the smoothed mean LH concentration in each of the treatment groups were significantly different from one another ( $P < 0.05$ ). The mean values for LH peak frequency and LH peak amplitude in ewes with 2-cm capsules were not different from those with 8-cm capsules, but the values in ewes with no capsules were significantly higher than those in either of the other two groups ( $P < 0.05$ ), indicating that the oestradiol implants led to a suppression of both LH peak frequency and amplitude. With respect to the number of progesterone envelopes both the overall mean and the smoothed mean LH concentration for each of the dose groups were different from one another ( $P < 0.05$ ). For LH peak frequency, the mean values in ewes with 1 envelope were not significantly different from those without implants but both the aforementioned had significantly higher frequencies than those with 3 envelopes (3 vs 1 or 3 vs 0) envelopes; ( $P < 0.01$ ).

**Table 1.** Effect of dose of oestradiol (length of capsule, cm) or progesterone (no. of implants) on the pulsatile characteristics of LH with respect to genotype in ovariectomized Booroola ewes (N = 5 ewes/genotype per treatment)

		Overall mean (ng/ml)	Smoothed mean (ng/ml)	LH peak frequency (peaks/12 h)	LH peak amplitude (ng/ml)
<b>Oestradiol</b>					
0	FF	3.7 ± 0.6	3.0 ± 0.5	11.2 ± 1.2	2.9 ± 0.7
	++	3.4 ± 0.2	2.8 ± 0.2	11.8 ± 2.1	2.4 ± 0.1
2	FF	1.8 ± 0.2	1.7 ± 0.2	5.8 ± 0.5	1.7 ± 0.3
	++	2.0 ± 0.5	1.9 ± 0.5	7.0 ± 0.7	1.0 ± 0.2
8	FF	0.6 ± 0.1	0.6 ± 0.1	5.2 ± 0.7	0.6 ± 0.1
	++	0.6 ± 0.1	0.6 ± 0.1	6.4 ± 0.8	0.6 ± 0.1
<b>Progesterone</b>					
0	FF	4.0 ± 0.6	3.4 ± 0.4	12.4 ± 1.9	2.9 ± 0.5
	++	4.0 ± 0.5	3.6 ± 0.4	11.8 ± 0.4	2.5 ± 0.6
1	FF	2.9 ± 0.3	2.6 ± 0.3	9.4 ± 1.2	3.1 ± 0.5
	++	2.6 ± 0.3	2.3 ± 0.3	8.2 ± 1.2	3.6 ± 0.8
3	FF	0.9 ± 0.1	0.8 ± 0.1	2.8 ± 0.4	3.3 ± 0.6
	++	0.7 ± 0.2	0.6 ± 0.2	2.2 ± 0.4	3.3 ± 0.9

For each treatment group, there were no significant effects of genotype with respect to each LH secretory characteristic.

#### *Effect of bFF on the plasma concentrations of FSH and LH (Exp. 3)*

The pretreatment mean concentrations of FSH in the FF (N = 20) and ++ ewes (N = 20) were  $16.2 \pm 1.4$  and  $11.6 \pm 1.0$  ng/ml respectively whereas those for LH in the FF and ++ ewes were  $3.5 \pm 0.4$  and  $2.8 \pm 0.3$  ng/ml respectively. The mean concentrations for FSH but not LH in FF ewes were significantly different from those in ++ ewes ( $P < 0.05$ ). The higher mean FSH values in Exp. 3 compared to Exp. 1 are probably due to time after ovariectomy (McNatty *et al.*, 1989). The data summarizing the mean FSH and LH concentrations over 6–12 h and over 18–24 h from the first bFF injection with respect to genotype and dose of bFF injected are shown in Fig. 5. When the effects of genotype (i.e. ++, FF) and dose (i.e. 0, 5 ml, 1 ml, 2.5 ml and 5 ml bFF) were examined by 2-way ANOVA, at 6–12 h or after 18–24 h there were significant effects with respect to dose ( $P < 0.05$ ) but no effects of genotype for both FSH and LH and no significant interactions were noted.



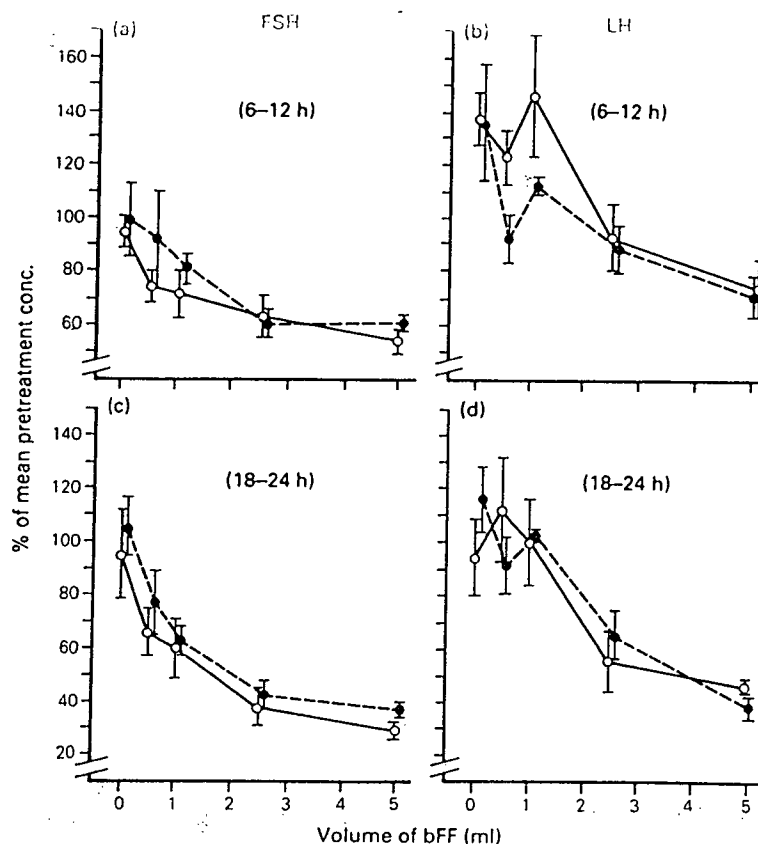


Fig. 5. Effect of different doses of bovine follicular fluid (bFF) on the plasma concentrations of FSH and LH in ovariectomized ewes with respect to Booroola genotype (FF,  $\circ$ — $\circ$ ; ++,  $\bullet$ — $\bullet$ ); 4 ewes/genotype per dose of bFF) at 6–12 h (a, b) and 18–24 h (c, d). Values are mean  $\pm$  s.e.m. for 5 ewes/genotype per bFF dose.

Doses of bFF at 2.5 ml or 5 ml s.c. suppressed FSH concentrations in ewes of both genotypes by  $\sim$ 40% between 6 and 12 h and by  $\sim$ 65% between 18 and 24 h. Moreover, the highest dose of bFF (i.e. 5 ml) suppressed LH concentrations in both genotypes by  $\sim$ 20% between 6 and 12 h and by  $\sim$ 45% between 18 and 24 h (Fig. 5). Overall bFF was effective even at the lowest dose (i.e. 0.5 ml) in suppressing FSH concentrations compared to the zero dose ( $P < 0.05$ ). In contrast, bFF was only effective in suppressing LH concentrations at the 2 highest doses (i.e. 2.5 ml and 5.0 ml; both ( $P < 0.05$ ) relative to the zero dose.

### Discussion

In this study we have examined the effects of different doses of oestradiol, progesterone or bFF in modulating the plasma concentrations of FSH and LH over time (i.e.  $\leq$  6 days) in ovariectomized FF and ++ Booroola ewes. The major finding is that the FF and ++ genotypes respond in a similar manner to each of the above substances. That is, no overall genotype differences were observed even though there were significant effects of the steroids or bFF with respect to dose. Thus the high ovulation rates in Booroola FF ewes compared to ++ ewes are unlikely to be related to gene-specific differences in the sensitivity of the neurohypophysis to ovarian hormones.

Implantation of oestradiol or of progesterone caused a rapid lowering of plasma LH concentrations after 2–5 h coincident with a measurable increase in the plasma concentrations of oestradiol or progesterone. Implantation of oestradiol but not progesterone provoked the release of LH to peak concentrations some 10 h later. The timing of this 'surge' and the peak characteristics (i.e. area and height) revealed no differences between the genotypes. These findings are consistent with those reported by Bindon *et al.* (1984) who showed that the timing of the preovulatory LH surge and LH peak concentrations were similar in Booroola and control Merino ewes, notwithstanding their different ovulation rates. Although implantation of progesterone did not provoke an LH 'surge' there was at least a partial recovery of the acute suppressive effects of progesterone some 20 h later; this was much more obvious after the implantation of 1 progesterone envelope than with 3 envelopes. From 30 to 50 h after progesterone implantation, the plasma LH concentrations, although fluctuating, had stabilized to some extent but were significantly lower overall than the untreated controls ( $P < 0.01$ ; Fig. 4). The lower but variable plasma LH concentrations are probably the result of the exogenous progesterone reducing LH peak frequency without altering LH peak amplitude (Table 1). In contrast, the lower and much less variable LH concentrations 30–50 h after oestradiol implantation are probably due to the oestradiol inhibiting LH peak amplitude as well as LH peak frequency (Table 1; Martin, 1984). Although there was no overall effect on genotype, there was a divergence in the mean LH concentrations between the genotypes at 30–50 h after implantation of the 8-cm but not the 2-cm oestradiol capsules. The higher mean values in the FF genotype were due to 2/5 animals showing a level of LH suppression of ~90% of the pretreatment mean whereas the remaining 3 animals displayed a level of suppression similar to that in the ++ genotype (i.e. 35% of the pretreatment mean). When the LH concentrations in these 5 animals were examined 6 days after implantation (Fig. 3), the above-mentioned discrepancy had completely disappeared. It seems unlikely that the apparent but not statistically significant difference at 30–50 h after implantation has any physiological significance.

Implantation of oestradiol caused a similar overall pattern of suppression for FSH as that observed for LH. However, there was a slower rate of suppression of FSH compared to LH, and the magnitude and duration of the subsequent FSH 'surge' were both smaller than were observed for LH. Implantation of progesterone had no significant suppressive effect on the plasma concentrations of FSH. This finding supports those of Hamernik *et al.* (1987) and Martin *et al.* (1988) and suggests that at concentrations  $\leq 3$  ng/ml, progesterone has no influence on FSH secretion in female sheep. Again, there were no significant gene-specific differences.

The effects after 6 days of steroid implantation were generally similar to those observed after 30–50 h. The major difference between these times was that the plasma concentrations of LH after oestradiol or progesterone implantation or FSH after oestradiol implantation were lower after 6 days than after 30–50 h (see text and Figs 1–4). Nevertheless, the longer exposure to progesterone or oestradiol did not unmask any gene-specific differences, in tonic LH and FSH secretion or in the pulsatile characteristics of LH secretion.

Bovine follicular fluid at all doses between 0.5 and 5 ml caused a significant suppression of FSH concentrations at 6–12 h and also at 18–24 h after treatment. Moreover, ewes of the FF and ++ genotypes responded similarly, in contrast to the data of Cummins *et al.* (1983) who reported that the plasma FSH concentrations were more readily suppressed in Booroola Merino ewes compared to those found in control Merino ewes (not of the Booroola strain) during the first day of treatment with charcoal-treated ovine follicular fluid. However, these data may not be related to the F gene. With regard to plasma LH concentrations, high doses of bFF (i.e. 2.5 ml and 5 ml per injection) also caused a significant suppression, but, as with FSH, no F gene-specific effects were observed.

In the present study most groups of FF animals had slightly higher mean hourly gonadotrophin concentrations than did those of the ++ genotype before steroid or bFF treatment but they were only significantly higher on one occasion (FSH in Exp. 3). This, at first glance, seems inconsistent with the results of previous studies (McNatty *et al.*, 1989) in which mean daily FSH and LH concentrations were found overall to be significantly higher in FF than in ++ ovariectomized

ewes. However, in these animals the genotypic differences were obvious only in the frequency of higher FSH and LH values in FF compared to ++ ewes over a long (i.e. 30-day) sampling period. The present experiments, which were designed to detect differences in gonadotrophin concentrations over relatively short periods of time, are therefore inappropriate for testing subtle differences which might become apparent in long-term sampling. Nevertheless, the present study has demonstrated the absence of any measurable genotypic differences in the positive and/or negative feedback effects to ovarian hormones at the level that can be observed between Finnish Landrace and Scottish Blackface ewes (Webb *et al.*, 1985a). Although Booroola FF ewes are more likely to have a higher frequency of elevated FSH and LH concentrations compared to ++ ewes (McNatty *et al.*, 1987, 1989), as is the case for Finnish Landrace compared to Scottish Blackface ewes (Webb *et al.*, 1985a), the mechanisms which underlie these differences may not be the same.

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## THE PHYSIOLOGY OF THE BOORoola EWE

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### INTRODUCTION

Booroola ewes with the fecundity (F) gene are characterized by their higher ovulation rate (OR) relative to ewes without this gene (Bindon et al., 1982; Davis et al., 1982). The mechanism as to how the F gene influences OR remains unknown. Studies with Booroola ewes which were homozygous (FF) or heterozygous (F+) and non-carriers (++) have demonstrated differences attributable to the F gene both before and after puberty in ovarian follicular development, the pituitary hormone concentrations in plasma and in the pituitary response to exogenous gonadotrophin-releasing hormone (GnRH) (Tassell et al., 1983; Bindon, 1984; McNatty & Henderson, 1988; Braw-Tal & Gootwine, 1989). In contrast to the ewe, there are no obvious physical characteristics which distinguish Booroola rams of the different genotypes. Until recently no endocrine or biochemical differences with respect to F gene expression have been noted (Bindon et al., 1985). However, Price et al. (1990) have found differences in pituitary hormone concentrations in plasma and also in the pituitary response to GnRH.

The purpose of this review is to summarize recent Australian and New Zealand (N.Z.) research on hypothalamic-pituitary and ovarian function in Booroola ewes. Also summarized here are the recent Australian studies on Booroola rams, whereas the comparable N.Z. studies are reported elsewhere in these proceedings by Price et al. (1990).

### ANIMALS

In N.Z. only two sources of animals have been used for physiological studies, namely the Booroola Merino and the Booroola Merino x Romney, with most animals being between 5 and 9 years old. All animals together with information

about their pedigrees, lifetime OR, etc. were supplied by George Davis, Kaye Isaacs and their colleagues at the Invermay Agricultural Research Centre, Mosgiel, N.Z. Most of the FF and ++ ewes were generated from 28 FF sires and 31 ++ sires respectively. For the study on the hypothalamic-pituitary disconnected sheep the FF (n=21) and ++ (n=21) ewes were generated from 16 and 12 FF and ++ sires respectively. The FF ewes were on average 1.5 kg lighter than the ++ ewes but this difference in liveweight never reached statistical significance in any of the studies due to large variation between individual ewes.

Australian studies are based on mixed-age Booroola Merino genotypes (FF and F+) drawn from the CSIRO selection flock where eight new FF or F+ sires are used each year. Ewes carrying the F gene have been compared with random bred (Control) Peppin Merinos run in the same environment as the Booroola flock since 1974. Booroola gene carriers have also been compared with ewes from the CSIRO 'T' Merino flock, selected for litter size since 1954 (Turner, 1978) and maintained in the same environment as the Booroola flock since 1959.

Booroola ram investigations reported here are based on progeny of Border Leicester rams and either Booroola Merino ewes of known F genotype (i.e., FF, F+ or ++) or Control Merino ewes.

#### THE HYPOTHALAMUS: IMMUNOREACTIVE GnRH CONCENTRATIONS AND PORTAL SECRETORY CHARACTERISTICS

The immunoreactive GnRH concentrations in various hypothalamic and extrahypothalamic areas of the brains from intact and ovariectomized FF, F+ and ++ Booroola Merino ewes have been investigated by Gale et al. (1988). The antiserum used to measure GnRH (CRR 11B73; supplied by Dr. Y.F. Chen and Dr. V.D. Ramirez, University of Illinois, IL, U.S.A.) requires the complete decapeptide sequence for binding although CRR 11B73 does not distinguish the decapeptide from GnRH prohormone molecules. In both ovary intact and ovariectomized (OVX;  $\geq 5$  months previously) Booroola ewes, the highest concentrations of GnRH were found in the median eminence (ME; Table 1);

Table 1. GnRH concentrations (pg/mg tissue)\* in the median eminence of intact and ovariectomized (OVX) ++, F+ and FF Booroola ewes

Ewes	Booroola Genotype		
	++	F+	FF
Intact	1049 $\pm$ 140 (18)	1138 $\pm$ 178 (14)	892 $\pm$ 110 (9)
OVX	1686 $\pm$ 228** (12)	1908 $\pm$ 354 (9)	1535 $\pm$ 304 (11)

\*Values are means + s.e.m.. Number of ewes per genotype is indicated in square brackets.

\*\*p < 0.05 when compared with intact animals of the same genotype (Wilcoxon test). Data from Gale et al. (1988).

substantially less immunoreactive GnRH was found in the medial basal hypothalamus (i.e., 13-75 pg GnRH/mg tissue), preoptic area (POA; 0.2-8.0 pg GnRH/mg tissue) and organum vasculosum of the lamina terminalis/supraoptic nuclei region (<0.1-19.1 pg GnRH/mg tissue) with only low concentrations (<6 pg GnRH/mg tissue) being found in the septal nuclei, frontal cortex, pineal or pituitary gland. In OVX Booroola ewes the GnRH concentrations in the ME were approximately twice those in intact ewes whereas in the POA they were barely measurable. In all instances no differences were noted, between F genotypes. However, studies on rates of pro-GnRH synthesis are needed before this source of F gene difference can be discounted.

Preliminary information on the GnRH secretory characteristics in hypophyseal portal blood of OVX Booroola Merino or Romney ewes (n=6 ewes/genotype) has been obtained. Portal blood was collected every 2.5, 5.0 or 10 min for 7 h from conscious ewes by the method of Clarke & Cummins (1982). The GnRH immunoassay characteristics and GnRH peak detection methodology were similar to those described by Li *et al.* (1989). The results of these studies are summarized in Table 2.

Table 2. GnRH secretory characteristics in portal blood of OVX ++ and FF Booroola ewes (n=6 ewes/genotype). Values are means s.e.m.

Genotype	GnRH Secretory Characteristics			
	Concentration (pg/ml)	Secretion rate (pg/min)	GnRH pulses per 7 h	Peak amplitude (pg/ml)
++	1.2 ± 0.2	0.6 ± 0.1	13.0 ± 2.0	3.1 ± 1.3
FF	1.2 ± 0.1	0.8 ± 0.1	12.6 ± 0.5	2.9 ± 0.5

In summary, no evidence has been found for differences in GnRH concentration in any brain areas measured that can be attributed to the F gene. Moreover, preliminary evidence (Table 2) suggests that the GnRH secretory characteristics in the different genotypes are similar, although firm conclusions must await appropriate studies of intact, rather than ovariectomized animals.

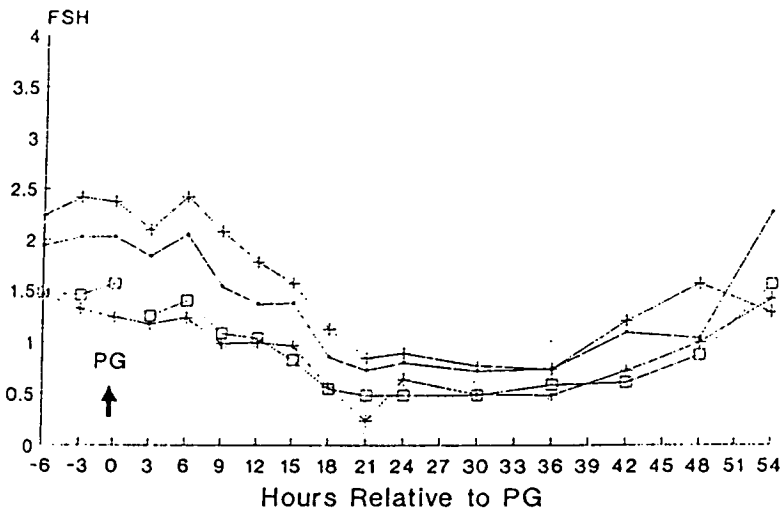
#### THE PITUITARY:

##### Gonadotrophin concentrations in plasma and pituitary tissue

Intact ewes carrying the F gene have higher plasma concentrations of FSH than non-carriers during the normal oestrous cycle and anoestrus (Bindon, 1984; McNatty *et al.*, 1987a). This has been confirmed in recent Australian studies following prostaglandin (PG) induced luteolysis (Fig. 1). The intact Booroola ewe is also characterized by elevated FSH content and concentration in pituitary tissue (Robertson *et al.*, 1984). In Booroola ewe lambs plasma FSH but not LH is significantly higher than in lambs without the F gene (Bindon *et al.*, 1985b; Braw-Tal & Gootwine, 1989; Montgomery *et al.*, 1989).



Fig. 1. Mean plasma FSH concentrations (ng/ml NIAMDD-oFSH-RP1) in Booroola FF (+; n=20), Booroola F+ (■ n=12), Control Merino (\* n=12) and CSIRO 'T' Merino (□ n=12) ewes studied at 3-hr intervals from 6 hr before PG injection to 24 hr after PG injection and then at 6-hr intervals until 54 hr after PG. The onset of oestrus occurred at  $54 \pm 4$  hr after PG injection.



Following ovariectomy of adult ewes in N.Z. there remain F-specific differences in plasma FSH and LH (McNatty et al., 1989a). Comparable studies in Australia have not confirmed this point. Table 3 shows one of two recent investigations by B.M. Bindon and M.A. Hillard (unpublished) which show that the plasma FSH concentrations following ovariectomy were similar in Booroola, Control Merino and 'T' Merino genotypes. Even as late as 100 days after castration (Table 3) FF and F+ ewes had similar plasma FSH (and LH) concentrations and these were indistinguishable from the other two Merino strains examined. In this study additional plasma FSH and LH concentrations were measured at 4 x 1-hourly intervals on each of days 99, 100 and 101 following ovariectomy to look for subtle differences in the means and variances between the four genotypes. There were no consistent differences between the genotypes. Overall mean FSH for the 14 FF ewes (i.e., over the 12 sampling times) was  $13.2 \pm 0.7$  ng/ml compared to  $12.6 \pm 0.9$  ng/ml for the 10 Control Merinos. This difference is not significant, but further analyses of the complete data set may be worthwhile.

In the N.Z. study, 30 samples were obtained at 2-day intervals from ovariectomy and the mean FSH values were significantly higher in FF compared to ++ ewes on 3/30 sampling days. However, the overall mean for the FF ewes (i.e., over all sampling times) was different from those for the ++ ewes ( $p < 0.02$ ). Partial autocorrelation analyses for each ewe over time revealed that most contributed to the greater frequency of higher FSH values in FF compared to ++ ewes, with no sheep or sire effects being noted. The inconsistency between the Australian and N.Z. FSH data in OVX ewes may be resolved by repeating the investigations in a third environment, such as France.

Table 3. Postcastration changes (means  $\pm$  s.e.m.) in plasma FSH (NIAMDD-oFSH-RP1; ng/ml) and LH (NIH-LH-S23; ng/ml) in Booroola, Control and 'T' Merino genotypes (B.M. Bindon and M.A. Hillard, unpublished)

Genotype	N	Time after ovariectomy (days)							
		0*		8		22		100	
		FSH	LH	FSH	LH	FSH	LH	FSH	LH
Booroola FF	14	3.1 $\pm 0.3$	1.6 $\pm 0.4$	7.6 $\pm 0.4$	4.0 $\pm 0.4$	7.6 $\pm 0.4$	3.2 $\pm 0.$	12.8 $\pm 0.5$	7.1 $\pm 0.9$
Booroola F+	7	2.9 $\pm 0.3$	1.4 $\pm 0.2$	7.9 $\pm 0.7$	4.9 $\pm 1.$	7.9 $\pm 0.6$	5.0 $\pm 1.3$	13.9 $\pm 2.5$	8.0 $\pm 2.1$
Control	10	2.8 $\pm 0.3$	1.3 $\pm 0.3$	7.7 $\pm 0.4$	3.9 $\pm 0.4$	7.0 $\pm 0.9$	2.8 $\pm 0.$	14.2 $\pm 1.3$	6.8 $\pm 1.2$
'T' Merino	10	2.3 $\pm 0.2$	1.0 $\pm 0.1$	9.2 $\pm 0.6$	5.0 $\pm 0.8$	8.0 $\pm 0.6$	3.5 $\pm 0.6$	12.8 $\pm 1.4$	5.9 $\pm 1.1$

Ewes ovariectomized on Days 10-12 of the oestrous cycle.

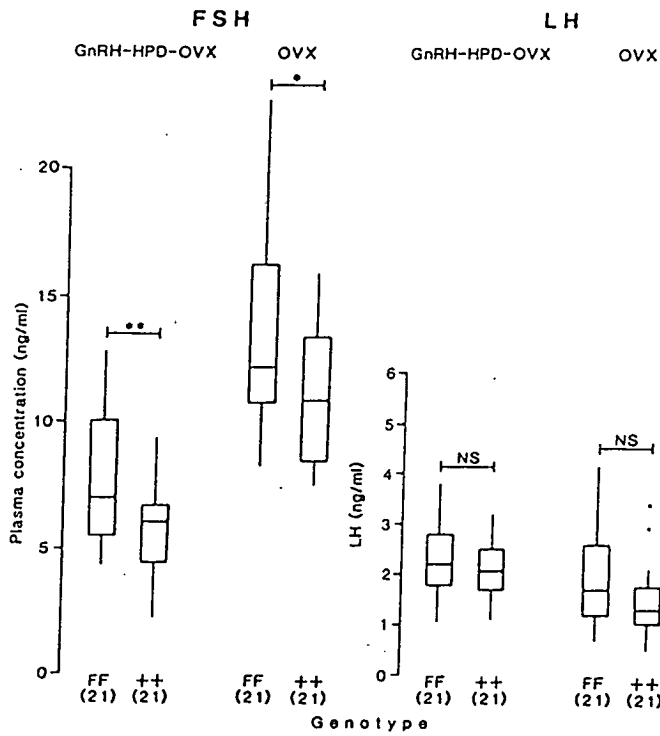
#### GnRH receptor binding

F gene effects on plasma gonadotrophin concentrations raised the possibility that the gene may act via differences in GnRH receptor binding characteristics. Recent studies of pituitary glands of both intact or OVX ewes (Fleming *et al.*, 1990) found a single class of GnRH binding sites. There were no F-specific differences in either the equilibrium association constant ( $K_a$ ) or maximal binding capability ( $B_{max}$ ), despite significant differences between intact and OVX ewes (FF versus + +) in plasma FSH concentration (Table 4).

#### GnRH-induced gonadotrophin secretion in hypothalamic-pituitary disconnected ovariectomized (HPD-OVX) Booroola ewes

The HPD-OVX Booroola ewe prepared as described by Clarke *et al.* (1983; 1984) is an experimental animal deficient in endogenous GnRH secretion and devoid of ovarian hormones but having a functionally intact pituitary gland: the plasma FSH and LH concentrations in HPD-OVX ewes are < 10% of those in OVX ewes with no gene differences in the residual plasma concentrations of pituitary hormones even after months of sampling. Using this model it is possible to test whether the differences in plasma FSH and LH concentrations due to the F gene in OVX Booroola ewes (McNatty *et al.*, 1989a) result from differences in the pituitary responsiveness to exogenous GnRH. The results of the HPD-OVX study are summarized in Fig. 2. Also included in Fig. 2 is a summary of the mean FSH and LH data for OVX ewes collected over the same time course as for the GnRH-HPD-OVX animals. These results are interpreted to show that the FSH but not LH concentrations in both the GnRH-HPD-OVX and OVX FF ewes were significantly higher than those in + + ewes ( $p < 0.01$  for GnRH-HPD-OVX ewes;  $p < 0.05$  for OVX ewes). No F gene effects were noted in pituitary weights or pituitary FSH or LH contents in either the HPD-OVX or OVX ewes.

Fig. 2. Box and Whisker plots of mean FSH and LH plasma concentrations from hypothalamic pituitary-disconnected OVX Booroola FF and ++ ewes administered with 250 ng GnRH in saline + 0.1% ovine serum albumin (iv) once every two hours for 36 days (i.e., GnRH-HPD ewes) or from a different flock of OVX Booroola FF and ++ ewes. Blood samples were collected every 2nd or 3rd day after OVX or from the onset of GnRH treatment for 36 days and one overall mean value for FSH and LH from each ewe obtained. \*\* $p < 0.01$ , \* $p < 0.05$ , NS = not significantly different (2 sample t-test). In the GnRH-HPD-OVX ewes the mean ( $\pm$  s.e.m.) FSH/LH concentrations before GnRH treatment were  $1.0(\pm 0.1)/0.4(\pm 0.04)$  and  $1.1(\pm 0.1)/0.5(\pm 0.1)$  ng/ml respectively for FF (n=21) and ++ ewes (n=21). In the OVX ewes the mean ( $\pm$  s.e.m.) FSH/LH concentrations before OVX were  $1.8(\pm 0.1)/0.3(\pm 0.1)$  and  $1.2(\pm 0.1)/0.3(\pm 0.1)$  ng/ml respectively for FF (n=21) and ++ ewes (n=21); the FSH but not LH concentrations were significantly higher in the FF compared to ++ ewes ( $p < 0.01$ ).



Overall the FSH but not LH concentrations were lower in GnRH-HPD-OVX compared to those in the OVX ewes. These results when taken together with those in Table 4 suggest that Booroola ewes with the F gene are more responsive to GnRH with respect to FSH secretion, but that this difference in responsiveness is not expressed at the level of the GnRH receptor. Importantly, this difference in responsiveness to GnRH is expressed only in the absence of ovarian hormones.

Table 4. Plasma FSH and LH concentrations (ng/ml) and equilibrium association constants ( $K_a$ , M<sup>-1</sup>) and maximal binding capacity ( $B_{max}$ , fmol/mg pituitary) for the binding of [<sup>125</sup>I]-D-Ala<sup>6</sup>-GnRH-ethylamide to pituitary receptors in intact and OVX homozygous (FF) or non-carriers (+ +) of the F gene

Reproductive status †	Booroola genotype	FSH	LH	$K_a$	$B_{max}$	n
Intact	FF	1.6 ± 0.1**	0.2 ± 0.05	1.8 ± 0.2	0.10 ± 0.02	18
	+ +	1.2 ± 0.1	0.2 ± 0.04	1.5 ± 0.1	0.14 ± 0.02	20
OVX	FF	22.3 ± 2.9*	2.5 ± 0.4	2.0 ± 0.3	0.08 ± 0.01	15
	+ +	14.9 ± 1.8	1.7 ± 0.3	2.1 ± 0.5	0.07 ± 0.01	14

† The pituitary glands from the intact ewes were recovered on either Day 4 or 12 of the oestrous cycle.

\*\*  $p < 0.01$  and \*  $p < 0.05$  with respect to + + intact and ovariectomized ewes respectively. All other comparisons for intact and OVX ewes separately were not significantly different. Data from Fleming *et al.* (1990).

## THE OVARY:

### Ovarian Morphology

Before puberty quantitative as well as qualitative differences have been noted in the ovarian composition (i.e., in the amounts of stromal and follicular tissue) and in antral follicle populations between F gene carriers and + + ewes or similarly aged non-Booroola ewes (Tassell *et al.*, 1983; McNatty *et al.*, 1987b; Braw-Tal & Gootwine, 1989). However, it is still not clear whether these differences are due to the F gene, litter size or breed. Nevertheless, in sexually mature Booroola ewes, a consistent feature is that the total antral follicle populations and proportions of non-atretic follicles are similar but that the ovarian follicles ovulate at a significantly smaller diameter in FF and F+ ewes than do those in + + ewes (Driancourt *et al.*, 1985; McNatty *et al.*, 1985a, 1986a). In FF ewes, the largest oestrogen-enriched, nonatretic follicles (i.e., presumptive preovulatory follicles; McNatty, 1982) have diameters of 3-4.5 mm whereas in + + ewes such follicles have diameters exceeding 5 mm, with those for F+ ewes being in between. A second important feature is that there are fewer granulosa cells present in nonatretic antral follicles from the FF or F+ genotype compared to similar sized follicles from + + ewes: these characteristics are evident some 68-70 days after hypophysectomy, at different times during the oestrous cycle and during anoestrus (McNatty *et al.*, 1986a, 1987a and unpublished data). This difference in the population of granulosa cells and/or in the size of the largest nonatretic follicle also occurs before puberty (McNatty *et al.*, 1987b).

In FF, F+ and + + Booroola ewes based on classifications of  $\geq 5$ , 3 or 4 and 1-2 ovulations respectively, the total number of granulosa cells in the oestrogen-enriched follicles per animal was found to be similar for each genotype (i.e.,  $5.4 \times 10^6$ ) as were the ovarian steroid (i.e., androstenedione, testosterone and oestradiol) secretion rates (McNatty and Henderson, 1987). After ovulation on Days 4, 10 and 12 of the oestrous cycle, the plasma progesterone concentrations, total luteal weight and cell composition in FF and + + ewes were similar notwithstanding the difference in OR (Bindon, 1984;

Niswender et al., 1990; ++ ewes, mean OR = 1.6; FF ewes, mean OR = 5.5). Thus despite marked differences in ovarian morphology the hypothalamus and pituitary appears not to be exposed to any different steroid milieu in different Booroola genotypes.

In the FF and F+ ewes, the preovulatory follicles and corpora lutea (CL) are of similar diameter and likewise the CL are of similar diameter and/or weight (McNatty et al., 1986a; Table 5). In FF ewes, no significant change in mean CL weight was noted as OR increased from 3-5 to >5 although greater variability in the weights of individual CL was apparent. In contrast to Booroola FF ewes, there was a wide range in the diameters of the presumptive preovulatory follicles, higher concentrations of plasma progesterone, higher mean CL weights and greater variation in the weights of individual CL of superovulated Romney or Booroola ++ ewes (Kelly et al., 1983; Henderson et al., 1988; Table 5).

Table 5. Mean weight of individual corpora lutea (CL) per ewe and mean coefficient of variability (CV) of CL weight per ewe with respect to OR in Booroola ++ and FF ewes and in superovulated Romney ewes

OR	Booroola genotype ++		FF		Superovulated Romney ewes	
	Mean CL weight / ewe (g)	Mean CV of CL weight / ewe (%)	Mean CL weight / ewe (g)	Mean CV of CL weight / ewe (%)	Mean CL weight / ewe (g)	Mean CV of CL weight / ewe (%)
2	0.5 ± 0.02 (16)	11 ± 2	-	-	0.55 ± 0.01a (46)	15 ± 1a
3-5	-	-	0.18 ± 0.02a (14)	10 ± 1b	0.41 ± 0.02b (11)	29 ± 3b
>5	-	-	0.14 ± 0.01a (10)	21 ± 5c	0.34 ± 0.01c (13)	27 ± 3b

Values are means + s.e.m.; ( ) = number of ewes; values in columns with different alphabetical superscripts are significantly different ( $p < 0.05$ ; ANOVA). CL from all animals recovered between days 8-12 of the oestrous cycle (unpublished data from K.P. McNatty & K.M. Henderson).

#### Gonadotrophin responsiveness, cAMP synthesis and catabolism

Ovarian follicles in F gene carriers either mature earlier or develop an earlier sensitivity to FSH (or LH) with regard to cAMP synthesis (Henderson et al., 1985, 1987). Table 6 shows that the Booroola F gene influences basal cAMP content and the cAMP response to gonadotrophins from the earliest phases of antral follicle growth (i.e., from 0.11 mm diameter). However, there is no evidence from studies on larger diameter follicles (i.e., > 1 mm diameter) to suggest that the aforementioned differences in cAMP content are due to F-specific differences in FSH or LH receptor binding characteristics (i.e., Bmax or Kd), in granulosa cells or LH receptor binding characteristics or in theca interna or luteal cells (McNatty et al., 1986b, 1989c). The only differences worth noting here are that the binding characteristics of FSH to granulosa cells remain unchanged throughout antral follicle growth but that LH receptors are acquired

at smaller follicle diameters (i.e., < 5 mm) in FF or F+ ewes than in ++ ewes (i.e.,  $\geq 5$  mm diameter) (Fig. 3). The latter observation is not unexpected given that preovulatory follicles in F+ or FF ewes rarely exceed 4.5 mm diameter.

Table 6. Cyclic AMP content (pmol) in follicular tissue at zero time and after incubation with LH and FSH (both 1  $\mu\text{g/ml}$ ) for 1 h with respect to Booroola genotype and follicular diameter

Follicle diam. (mm)	Time (h)	Genotype		
		++	F+	FF
0.13-0.52 (n)	0	0.13[0.09,0.16]a (50)	0.17[0.15,0.20]b (50)	0.20[0.16,0.24]b (34)
	1	0.14[0.12,0.16]b (114)	0.26[0.39,0.57]b (112)	0.35[0.23,0.48]c (62)
0.53-1.00 (n)	0	0.40[0.31,0.51]b (39)	0.48[0.39,0.57]b (42)	0.40[0.32,0.49]b (32)
	1	0.32[0.26,0.39]b (86)	0.80[0.67,0.95]c (112)	1.14[0.90,1.41]d (73)

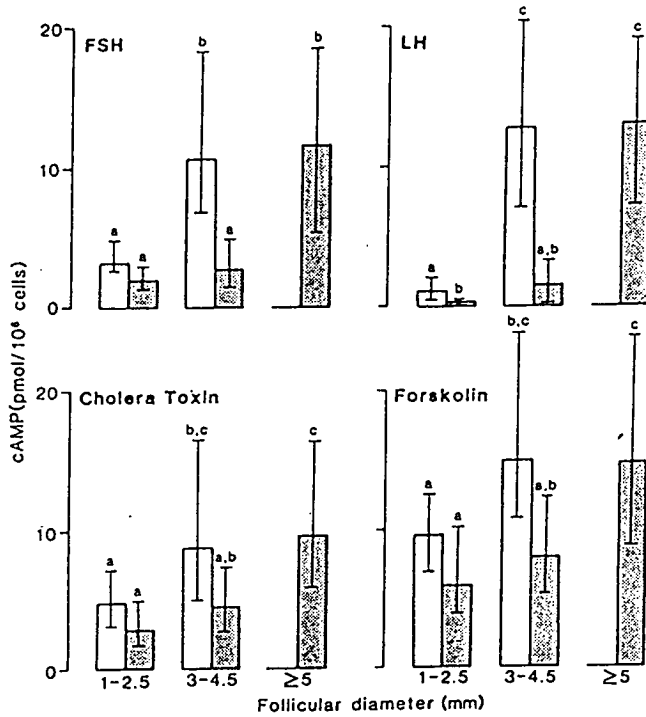
Values are geometric means [and 95% confidence limits], n = number of follicles. For each follicular diameter, the values in rows or columns having a different superscript are significantly different from one another ( $p < 0.025$ ). From McNatty *et al.* (1985b)

If it is assumed that 1-2.5 mm and 3-4.5 mm diameter follicles in FF ewes are respectively at similar phases of maturation to 3-4.5 mm and > 5 mm diameter follicles in ++ ewes, no significant differences were found at any dose of FSH, cholera toxin, forskolin, forskolin + cholera toxin, pertussis toxin or LH with respect to granulosa cell cAMP synthesis (Fig. 3; McNatty *et al.*, 1990). Moreover, no F gene effects were noted with respect to the rates of cAMP synthesis (McNatty *et al.*, 1990), or cAMP catabolism when assessed on a per cell basis (McNatty *et al.*, 1989d, 1990). Thus there is no evidence to suggest that the F gene has any direct influence on LH or FSH receptors or on the gonadotrophin, cholera toxin, forskolin or pertussis toxin sensitive components of the cellular cAMP generating system.

#### Ovarian sensitivity to exogenous gonadotrophins in vivo

If the F gene alters the responsiveness of individual follicles to gonadotrophins this should be reflected in altered slope or elevation of ovulatory dose responses to exogenous FSH in Booroola ewes. This has been investigated by Bindon *et al.* (1987) (see Table 7). These data show that Booroola FF and F+ ovaries were not more responsive to FSH of varying purity compared to Control Merino ovaries. Indeed the converse tended to be true. Comparable studies with pregnant mare's serum gonadotrophin (PMSG) led to the conclusion that the F gene caused increased sensitivity to this gonadotrophin (Kelly *et al.*, 1983; Piper *et al.*, 1982). It is difficult to interpret these results in relation to Table 7, because PMSG is a complex hormone and is not simply a mixture of FSH and LH.

Fig. 3. Effects of FSH (1 mg/ml), cholera toxin (0.5 µg/ml), forskolin (10<sup>-4</sup> M) and LH (1 µg/ml) on granulosa cell cAMP synthesis with respect to Booroola genotype (n=10 ewes/genotype). White histograms = cells from FF ewes, hatched histograms = cells from ++ ewes. The granulosa cells were recovered from nonatretic follicles (see McNatty et al., 1986a for classification system). Values are geometric means and vertical lines the 95% confidence limits. The incubation time was 45 min. For each treatment (i.e., FSH, cholera toxin etc.), the cAMP values not sharing a common alphabetical superscript indicate a significant difference (p<0.05; paired or multiple t-tests on loge-transformed data to equalize variances) between follicles of different size and/or genotype.



**Steroid synthesis**

During antrum formation (i.e., 0.1-0.2 mm diam.) sheep follicles have the capacity to synthesize progesterone and androstenedione. In larger follicles (i.e., between 0.3-0.5 mm diam.) they acquire the capacity to synthesize testosterone and oestradiol-17β (McNatty and Henderson, 1987). Thus when follicles develop beyond 0.5 mm diameter some are capable of synthesizing all the above steroids in vitro (McNatty et al., 1985b). In Booroola ewes, significantly higher proportions of follicles in FF and F+ compared to ++ ewes

were capable of synthesizing steroids (McNatty *et al.*, 1985b). This is consistent with the presence of higher cAMP contents and maturation at smaller follicular diameters. In larger follicles (i.e.,  $\geq 1$  mm diameter) granulosa cells were identified as the sole source of follicular oestradiol and the ability of these cells to aromatize androgens to oestradiol reached peak activity in 3-4.5 mm, 3-5.5 mm and  $\geq 5$  mm diameter follicles in the FF, F+ and ++ genotypes respectively (McNatty *et al.*, 1986a).

Table 7. Genotype effects on ovulation response to three different FSH preparations

Hormone	Potency units/mg		Sheep genotype*	FSH dose level**			
	FSH †	LH †		0	1	2	3
FSH-P (BurnsBiotech)	2.1	0.05	Booroola	3.4 ± 1.0	3.6 ± 1.0	4.5 ± 1.0	11.6 ± 1.0
			Control	0.8 ± 1.1	1.0 ± 1.1	3.5 ± 1.1	9.5 ± 1.1
FSH-ovine (Heriot)	4.5	0.009	Booroola	3.4 ± 1.2	3.3 ± 1.2	3.8 ± 1.0	3.3 ± 1.1
			Control	0.8 ± 1.1	1.5 ± 1.0	1.2 ± 1.0	5.6 ± 1.1
FSH-porcine (Heriot)	2.3	0.002	Booroola	2.9 ± 1.2	3.5 ± 1.3	3.6 ± 1.3	7.9 ± 1.3
			Control	1.5 ± 1.1	1.8 ± 1.1	4.3 ± 1.1	7.8 ± 1.1

Data from Bindon *et al.* (1987).

- \* Booroola = FF/F+; Control = Merino
- \*\* FSH-P dose level 1 = 4 mg; 2 = 8 mg; 3 = 16 mg given as 8 injections over 4 days.  
FSH-ovine 1 = 2; 2 = 4; 3 = 8 mg given as 8 injections over 4 days.  
FSH-porcine 1 = 6; 2 = 12; 3 = 24 mg given as 8 injections over 4 days.
- † Radioreceptor assay potency of FSH (units NIH-FSH-S1 per mg) and LH (units NIH-LH-S23 per mg).

Previous studies in Booroola ewes also identified the theca interna as the major source of androstenedione and to a lesser extent of testosterone and progesterone and that the synthesis of these steroids was influenced by LH (McNatty *et al.*, 1986a). However no F-specific differences were noted in LH-induced cAMP or steroid synthesis when the results were expressed on a per unit weight basis. Also no differences were found in the follicular fluid concentrations of progesterone or androgens suggesting that the theca interna is functionally similar in all Booroola genotypes (McNatty *et al.*, 1986a).

#### Inhibin in ovarian cytosol and follicular fluid

Bio-active inhibin (Scott *et al.*, 1980) has been measured on two occasions in ovarian cytosol from Booroola (FF and F+) and Control Merinos (Table 8). The Booroola ovary clearly contains less inhibin. In more recent studies, both bio-active and immunoactive inhibin concentrations have been measured in pools of follicular fluid from the ovaries of Booroola (FF/F+), Control and 'T' Merinos. The results in Table 9 suggest no difference between genotypes in mean inhibin concentrations in follicular fluid, but the significantly reduced follicular fluid volumes in the Booroola ovary would still account for a lower ovarian immunoactive inhibin content in this genotype. Both Booroola and Control Merinos had significantly lower ( $p < 0.05$ ) mean bio-active inhibin



concentrations in follicular fluid than 'T' Merinos. It may be that 'T' ewes have a qualitatively different inhibin in the ovary.

These data would suggest that the lower total ovarian bio-active inhibin content of Booroola ewes (Table 8) cannot be explained by the average bio-inhibin concentrations in follicular fluid. Presumably it is due to the presence of smaller follicles in FF/F+ animals compared to controls (Bindon, 1984; McNatty et al., 1986a).

**Table 8.** Estimates of bio-active inhibin content of ovaries from Booroola and Control Merinos

Contrast	Genotype	No. of ewes	No. of ovarian cytosol pools assayed	Mean $\pm$ s.e.m. inhibin content (units/ovary)	Reference
Ewe aged 6 years, mid-luteal phase	Booroola	10	10	400 $\pm$ 60*	Cummins et al., 1983
	Control	10	10	1230 $\pm$ 130	
Ewes aged 2-8 years, unknown day of cycle; ovaries pooled within genotype	Booroola	71	1	830	Cummins, 1984
	Control	63	1	1630	

\* Difference between Booroola and Control significant ( $p < 0.001$ ).

**Table 9.** Inhibin bio- and immunoactivity in pools of follicular fluid from Booroola, Control Merino and CSIRO 'T' Merino ewes on Day 3 of the oestrous cycle (from B.M. Bindon, J.K. Findlay and T. O'Shea, unpublished)

Genotype	Mean volume follicular fluid per ovary (ml)	Inhibin concentration		Ovarian inhibin content (x103)	
		RIA* (ng/ml)	Bioassay** (U/ml)	RIA (ng)	Bioassay (U)
Booroola (FF/F+)	25	21346	12400 (9000-13800)	533	310
Control Merino	67	19158	8260 (5680-11900)	1283	553
CSIRO 'T' Merino	77	20112	17700 (12600-24300)	1548	1363

\* ng bovine 32 kD inhibin/ml (see Findlay et al., 1990).

\*\* U = 1 unit of ovine testicular lymph inhibin standard per ml. There were 175, 174 and 75 ovaries sampled from Booroola (FF/F+), Control Merino and CSIRO 'T' Merinos respectively to obtain one pool of follicular fluid from each for assay. Range of values in inhibin bioassay refer to 95% confidence limits.

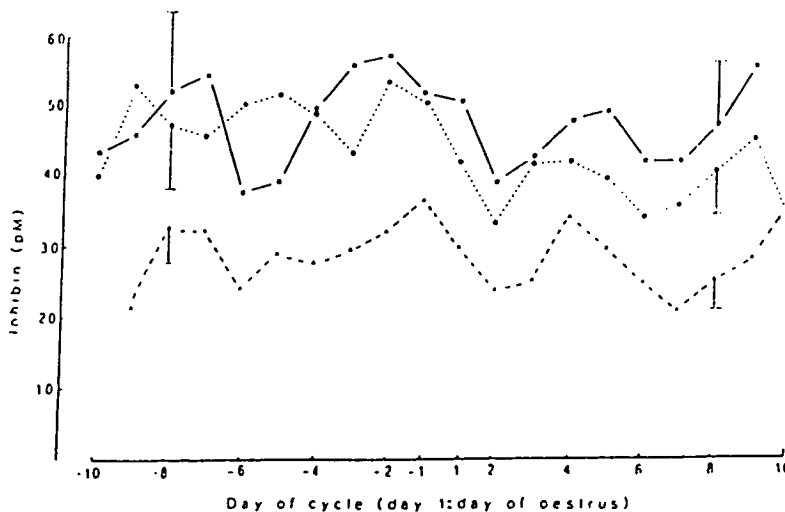
In N.Z. studies *in vitro* inhibin synthesis by granulosa cells from Booroola FF and ++ ewes has been assessed using a sheep pituitary cell bioassay (Henderson et al., 1989) and the WHO/NIH Inhibin Reference Standard 86/690. Serial dilutions of granulosa cell incubation media from FF or ++ genotypes generated FSH dose response lines which were parallel with one another and also with the WHO/NIH Inhibin Standard. Inhibin bioactivity was detectable after a 24-h culture of granulosa cells recovered from FF follicles  $\geq 1$  mm

diameter and from ++ follicles  $\geq 3$  mm diameter. Cells from FF follicles 3-4.5 mm diameter ( $n=7$ ) produced similar levels of inhibin as did cells from ++ follicles  $\geq 5$  mm diameter ( $n=11$ ). Irrespective of genotype the inhibin output (i.e., geometric mean (and 95% confidence limits)) was 4.9 (3.1, 10.1) units/106 cells in these follicles. Likewise cells from FF follicles 1-2.5 mm diameter ( $n=13$ ) produced similar levels of inhibin to that of cells from ++ follicles, 3-4.5 mm diameter ( $n=13$ ) with the overall inhibin output being 0.8 (0.5, 1.3) units/106 cells. The inhibin outputs in either 1-2.5 mm diameter FF and/or 3-4.5 mm diameter ++ follicles were significantly lower than in the correspondingly larger FF and ++ follicles respectively ( $p < 0.005$ ). Also for each genotype separately and collectively there were significant linear relationships between granulosa cell aromatase activity (expressed as ng oestradiol/106 cells per 3 h) and inhibin secretory activity (i.e., overall  $R=0.66$ ,  $p < 0.001$ ,  $n=34$ ). Similar conclusions were also reached when granulosa cell inhibin output was assessed by inhibin RIA using the method of Findlay *et al.* (1990).

#### Immunoactive inhibin in plasma

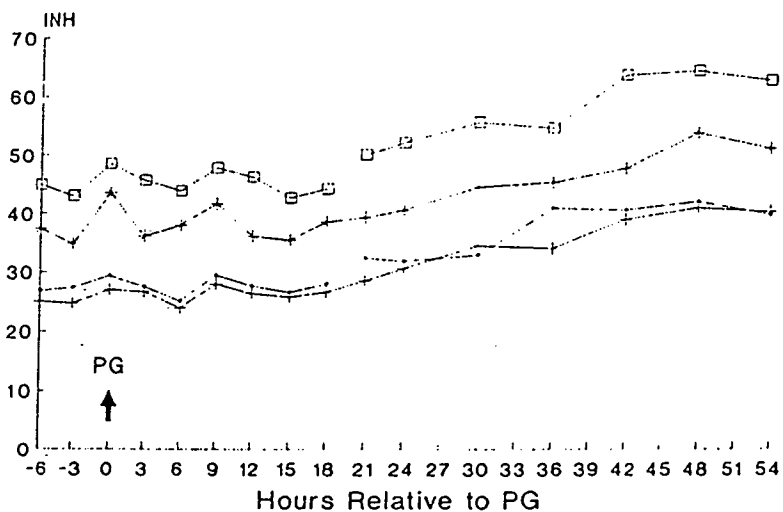
Inhibin concentrations in plasma are more meaningful measures of ovarian inhibin feed-back than either total ovarian or follicular fluid inhibin concentrations. Plasma concentrations require no judgement about the significance of ovarian cellular or plasma clearance rates of inhibin and thus are of greater significance with respect to understanding its relationship to FSH synthesis and release. In two recent investigations (B.M. Bindon and J.K. Findlay, unpublished) plasma immunoactive inhibin concentrations (Findlay *et al.*, 1990) have been measured in Booroola (FF), Control and 'T' Merinos daily throughout the oestrous cycle (Fig. 4). Even in this small data set ( $n=4$  ewes per genotype) it is evident that plasma inhibin concentrations are lower in Booroola than the other two genotypes (e.g.,  $p < 0.025$  on Day 2 and  $p < 0.05$  on Day 7).

Fig. 4. Mean plasma immunoactive inhibin concentrations (pM) in daily samples from four ewes/genotype during the oestrous cycle of Booroola FF (♦), Control Merino (■) and CSIRO 'T' (●) ewes. s.e. terms included for days -8 and +8 (unpublished data of B.M. Bindon, J.K. Findlay and M.A. Hillard).



More convincing differences are seen during intensive plasma inhibin concentration measurements in the follicular phase following prostaglandin (PG)-induced luteolysis of FF ( $n=20$ ) and F+ ( $n=12$ ) Booroola ewes and Control ( $n=12$ ) and 'T' Merino ewes ( $n=12$ ; Fig. 5). (Ovulation rates were measured in these ewes in successive cycles before and after PG-induced luteolysis. For FF, F+, Control and 'T' flocks the mean ( $\pm$  s.e.) ovulation rates were  $6.65 \pm 0.2$  and  $6.20 \pm 0.3$ ,  $3.25 \pm 0.3$  and  $3.67 \pm 0.4$ ,  $1.58 \pm 0.3$  and  $1.42 \pm 0.4$ , and  $2.08 \pm 0.3$  and  $2.25 \pm 0.4$  respectively.

Fig. 5. Mean plasma inhibin concentrations (pM) in Booroola FF ( $\bullet$ ;  $n=20$ ), Booroola F+ ( $\square$ ;  $n=12$ ), Control Merino (W;  $n=12$ ) and CSIRO 'T' Merino (r;  $n=12$ ) ewes studied at 3-hr intervals from 6 hr before PG injection to 24 hr after PG injection and then at 6-hr intervals until 54 hr after PG. The average onset of oestrus occurred at  $54 \pm 4$  hr after PG.



The Booroola genotypes (FF and F+) have significantly ( $p < 0.01$ ) reduced plasma inhibin concentrations throughout the period of study in comparison with Control and 'T' Merinos. This is what you would predict from the results in Table 9, but is at variance with the total granulosa cell population of oestrogenized follicles and their inhibin synthetic capability in Booroola ovaries. It is important to note that FF and F+ ewes were not significantly different. This means that one copy of the F gene had no measurable effect on plasma inhibin concentration. The differences in average plasma inhibin concentrations in Control and 'T' Merinos approached significance ( $p = 0.07$ ).

The inhibin data relate to some extent to the plasma FSH concentrations measured in the same animals (Fig. 1). Booroola ewes have low plasma inhibin and elevated plasma FSH during the critical period of pre-ovulatory follicle growth. In this study the average between-sheep, within-genotype correlation between FSH and inhibin was negative and significant ( $r = -0.449$ ;  $p < 0.01$ ). Ewes with lowest inhibin concentrations had the highest FSH concentrations.

## Response to inhibin immunization

Reduced total ovarian and plasma inhibin concentrations in Booroola gene carriers provide some grounds for thinking that Booroola ewes would not show further increases in ovulation rate in response to active immunization against inhibin(s). (That is, if these animals are already deficient in inhibin perhaps they should fail to show any change in ovulation rate in response to inhibin immunization?) Recent experiments (T. O'Shea and B.M. Bindon, unpublished; Tables 10 and 11) do not confirm this notion. It is clear that Booroola ewes are in fact more responsive than Control Merinos to immunization against either native bovine inhibin (Table 10) or a synthetic fragment of the  $\alpha$ -subunit of porcine inhibin (Table 11).

Table 10. Ovulation rate (means  $\pm$  s.e.) of Booroola (FF and F+) and Control Merino ewes after immunization with native bovine inhibin (from T O'Shea and B M Bindon, unpublished results)

Ewe genotype	n	Immunogen	Ovulation rate*		
			Before	After	Change
Control Merino	22	Nil	1.14 $\pm$ 0.07	1.29 $\pm$ 0.1	+0.10 $\pm$ 0.10
	20	Bovine inhibin	1.15 $\pm$ 0.08	4.10 $\pm$ 0.7	+2.95 $\pm$ 0.72
Booroola (FF and F+)	15	Nil	4.40 $\pm$ 0.5	4.33 $\pm$ 0.6	-0.07 $\pm$ 0.23
	16	Bovine inhibin	3.88 $\pm$ 0.4	10.13 $\pm$ 0.8	+6.25 $\pm$ 0.83

\* Ovulation rate measured before primary immunization and 49 days after primary immunization (that is, 20 days after booster immunization).

Table 11. Ovulation rate changes following immunization (- or +) of Control Merino and Booroola ewes against synthetic porcine  $\alpha$ -inhibin fragment (from T. O'Shea & B.M. Bindon, unpublished)

Ewe genotype	n	Immunogen	Ovulation rate*		
			Before	After	Change
Booroola	16	-	5.19 $\pm$ 0.50	4.93 $\pm$ 0.36	-0.27 $\pm$ 0.37
(FF and F+)	16	+	4.67 $\pm$ 0.36	8.40 $\pm$ 1.60	4.23 $\pm$ 1.83
Control Merino	15	-	1.27 $\pm$ 0.15	1.93 $\pm$ 0.17	0.71 $\pm$ 0.16
	15	+	1.20 $\pm$ 0.11	2.87 $\pm$ 0.33	1.67 $\pm$ 0.33

\* That is, ovulation rate measured before primary immunization or eight weeks after booster immunization given 34 days after primary.

Ewes were immunized (primary and booster) with either 100  $\mu$ g human serum albumin in Freund's Complete Adjuvant (-) or 100  $\mu$ g HSA conjugated to 50  $\mu$ g porcine INH ( $\alpha$ 1-32) in Freund's Complete Adjuvant (+).

## CONCLUSION

The most consistent finding from Australian and N.Z. studies of the Booroola ewe is that the F gene causes an elevation of plasma FSH during the follicular phase of the oestrous cycle (McNatty *et al.*, 1987a; Fig. 1 of this paper). This finding accords well with the view that the major determinant of ovulation rate

in the sheep is the amount of FSH reaching the ovary during this period (e.g., Henderson et al., 1988). The question is whether this results from a direct effect of the F gene on FSH secretion at the hypothalmo-pituitary axis or from an indirect effect operating via feed-back secretions from the ovary.

The data reviewed in this paper support both possibilities: the N.Z. studies show (Fig. 2) that differences in plasma FSH attributable to the F gene persist after ovariectomy and can be reproduced by GnRH treatment of HPD-OVX Booroola ewes. This implies that the effects of the F gene are independent of the presence of the ovary or its secretions.

The Australian studies, on the other hand, favour the second possibility for these reasons: First, the plasma FSH differences due to the presence of the F gene seen in intact ewes did not persist following ovariectomy (Table 3). Second, there is convincing evidence that ewes carrying the F gene have significantly lower concentrations of plasma inhibin, the major ovarian feed-back regulator of FSH secretion (Fig. 5). There is not yet sufficient evidence to conclude that the apparent inhibin deficiency of Booroola ewes is a direct effect of the F gene. It may be a secondary effect of the developmental abnormality of the Booroola ovary which leads to its follicles reaching maturity at smaller size, with fewer granulosa cells and reduced antral fluid secretion.

These conclusions are probably a vast oversimplification of a very complex genetic phenomenon. The ovary and the pituitary gland are logical sites of action for a major gene which only appears to affect ovulation rate. However, the F gene may act at some other site outside the range of possibilities covered by our present knowledge of ovarian physiology.

With respect to future physiological research in the Booroola ewe, further studies of intra-ovarian factors and FSH in regulating ovarian follicle development, the regulation of pituitary FSH synthesis and secretion together with studies of the pituitary-gonadal axis during foetal development seem warranted.

In the Booroola male the recent studies of Price et al. (1990) provide some encouragement for the idea that the F gene has a measurable effect on the reproductive endocrinology of the ram. Under Australian conditions the conclusion remains that F gene expression is limited to the ewe.

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## Inhibin production *in vitro* by granulosa cells from Booroola ewes which were either homozygous or non-carriers of a fecundity gene influencing their ovulation rate

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**Summary.** The production of inhibin by granulosa cells was studied *in vitro* using cells from follicles of various sizes and health. Follicles were recovered on Days 10–13 of the oestrous cycle, from Booroola × Romney ewes which were homozygous (*FF*) carriers or non-carriers (*++*) of the fecundity (*F*) gene. Inhibin was measured using a bioassay based on the suppression of follicle-stimulating hormone (FSH) output by cultured pituitary cells from ovariectomized Romney ewes and, in some instances, for comparative purposes, by radioimmunoassay also. Geometric mean inhibin production by granulosa cells from nonatretic follicles increased with increasing follicle diameter, during the first 24 h of culture, for both genotypes. The geometric mean production of inhibin by cells from nonatretic 3–4.5 mm diameter *FF* follicles (the largest follicles found in *FF* ewes), was significantly higher ( $P < 0.05$ ) than that by cells from non-atretic 3–4.5 mm diameter *++* follicles, but similar to that of cells from non-atretic  $\geq 5$  mm diameter *++* follicles. The production of oestradiol-17 $\beta$  by cells cultured in the presence of testosterone (1  $\mu\text{g/ml}$ ) followed a pattern similar to cellular inhibin production. There was a positive linear correlation between inhibin and oestradiol-17 $\beta$  production during the first 24 h of culture, for both genotypes. In addition to acting as a substrate for oestradiol-17 $\beta$  synthesis, testosterone generally had a slight, stimulatory effect on inhibin production. Irrespective of follicle size, or genotype, no detectable amounts of inhibin were produced by granulosa cells from atretic follicles during the first 24 h of culture, or by cells from nonatretic or atretic follicles during the second 24 h of culture.

These studies show that the highest mean amounts of inhibin produced by granulosa cells *in vitro* are similar for both genotypes. Moreover, they are achieved with cells from the largest nonatretic follicles in both *++* (i.e.  $\geq 5$  mm diameter) and *FF* (i.e. 3–4.5 mm diameter) ewes.

**Keywords:** Booroola ewes; inhibin; granulosa cells; oestradiol-17 $\beta$

### Introduction

Studies of high fecundity Booroola ewes have shown that, in ewes bearing the fecundity (*F*) gene, high plasma concentrations of follicle-stimulating hormone (FSH) occur more frequently and follicles attain ovulatory maturity earlier, i.e. at a smaller diameter, relative to non-carriers of the gene (McNatty & Henderson, 1987). There is increasing evidence that inhibin may be important in regulating plasma FSH concentrations in sheep (Martin *et al.*, 1988; Findlay *et al.*, 1989; Mann *et al.*, 1989) and thereby follicular activity. Inhibin is therefore a possible candidate for mediating

the effects of the Booroola *F* gene. There may be differences between the Booroola genotypes in inhibin production by granulosa cells, these cells being the principal site of ovarian inhibin production (Henderson & Franchimont, 1983; Mann *et al.*, 1989; Rodgers *et al.*, 1989; Torney *et al.*, 1989). The purpose of this study was to investigate inhibin production by granulosa cells *in vitro* using cells from follicles of various sizes and health obtained from Booroola × Romney ewes which were homozygous (*FF*) or non-carriers (*++*) of the *F* gene. Inhibin was measured using a bioassay based on the suppression of FSH output by ovine pituitary cells *in vitro* (Henderson *et al.*, 1989) and by radioimmunoassay, for comparative purposes, in some instances.

## Materials and Methods

**Sheep.** The Booroolas were 5–8 years old *FF* ( $n = 16$ ) and *++* ( $n = 16$ ) Booroola × Romney ewes which had been segregated on the basis of at least one ovulation-rate recording of  $\geq 5$  and 1 or 2, respectively (Davis *et al.*, 1982). The ewes were grazed on open pasture, and run with a vasectomized ram fitted with a marking harness to detect oestrous activity. Granulosa cells were recovered at Days 10–13 of the oestrous cycle. Pituitary cells for the inhibin bioassay were obtained from Romney ewes which had been ovariectomized for 4–8 weeks.

**Reagents.** Collagenase (type IV), deoxyribonuclease I, gentamicin, glutamine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), oestradiol-17 $\beta$ , sodium bicarbonate, sodium pyruvate and testosterone were obtained from Sigma Chemical Co., St Louis, MO, USA. Bovine serum albumin (BSA) was obtained from Immuno Chemical Products Ltd, Auckland, New Zealand; dispase from Boehringer Mannheim GmbH, Mannheim, W. Germany; amphotericin B from E.R. Squibb & Son Inc., Princeton, NJ, USA; penicillin from Glaxo Laboratories Ltd, Greenford, UK; Medium 199 with Earle's salts, Dulbecco's modified Eagle medium and non-essential amino acids from Gibco Laboratories, Grand Island, NY, USA. The sheep FSH radioimmunoassay kit and sheep LH preparations (NIDDK-oLH-1-3 and NIDDK-oLH-24) were gifts from the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, University of Maryland, USA. The ovine serum used in the granulosa and pituitary cell cultures was from a single batch, described previously (Henderson *et al.*, 1989), obtained from 4 ovariectomized Romney ewes.

A single pool of ovine follicular fluid was used as an inhibin reference standard in the inhibin bioassay. The fluid was collected from ovaries of sheep slaughtered at a local abattoir, and treated with dextran-coated charcoal, as described previously (Henderson *et al.*, 1986). The inhibin bioactivity of the pool of follicular fluid was calibrated in the inhibin bioassay against an inhibin standard (code no. 86/690), prepared from pig follicular fluid, and provided by the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK. The dose–response curve of the sheep follicular fluid pool paralleled that of the inhibin standard. The potency of the ovine follicular fluid pool in the bioassay, in terms of the inhibin standard (86/690), was 10.4 Units/ $\mu$ l, with 95% confidence limits of 7.9 and 12.9 U/ $\mu$ l. The calibrated ovine follicular fluid pool was then routinely used as the inhibin standard in subsequent bioassays, and all inhibin bioactivity results were expressed in terms of the 86/690 standard.

**Ovarian dissections.** Pairs of ovaries were recovered from Booroola ewes at Days 10–13 of the oestrous cycle (Day 0 is day of oestrus). All the corpora lutea were dissected out and counted. All antral follicles  $\geq 1.0$  mm in diameter were individually dissected from each pair of ovaries, their diameters measured and then placed into sterile Medium 199 with Earle's salts (Eagle, 1959), supplemented with Hepes buffer (20 mM), gentamicin (50  $\mu$ g/ml) and 1.0% bovine serum albumin. Assessment of the health of each follicle and the harvesting of granulosa cells was performed as described previously (McNatty *et al.*, 1984). Follicles considered to be non-atretic were those with visible thecal capillaries when viewed at  $\times 10$  magnification under a dissecting microscope, no debris in the follicular fluid, an oocyte of healthy appearance,  $\geq 26\%$  of the maximum number of recoverable granulosa cells for a follicle of a given size, and a pink to red theca interna. Follicles were considered to be atretic when one or more of these 5 criteria was not applicable.

**Granulosa cell cultures.** Preliminary studies with cells from non-atretic follicles indicated that approximately  $1 \times 10^6$  granulosa cells/culture were required to produce sufficient amounts of inhibin for determination by the bioassay. Therefore, for each pair of ovaries it was often necessary to pool cells from follicles of a similar size and classification. Pools were composed of cells from either non-atretic or atretic follicles of diameters 1–2.5 mm, 3–4.5 mm and  $\geq 5$  mm. After collecting the cells and pooling as appropriate, the cells were centrifuged at 200 g for 10 min and resuspended in Dulbecco's Modified Eagle Medium containing glutamine (1%), non-essential amino acids, sodium pyruvate (1 mM), sodium bicarbonate (3.7 mg/ml), gentamicin (50  $\mu$ g/ml), amphotericin B (2.5  $\mu$ g/ml) and penicillin (100 units/ml), pH 7.4 (Medium DM). The cells were centrifuged again (200 g, 10 min) and finally resuspended in Medium DM containing 10% ovariectomized sheep serum (Medium DMS). The cells were counted using a haemocytometer, and the volume of Medium DMS was adjusted to give approximately  $2 \times 10^6$  cells/ml. Samples of 0.5 ml were pipetted into individual round wells (16 mm diam.) of multiwelled tissue-culture dishes (Nunc, Kamstrup, Denmark). A further 0.5 ml Medium DMS alone or Medium DMS containing 2  $\mu$ g testosterone/ml was

added. The limited number of cells available meant that generally only duplicate cultures of each treatment were established. The cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 48 h, with the medium being renewed after 24 h. At the end of the culture period, the cells were washed thoroughly with saline and stored frozen until assayed for protein by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard. In some instances, samples of granulosa cells for culture were homogenized in 1 ml of Medium DMS and the media stored frozen immediately so that the endogenous cellular inhibin content could be determined. The 1-ml samples of spent granulosa cell culture medium were divided into two 0.5 ml aliquots. One aliquot was stored frozen at -20°C until subsequent determination of oestradiol-17 $\beta$ , and in some instances determination of inhibin by radioimmunoassay. The other aliquot, for inhibin bioassay, was incubated with 0.5 ml of aqueous dextran T-70 (0.02% w/v)-coated charcoal (0.2% w/v) for 16 h at 4°C. Following centrifugation at 3000 g, the supernatant was stored frozen at -20°C.

**Inhibin bioassay.** The bioassay of inhibin activity was based on the suppression of basal FSH output by cultured anterior pituitary cells from ovariectomized ewes as described previously (Henderson *et al.*, 1989). Briefly, dispersed cells were obtained from anterior pituitaries following enzymic treatment with collagenase, dispase and deoxyribonuclease. Samples of  $1 \times 10^6$  dispersed cells were cultured in 1 ml Medium DMS in multiwelled tissue culture dishes (Nunc, Kamstrup, Denmark) for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The medium was then replaced with fresh Medium DMS (1 ml) containing added dextran-charcoal-treated granulosa cell culture medium or the ovine follicular fluid inhibin standard. Triplicate cultures of each dose of each treatment were usually established. Incubation was continued for a further 48 h with the medium being renewed after 24 h with a fresh 1 ml medium containing the same additions of granulosa cell culture medium or inhibin standard. Spent media were stored frozen at -20°C until assayed for gonadotrophins. At the end of the culture period, the cells were washed thoroughly with saline (0.154 M) and stored frozen until assayed for protein by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard. In the pituitary cell culture medium, the concentration of granulosa cell culture medium ranged from 2.5 to 10%. Culture medium (DMS) with and without added testosterone (1  $\mu$ g/ml) incubated in the absence of granulosa cells, and then treated with dextran-coated charcoal, served as control medium for adding to the pituitary cell culture medium. The calibrated ovine follicular fluid inhibin standard was added to the pituitary cell cultures at doses of 0.04 to 5.2 units/ml. Inhibin bioactivity of the granulosa cell culture media was expressed relative to the activity of the inhibin standard by interpolation from standard curves generated by plotting FSH output (as a percentage of that of control pituitary cultures) versus the logarithm of the concentration of the ovine follicular fluid standard. Inhibin bioactivity in the culture medium was then normalized with respect to either the number of granulosa cells initially cultured, or the protein content of the granulosa cells at the end of their 48-h culture period.

**Radioimmunoassays.** Gonadotrophin concentrations in pituitary cell culture medium were measured by specific radioimmunoassays and normalized with respect to the pituitary cell protein. FSH was measured using the sheep FSH radioimmunoassay kit and protocol provided by the National Hormone and Pituitary Program, NIDDK, University of Maryland, USA. The FSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113), the ovine FSH for iodination was NIAMDD-oFSH-I-1 (AFP-5679C) and the reference preparation was NIAMDD-oFSH-RP-1 (biopotency  $75 \times$  NIH-FSH-S1). The sensitivity of the assay was 0.05–0.1 ng/assay tube and the intra- and inter-assay coefficients of variation were <10%. LH was measured using the specific radioimmunoassay described previously (McNatty *et al.*, 1981). The antiserum used was generated in a rabbit against NIH-LH-S11 and was used at an initial dilution of 1:40 000. The ovine LH reference preparation was NIADDK-oLH-24 and the iodinated tracer was NIADDK-oLH-I-3. The sensitivity of the assay was 0.025–0.05 ng per assay tube, and the intra- and interassay coefficients of variation were <11%.

Inhibin concentrations in granulosa cell culture medium were measured by radioimmunoassay using reagents provided by Dr D. M. Robertson, Department of Anatomy, Monash University, Clayton, Victoria, Australia. The methodology was as previously described and found suitable for measurement of inhibin concentrations in bovine granulosa cell culture medium (McLachlan *et al.*, 1986). The inhibin antiserum (no. 1989) was generated in a rabbit against pure bovine inhibin of  $M_r$  31 000. The antiserum exhibited insignificant (<1%) cross-reaction with ovine LH, FSH, growth hormone and prolactin, bovine activin-A, porcine transforming growth factor- $\beta$ , Müllerian inhibiting substance, the reduced and alkylated  $\alpha$ - and  $\beta$ -subunits of bovine inhibin, and FSH-suppressing protein. However, the antiserum displayed considerable cross-reaction with bovine pro- $\alpha$ C-subunit (288%; Robertson *et al.*, 1989). Purified bovine inhibin of  $M_r$  31 000 was used to prepare the iodinated tracer. The inhibin reference preparation was the same ovine follicular fluid pool used as a standard in the inhibin bioassay. It was calibrated in the radioimmunoassay against the pig follicular fluid reference inhibin preparation (code no. 86/690) provided by the National Institute for Biological Standards and Control, Poters Bar, Hertfordshire, UK. Both preparations gave parallel dose-response curves in the radioimmunoassay. The potency of the ovine follicular fluid pool in the radioimmunoassay was 230 Units  $\mu$ l. The sensitivity of the radioimmunoassay was 230 mU/tube and the intra- and interassay coefficients of variation were 7% and 13%, respectively.

Oestradiol-17 $\beta$  was measured directly in aliquants of granulosa cell culture medium by a specific radioimmunoassay as described previously (McNatty *et al.*, 1984). The sensitivity of the assay (per tube) was 5 pg and the intra- and interassay coefficients of variation were <11%.

The inhibin and oestradiol-17 $\beta$  values were normalized with respect to either number of granulosa cells at the start of culture, or granulosa cell protein at the end of culture.

## Results

### Number of corpora lutea and follicles in *FF* and *++* ewes

Table 1 shows the number of corpora lutea and the distribution of follicles in each genotype. The mean number of total and nonatretic follicles per ewe, 1–4.5 mm in diameter, did not differ significantly between the genotypes ( $P > 0.05$ , Student's *t* test). Follicles  $\geq 5$  mm in diameter were present in the ovaries of *++* but not *FF* ewes.

Table 1. Number of corpora lutea per ewe, and distribution of follicles with regard to health and size in *FF* and *++* ewes

Genotype	No. of corpora lutea/ewe	No. of follicles per ewe of diameter (mm)					
		1–2.5		3–4.5		$\geq 5$	
		Total	Nonatretic	Total	Nonatretic	Total	Nonatretic
<i>++</i>	1.6 $\pm$ 0.1	39 $\pm$ 5	16 $\pm$ 2	4.7 $\pm$ 1.0	2.6 $\pm$ 0.6	1.7 $\pm$ 0.3	1.1 $\pm$ 0.2
<i>FF</i>	6.1 $\pm$ 0.5	31 $\pm$ 4	15 $\pm$ 3	3.1 $\pm$ 0.6	2.4 $\pm$ 0.5	0	0

Values are arithmetic means  $\pm$  s.e.m. for 16 ewes per genotype.

### Inhibin and oestradiol-17 $\beta$ production by granulosa cells

In preliminary studies, FSH output by cultured pituitary cells was suppressed by the ovine follicular fluid inhibin standard and granulosa cell culture medium to a greater extent during the second 24-h period of exposure to these agents than during the first 24 h. This confirms previous findings of the effects of ovine follicular fluid (Henderson *et al.*, 1989). Inhibin bioactivity was therefore subsequently estimated only from the effects of the inhibin standard and granulosa cell culture medium samples during the second 24-h period of their exposure to the pituitary cells.

Figure 1 shows the effect on FSH output by cultured pituitary cells of increasing amounts of the ovine follicular fluid inhibin standard, and increasing volumes of granulosa cell culture medium pooled from cultures of granulosa cells obtained from nonatretic follicles of different sizes from *++* and *FF* ewes. FSH output was suppressed in a dose-dependent fashion by the inhibin standard, and all the pools of granulosa cell culture medium, although with Pool 1 50  $\mu$ l medium was no more effective than 25  $\mu$ l in suppressing FSH output. There was insufficient medium from Pool 4 to test at 100  $\mu$ l. The slopes of straight lines fitted to the dose-response data for the effects of the pools of granulosa cell media on FSH output did not differ significantly ( $P > 0.05$ ) from the corresponding slope for the inhibin standard. The slope values were (mean  $\pm$  s.d.): Pool 1, 10.4  $\pm$  2.4; Pool 2, 9.8  $\pm$  1.6; Pool 3, 7.0  $\pm$  0.9; Pool 4, 9.4  $\pm$  2.0; inhibin standard, 8.1  $\pm$  1.1.

While pituitary cell FSH output could be suppressed by the inhibin standard and granulosa cell culture medium pools, there was no consistent effect on LH output, which varied from 88 to 113% of that produced by control pituitary cultures. None of the treatments had any significant effect on the mean amounts of pituitary cellular protein determined at the end of the culture period, relative to control cultures. The suppression of FSH output was therefore unlikely to be a consequence of a toxic effect of the treatments.

Although pooled medium from the first 24 h of granulosa cell culture could suppress FSH output by pituitary cells (Fig. 1), pooled medium from the second 24 h of granulosa cell culture was without effect on FSH output, at doses of up to 100  $\mu$ l culture medium per pituitary culture. Having established that the dose-response curves of the pooled granulosa cell culture media, tested at 2 or 3 doses, paralleled that of the follicular fluid inhibin standard (Fig. 1), it was considered appropriate

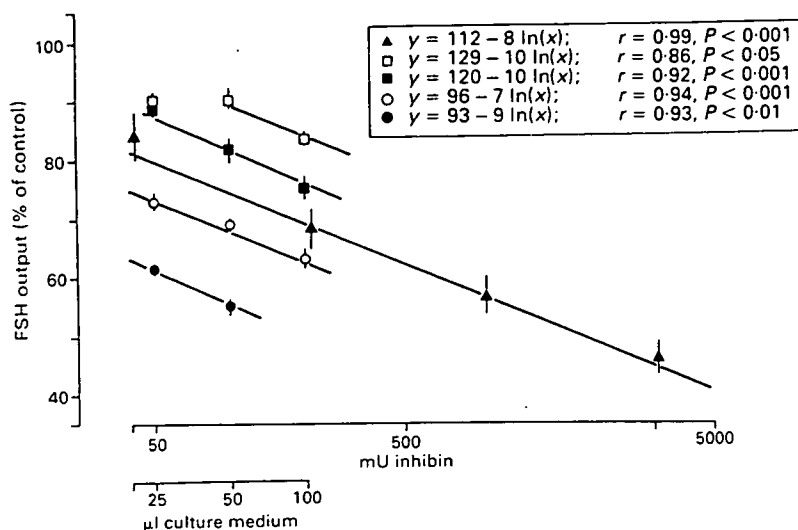
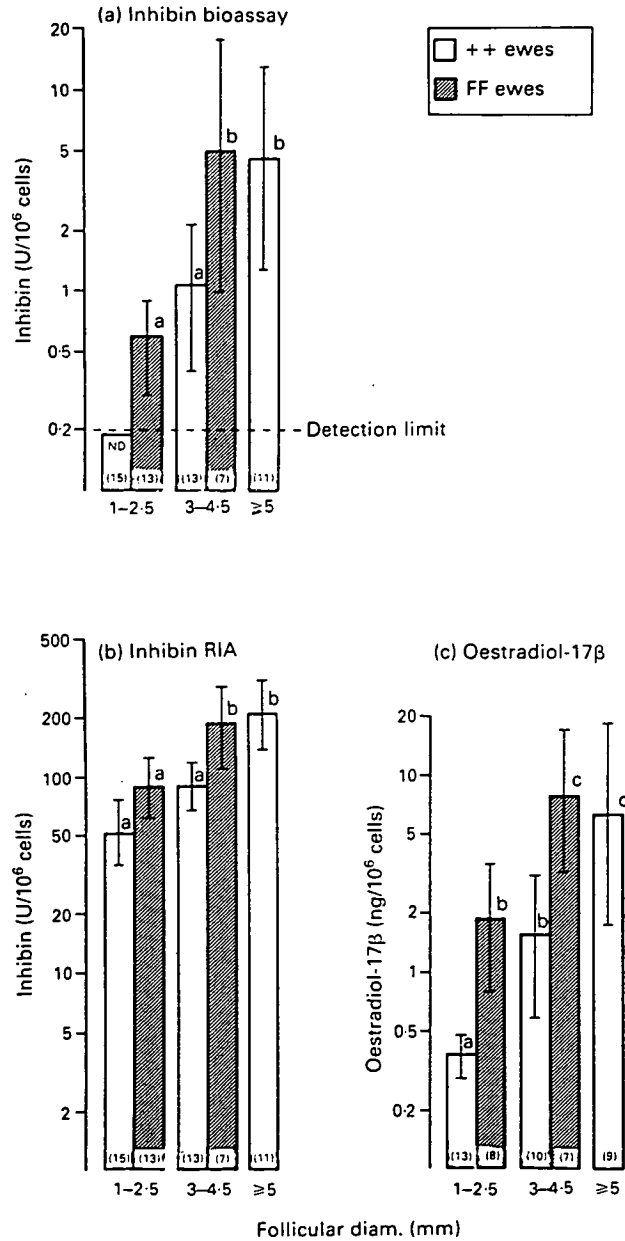


Fig. 1. Effect of increasing doses of inhibin standard ( $\blacktriangle$ ) and volumes of pooled granulosa cell culture medium on FSH output by cultured ovine pituitary cells during the second 24 h of treatment. Granulosa cell medium Pools 1 ( $\square$ ) and 4 ( $\bullet$ ) were derived from medium from the first 24 h of culture of cells from nonatretic follicles 3–4.5 mm and  $\geq$  5 mm in diameter, respectively from ++ ewes. Pools 2 ( $\blacksquare$ ) and 3 ( $\circ$ ) were derived from medium from the first 24 h of culture of cells from non-atretic follicles 1–2.5 mm and 3–4.5 mm in diameter, respectively, from FF ewes. Values for the pools of medium are means  $\pm$  s.e.m. for 3 replicate cultures. Values for the inhibin standard are means  $\pm$  s.e.m. for 8 replicate experiments. Mean FSH output has been expressed as a percentage of the mean output by appropriate control (zero dose) cultures. Linear regression lines have been fitted to the data (50 and 100  $\mu$ l data only for Pool 1) and the associated regression equations are shown, together with the correlation coefficients ( $r$ ) and significance.

to determine inhibin concentrations in individual granulosa cell cultures at a single dose-level. Triplicate 50- $\mu$ l aliquants of all medium samples from the first 24 h of culture, and representative samples from the second 24 h of culture were therefore assayed. Assaying a single volume of all the granulosa cell culture medium samples minimized the number of sheep which had to be ovariectomized and subsequently killed to provide pituitary cells for the bioassay.

Figure 2(a) shows the bioactive inhibin output, during the first 24 h of culture, by granulosa cells from nonatretic follicles of various sizes from FF and ++ ewes. Values presented have been normalized with respect to the number of granulosa cells initially cultured. However, similar results were obtained if inhibin output was normalized with respect to cellular protein determined at the end of the culture period. Within each genotype, the geometric mean bioactive inhibin output increased with increasing follicular diameter. Follicles  $\geq$  5 mm in diameter were not found in FF ewes. However, the geometric mean bioactive inhibin output by cells from 3–4.5 mm diameter FF follicles did not differ significantly ( $P > 0.05$ ) from that of cells from  $\geq$  5 mm diameter ++ follicles, and was significantly greater ( $P < 0.05$ ) than that of cells from 3–4.5 mm diameter ++ follicles. The bioactive inhibin output by granulosa cells from 1–2.5 mm diameter ++ follicles was undetectable ( $< 0.2$  U/ $10^6$  cells), whereas the inhibin output by cells from similarly sized FF follicles, although low, was measurable. A similar pattern of results emerged when the inhibin content of the same samples was measured by radioimmunoassay, although the absolute amounts of inhibin measured by the bioassay and radioimmunoassay differed markedly (Fig. 2b).

Inhibin bioactivity was not detectable in the medium homogenates of freshly isolated granulosa cells. Inhibin output by cultured cells probably therefore represents production of inhibin, rather than simply release of preformed intracellular inhibin. Irrespective of follicle size, or genotype,



**Fig. 2.** Inhibin output, measured by bioassay (a) and radioimmunoassay (b), and oestradiol-17 $\beta$  production (c) by granulosa cells from non-atretic follicles of various diameters from ++ and FF ewes. Data are for the first 24 h of granulosa cell culture. Oestradiol-17 $\beta$  production was determined in cultures treated with testosterone (1  $\mu$ g/ml). Inhibin output was determined in cultures not exposed to testosterone, and no detectable amounts of oestradiol-17 $\beta$  were produced by these cultures. Values are geometric means of (*n*) ewes with 95% confidence limits indicated by the vertical lines. Geometric mean values with different letters are significantly different ( $P < 0.05$ , analysis of variance in conjunction with Newman-Keuls multiple range test on data logarithmically transformed [ $\ln(x + 1)$ ] to equalize the variances). Bioactive inhibin output by cells from 1-2.5 mm diameter ++ follicles was not detectable (ND, i.e.  $< 0.2$  U/10<sup>6</sup> cells) and this group was excluded from the statistical comparisons.



no detectable amounts of bioactive inhibin were produced by cells from atretic follicles during the first 24 h of culture, or by cells from non-atretic or atretic follicles during the second 24-h period of culture.

Figure 2(c) shows oestradiol-17 $\beta$  production during the first 24 h of culture by granulosa cells from non-atretic follicles of various diameters from ++ and FF ewes. The results are similar to those for inhibin output. The geometric mean oestradiol-17 $\beta$  production by cells from 3–4.5 mm diameter follicles of FF ewes was similar to that of cells from  $\geq 5$  mm diameter follicles of ++ ewes. Geometric mean oestradiol-17 $\beta$  production by cells from 3–4.5 mm and 1–2.5 mm diameter FF follicles was significantly higher ( $P < 0.05$ ) than that of cells from correspondingly sized follicles from ++ ewes. Oestradiol-17 $\beta$  production by cells from nonatretic follicles fell markedly during the second 24 h of culture; geometric mean values being only 20–54% of values for the first 24 h of culture. Oestradiol-17 $\beta$  production during both 24-h periods of culture by cells from atretic follicles was low ( $< 0.2$  ng oestradiol-17 $\beta$ /10<sup>6</sup> cells), irrespective of follicle diameter or ewe genotype.

For each genotype, there was a significant linear correlation between bioactive inhibin output and oestradiol-17 $\beta$  production by granulosa cells during the first 24 h of culture (Fig. 3). There was no significant difference ( $P > 0.05$ ) between the values for the slopes (0.7 vs. 0.6) or the intercepts (0.5 vs. 0.8), indicating that the regression lines for the 2 genotype populations were coincidental.

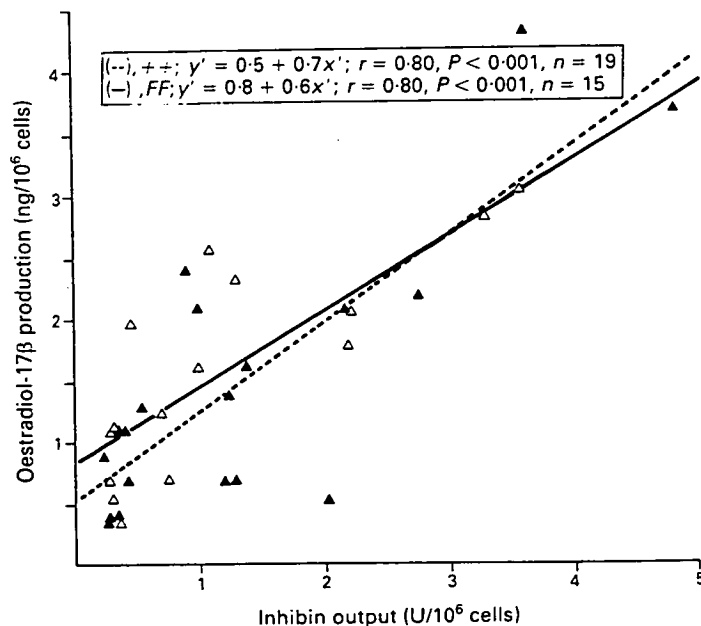


Fig. 3. Relationship between bioactive inhibin output and oestradiol-17 $\beta$  production during the first 24 h of culture by granulosa cells from 1–4.5 mm and 3– $\geq 5$  mm diameter nonatretic follicles from FF ( $\Delta$ ) and ++ ( $\blacktriangle$ ) ewes, respectively. Data have been transformed to logarithms  $y' = \ln(y + 1)$ ,  $x' = \ln(x + 1)$ , to equalize the variances. Linear regression lines have been fitted to the ++ and FF data separately, and the associated regression equations are shown, together with the correlation coefficients ( $r$ ), significance and  $n$  values.

#### Effect of testosterone on inhibin production

Testosterone (1  $\mu\text{g/ml}$ ) generally had a stimulatory effect on bioactive inhibin production by granulosa cells from both genotypes, during the first 24 h of culture. Overall, inhibin production by

testosterone-treated cultures was  $2.3 \pm 0.4$ -fold (mean  $\pm$  s.e.m.) and  $2.4 \pm 0.5$ -fold higher than that of corresponding control cultures for  $++$  and  $FF$  ewes, respectively ( $P < 0.05$ ,  $n = 19$  for  $++$  ewes;  $P < 0.01$ ,  $n = 15$  for  $FF$  ewes; paired Student's  $t$  test).

## Discussion

Follicles in  $FF$  ewes have a maximum diameter of 3–4.5 mm, whereas in  $++$  ewes preovulatory follicles are  $\geq 5$  mm in diameter (Table 1; McNatty & Henderson, 1987). Granulosa cells from 3–4.5 mm diameter  $FF$  follicles, however, share characteristics similar to those of cells from  $\geq 5$  mm diameter  $++$  follicles. The mean aromatase (i.e. oestradiol-17 $\beta$  synthetase) activity and mean FSH- and LH-stimulatable cyclic AMP production by granulosa cells from 3–4.5 mm diameter  $FF$  follicles is greater than that of cells from  $++$  follicles of similar size, but comparable to that of cells from  $\geq 5$  mm diameter  $++$  follicles (Fig. 2c; Henderson *et al.*, 1987; McNatty & Henderson, 1987). The present study demonstrates that inhibin output by granulosa cells from the two genotypes follows a similar pattern. In both genotypes, geometric mean inhibin output by cells from non-atretic follicles increased with increasing follicular diameter. Geometric mean inhibin output by cells from 3–4.5 mm diameter  $FF$  ewes was significantly higher ( $P < 0.05$ ) than that of cells from similarly sized  $++$  follicles, but comparable to that of  $\geq 5$  mm diameter  $++$  follicles. Oestradiol-17 $\beta$  production by the cells, cultured in the presence of testosterone, followed a similar pattern, and so confirmed previous findings (Henderson *et al.*, 1987).

The observed differences in granulosa cell inhibin production between the genotypes showed a similar pattern whether inhibin was measured by radioimmunoassay or bioassay (Figs 2a & b). However, the absolute amounts of inhibin measured by the two assays differed markedly, much lower values being measured by the bioassay relative to the radioimmunoassay. Previous comparisons of the bioassay and immunoassay of inhibin have also found that inhibin concentrations measured by bioassay can be considerably lower than those measured by radioimmunoassay (McLachlan *et al.*, 1986; Robertson *et al.*, 1988; Mann *et al.*, 1989). When discrepancies between bioassay and immunoassay data occur, particularly when the discrepancies are very large as in the present study, considerable caution must be exercised when interpreting the data. The disparity between the bioassay and immunoassay measurements of inhibin may be related to differences in the specificities of the two assays (Robertson, 1990). Granulosa cells may secrete, in addition to inhibin, other proteins such as activin and gonadotrophin-releasing hormone-like proteins, which stimulate pituitary FSH production, and follistatin, which suppresses FSH production. While these proteins would not be detected by the inhibin radioimmunoassay, they could influence the inhibin bioassay through their effects on FSH production. Thus, bioactive inhibin values could be artificially high or low depending on the relative amounts of other proteins in the granulosa cell culture medium influencing pituitary FSH output. In addition, granulosa cells may secrete subunits of inhibin, notably monomeric inhibin  $\alpha$  subunit, and inhibin subunit fragments and precursors, e.g. pro- $\alpha$ C-subunit, which are inactive in the inhibin bioassay, but highly reactive in the radioimmunoassay (Bicsak *et al.*, 1988; Knight *et al.*, 1989). Consequently, inhibin concentrations measured by the radioimmunoassay could be artificially high. Nevertheless, despite the differences in the absolute amounts of inhibin measured, both assays indicated that the highest mean amounts of inhibin produced by granulosa cells *in vitro* were similar for both genotypes. Moreover, it was achieved with cells from the largest non-atretic follicles in  $++$  (i.e.  $\geq 5$  mm diameter) and  $FF$  (i.e. 3–4.5 mm diameter) ewes.

It could be argued that the high ovulation rate of  $FF$  ewes, relative to  $++$  ewes, is a result of their higher plasma concentrations of FSH (McNatty *et al.*, 1987), allowing more follicles to reach ovulatory maturity in synchrony. The higher plasma FSH concentrations, in turn, being brought about by the  $F$  gene having a negative influence on ovarian inhibin production. Consistent with

this argument was the early finding of Cummins *et al.* (1983) that the bioactive inhibin content of ovaries collected from highly fecund Booroola  $\times$  Merino ewes was only one-third of that of control Merino ewes. Moreover, active immunization with inhibin immunogens, to immuno-neutralize circulating inhibin, raises ovulation rates in sheep (Henderson *et al.*, 1984; Cummins *et al.*, 1986; Forage *et al.*, 1987). As granulosa cells are the principal source of follicular inhibin production (Henderson & Franchimont, 1983; Rodgers *et al.*, 1989; Torney *et al.*, 1989), one might have expected mean cellular inhibin production *in vitro* by granulosa cells from *FF* ewes to be lower than in cells from *++* ewes. This, however, was not the case. It is possible that cellular inhibin production *in vitro* may not reflect granulosa cell inhibin production *in vivo*. Although plasma FSH concentrations differ between the genotypes *in vivo*, the granulosa cells from follicles of both genotypes were cultured in media containing the same low concentration of endogenous FSH (0.9 ng/ml) originating from the 10% ovariectomized sheep serum. The extent to which this endogenous FSH might have influenced the functioning *in vitro* of the cells from the 2 genotypes, if at all, is uncertain. Exogenous FSH does not influence oestradiol-17 $\beta$  production by ovine granulosa cells *in vitro*, while the effects on inhibin production *in vitro* have not been studied. However, previous studies with Booroola granulosa cells *in vitro* have indicated that  $\geq 10$  ng FSH/ml is required to stimulate production of cyclic AMP, an early event in FSH action (Henderson *et al.*, 1987). The present study was performed using ewes at Days 10–13 of the oestrous cycle. It is possible that differences in granulosa cell inhibin production between the genotypes may only occur at particular times of the oestrous cycle, e.g. during the preovulatory period, when ovulation rates can be markedly influenced by relatively small changes in plasma FSH concentrations (Henderson *et al.*, 1988). Differences in plasma FSH concentrations between the genotypes are, however, found during both the luteal and follicular phases of the oestrous cycle, and during anoestrus (McNatty *et al.*, 1987).

In sheep of both genotypes, granulosa cells from the largest nonatretic follicles produced the highest mean amounts of inhibin and oestradiol-17 $\beta$ . Such large antral follicles are also the major source of circulating inhibin in sheep (Mann *et al.*, 1989; Findlay *et al.*, 1990). Previous studies have shown that in *FF* Booroola ewes there are on average 4.8 large oestrogenic follicles, each containing  $1.1 \times 10^6$  granulosa cells (McNatty *et al.*, 1986). In *++* ewes, there are on average 1.5 large oestrogenic follicles with  $3.8 \times 10^6$  cells/follicle (McNatty *et al.*, 1986). The total number of granulosa cells present in these large non-atretic follicles is therefore similar for both genotypes ( $5.3$  vs.  $5.7 \times 10^6$  cells for *FF* vs. *++* ewes). The mean per cell production of inhibin *in vitro* by granulosa cells from large nonatretic follicles is also similar for both genotypes (Fig. 2). One might, therefore, anticipate that the amounts of inhibin secreted by the ovaries of both genotypes would be similar, as has already been found for oestradiol-17 $\beta$  (McNatty *et al.*, 1986), and *F*-gene-specific differences in plasma FSH may not be mediated through inhibin. Indeed, the ovary may not even be involved, as recent studies have demonstrated *F*-gene-specific differences in plasma FSH concentrations in ovariectomized Booroola ewes (McNatty *et al.*, 1989).

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**Section 5: Papers related to the manipulation of ovulation-rate**

Paper no.: 48

Authors: K.M. Henderson, P. Franchimont, M.J. Lecomte-Yerna, N.L. Hudson & K. Ball

Title: Increase in ovulation rate after active immunization of sheep with inhibin partially purified from bovine follicular fluid

Reference: Journal of Endocrinology (1984) 102, 305-309

# Increase in ovulation rate after active immunization of sheep with inhibin partially purified from bovine follicular fluid

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## ABSTRACT

Four Romney ewes were actively immunized with a partially purified preparation of inhibin derived from bovine follicular fluid and their ovulation rates in four successive oestrous cycles were compared with those of four ewes receiving adjuvant alone. The ovulation rates of the ewes immunized with the inhibin preparation were significantly higher than those of the control ewes ( $2.06 \pm 0.16$  (s.e.m.) vs  $1.31 \pm 0.06$  ovulations/ewe,  $n=4$ ). Plasma concentrations of FSH

and LH, measured in blood samples taken three times a week for 11 weeks, during which time each ewe was immunized three times, were not significantly different between the two treatment groups. These results suggest that active immunization with inhibin-enriched follicular fluid may be a potential means of increasing fecundity in sheep.

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## INTRODUCTION

The ovary of several primate and non-primate species produces a non-steroidal compound, named inhibin, which can selectively inhibit, both *in vivo* and *in vitro*, follicle-stimulating hormone (FSH) production by the pituitary gland (Franchimont, Henderson, Verhoeven *et al.* 1981; Channing, Anderson, Hoover *et al.* 1982; Grady, Charlesworth & Schwartz, 1982). Recent studies have demonstrated that ovarian inhibin is synthesized by granulosa cells (Erickson & Hsueh, 1978; Anderson & De Paolo, 1981; Henderson & Franchimont, 1981) and that androgens, FSH and follicle size and health each influence inhibin production by granulosa cells (Henderson & Franchimont, 1981, 1983; Henderson, Franchimont, Charlet-Renard & McNatty, 1984). However, the physiological role(s) of ovarian inhibin in the normal regulation of FSH production, and hence follicular maturation, remains uncertain. The purpose of this study was to immunize sheep actively with a partially purified preparation of inhibin, derived from bovine follicular fluid, in an attempt to neutralize any circulating inhibin, and to monitor the effects on ovulation rate and plasma gonadotrophins.

## MATERIALS AND METHODS

### Preparation of inhibin immunogen

Ovaries were obtained at slaughter from cows at a local abattoir. All antral follicles visible on the surface of the ovaries were punctured with a 20-gauge needle and the follicular fluid was aspirated under vacuum. The follicular fluids were pooled and cleared of debris and red blood cells by centrifugation at 1200 g for 15 min at 4°C. The supernatant fraction was freeze-dried and stored at -20°C. The powder was rehydrated as required by the addition of cold deionized water. Steroids were removed to undetectable levels by mixing for 16 h at 4°C with activated charcoal (Norit A, 1%; Fisher Scientific Co., Springfield, New Jersey, U.S.A.) pretreated with dextran T-70 (0.1%; Pharmacia Fine Chemicals AB, Uppsala, Sweden) followed by centrifugation at 3000 g (Henderson & Franchimont, 1981). An inhibin-enriched preparation was prepared from the supernatant fraction by subjecting it to affinity chromatography on Matrex gel red A (Amicon Corporation, Lexington, Massachusetts, U.S.A.) as described previously (Franchimont, Lecomte-Yerna, Henderson *et al.* 1983). Inhibin activity was monitored using a bioassay



based on the inhibition of luteinizing hormone releasing hormone-stimulated FSH secretion by cultured rat anterior pituitary cells as described and validated previously (Henderson & Franchimont, 1981). Inhibin potency was expressed relative to that of a standard reference preparation of inhibin derived from ovine testicular lymph which has been designated an arbitrary potency of 1 unit/mg (Eddie, Baker, Higginson & Hudson, 1979). Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The protein content of the inhibin preparation was 0.4% of the original follicular fluid and the inhibin preparation had a biological specific activity of 600 units/mg protein compared to 50 units/mg protein for the original follicular fluid. No detectable amounts of progesterone, oestradiol-17 $\beta$ , testosterone or androstenedione (measured by radioimmunoassay) were present in the inhibin preparation.

The inhibin-enriched preparation, dissolved in physiological saline to a concentration of 1 mg protein/ml, was emulsified at 4°C with an equal volume of Freund's complete adjuvant (Freund, Thomson, Hough *et al.* 1948) and used immediately. Ewes were injected subcutaneously into the gracilar or axillary region with 1 ml of this stable emulsion (inhibin-immunized ewes) or with 1 ml of a stable emulsion of equal volumes of saline and Freund's complete adjuvant (control ewes).

#### Sheep and immunizations

These studies were performed using eight parous New Zealand Romney ewes which had naturally synchronous oestrous cycles at the time of the first immunization. The ewes were grazed in open pasture and run with a vasectomized ram fitted with a marking harness, the ewes being examined daily for signs of oestrus. In April, on day 10 of the oestrous cycle, four ewes were immunized with the inhibin preparation and four with the control immunogen. The day of this first immunization was designated day 1 of the experiment. The immunizations were repeated monthly on days 30 and 64.

#### Blood sampling and laparoscopies

Immediately before the first immunization and three times a week thereafter (usually Monday, Wednesday and Friday mornings) for 11 weeks, a jugular venous blood sample (~2 ml) was obtained from each ewe. The plasma was separated by centrifugation (1200 g for 15 min at 4°C) and stored frozen (-20°C) until assayed for luteinizing hormone (LH) and FSH by radioimmunoassay. Immediately before each immunization and on day 48 of the experiment, an additional jugular venous blood sample (~50 ml) was

obtained from each ewe. The blood was allowed to clot and the serum separated and stored frozen (-20°C) until tested for the presence of antibodies to inhibin by a double-diffusion precipitation test. The number of ovulations (ovulation rate) in the four successive oestrous cycles occurring immediately after the first immunization was determined by laparoscope examination of the ovaries of each ewe, 9–12 days after each display of oestrous behaviour, and counting the number of corpora lutea present.

#### Immunoprecipitation test

A double-diffusion in agar precipitation test was used to detect the presence of antibodies to the inhibin preparation in the serum samples (Ouchterlony & Nilsson, 1973). Briefly, three rows of wells, 3 mm apart, were cut in a plate of agar gel. The middle row of wells was filled with 10  $\mu$ l aliquots of a solution of the inhibin preparation (2 mg/ml in phosphate-buffered saline) and the two outer rows filled with 10  $\mu$ l aliquots of undiluted serum. The serum and inhibin were allowed to diffuse towards each other for 16 h at room temperature in a humid atmosphere. The plates were then examined for lines of precipitation which were subsequently stained with Coomassie Brilliant blue R-250.

#### Gonadotrophin radioimmunoassays

Both FSH and LH were measured in sheep plasma by specific radioimmunoassays described previously (Scaramuzzi, Caldwell & Moor, 1970; Salamonsen, Jonas, Burger *et al.* 1973; McNatty, Gibb, Dobson *et al.* 1981). The standard FSH preparation was NIH-FSH-S11 and Papkoff preparation G4-150C was used as the iodinated tracer. The FSH antiserum was rabbit anti-human FSH (Butt, M94) and was used at an initial dilution of 1:8000. Its cross-reaction with ovine LH (NIH-LH-S21), ovine prolactin (NIH-P-S18), ovine growth hormone (NIH-GH-S11) and ovine thyroid-stimulating hormone (NIH-TSH-S8) was <0.5%. The LH antibody raised in a rabbit against NIH-LH-S11 was used at an initial dilution of 1:40 000. The LH antiserum exhibited low cross-reactions with other pituitary hormones: ovine prolactin (NIH-P-S12) 0.09%; ovine thyroid-stimulating hormone (NIH-TSH-S8) 2.4%; ovine growth hormone (NIH-GH-S11) 0.4%; ovine FSH (NIH-FSH-S10) 0.4%. The pituitary LH preparation for both the standard and the iodinated tracer was NIH-LH-S11. All the determinations of plasma FSH and LH were performed in single assays and the intra-assay coefficients of variation were 8.7 and 6.8% for the FSH and LH assays respectively. The detection limits were 20  $\mu$ g FSH/l plasma and 0.2  $\mu$ g LH/l plasma.

**RESULTS**

**Immunological response and oestrous cycle length**

Double-diffusion in agar precipitation tests indicated the presence of antibodies to the inhibin preparation in sera collected from all inhibin-immunized ewes on days 48 and 64 but not in sera collected before day 48. None of the serum samples collected from the control ewes contained demonstrable antibodies to the inhibin preparation.

The lengths of the oestrous cycles did not differ significantly (Wilcoxon rank sum test) between the inhibin-immunized and control ewes ( $15.9 \pm 0.1$  vs  $16.1 \pm 0.1$  days, for four oestrous cycles).

**Effect of inhibin immunization on ovulation rate**

Table 1 shows the ovulation rate of each ewe (as assessed by the number of corpora lutea present at laparoscopy) in the four successive oestrous cycles following the primary immunization. The inhibin-immunized ewes had significantly higher mean ovulation rates than the control ewes ( $2.06 \pm 0.16$  vs  $1.31 \pm 0.06$  for respective overall mean ovulation rates,  $n=4$ ;  $P < 0.05$ , Wilcoxon rank sum test). Ovulation rates of 2 or more occurred in only 5 of the 16 ovulations of the control ewes compared with 15 of the 16 ovulations of the inhibin-immunized ewes.

**Effect of immunization with the inhibin preparation on plasma gonadotrophin concentrations**

On none of the 33 days on which blood samples were taken was there a significant difference in plasma concentrations of either LH or FSH between the control ewes and those immunized with the inhibin

preparation (Wilcoxon rank sum tests). Moreover, the overall mean plasma concentrations of FSH and LH of the four inhibin-immunized ewes (116, 125, 142 and 227  $\mu\text{g FSH/l}$ ; 1.6, 2.3, 3.3 and 4.2  $\mu\text{g LH/l}$ ;  $n=33$ ) did not differ significantly (Wilcoxon rank sum test) from those of the four control ewes (88, 107, 107 and 214  $\mu\text{g FSH/l}$ ; 2.0, 2.5, 3.1 and 4.8  $\mu\text{g LH/l}$ ;  $n=33$ ).

**DISCUSSION**

This study showed that ovulation rate in sheep increased significantly by active immunization with a partially purified preparation of inhibin obtained from bovine follicular fluid. This finding provides further support for the notion that inhibin is physiologically important in the regulation of ovarian function in the ewe (Goodman, Pickover & Karsch, 1981; Cummins, O'Shea, Bindon *et al.* 1983). Although inhibin was not purified to homogeneity, the protein content of the inhibin preparation was only 0.4% of the starting follicular fluid.

The mechanism by which immunization with the inhibin preparation increased ovulation rate is unclear. Previous studies have suggested that plasma FSH concentrations may influence ovulation rate in sheep (Davis, Brien, Findlay & Cumming, 1981) and, indeed, exogenous administration of FSH will increase ovulation rate in ewes (Wright, Bondioli, Grammer *et al.* 1981). If inhibin has a physiological role in suppressing FSH production in sheep then one might have anticipated that immunization against inhibin may have raised plasma FSH concentrations through inhibin antibodies effectively 'neutralizing' circulating inhibin, thereby releasing the pituitary gland from the suppressive effect of inhibin. Raised plasma FSH concentrations may then have allowed additional follicles to

TABLE 1. Ovulation rates (OR) of control and inhibin-immunized ewes in the first four oestrous cycles after the primary immunization

Sheep no.	No. of CL (OR) in oestrous cycle:				Mean OR per ewe	Overall OR (mean $\pm$ S.E.M.)
	1	2	3	4		
<b>Controls</b>						$1.31 \pm 0.06$
1	1	1	2	1	1.25	
2	1	1	1	2	1.25	
3	2	1	1	1	1.25	
4	1	2	1	2	1.50	
<b>Inhibin-immunized</b>						$2.06 \pm 0.16^*$
5	2	2	2	2	2.00	
6	3	2	3	2	2.50	
7	1	2	2	2	1.75	
8	2	2	2	2	2.00	

\* $P < 0.05$  compared with control ewes (Wilcoxon rank sum test).  
CL, corpora lutea.

achieve ovulatory maturity. Immunization with the inhibin preparation, however, had no significant effect on plasma FSH concentrations, although the overall mean plasma FSH concentration was higher in the inhibin-immunized ewes (153 µg FSH/l) compared to the controls (129 µg FSH/l). Recent studies indicate that follicular inhibin is produced in largest amounts by large, healthy follicles and that LH-stimulated thecal androgens may be important in stimulating follicular inhibin production (Henderson & Franchimont, 1983; Henderson *et al.* 1984). Thus, follicular inhibin may only have a major role in regulating plasma FSH concentrations during a limited part of the oestrous cycle, perhaps only during the final stages of follicular maturation before the ovulatory LH surge when LH secretion increases and plasma FSH concentrations fall (Baird, Swanston & McNeilly, 1981). Frequent blood sampling throughout the follicular phase may have been more appropriate than the thrice weekly bleeds taken in the present study.

Immunization with the inhibin preparation produced a rapid effect. An increase in mean ovulation rate, relative to the control ewes, was observed in the first ovulations after the primary immunization (Table 1). Moreover, there was no further increase in ovulation rate in response to the booster immunizations with the inhibin preparation. Antibodies to the inhibin preparation could not be detected by the precipitation test until after the second immunization with inhibin. However, it is possible that lower concentrations of antibody, not detectable by this test, were present earlier, in amounts sufficient to account for the rapid effect that immunization with inhibin had on ovulation rate. It is also conceivable that the inhibin preparation was having a direct action on the pituitary gland. For instance, the continuous release of inhibin from the adjuvant could desensitize pituitary receptors for inhibin (Sairam, 1981; Steinberger, Seethalakshmi, Kessler & Steinberger, 1982), causing the pituitary gland to become refractory to circulating inhibin thereby allowing increased secretion of FSH. Also, direct action of inhibin on the ovary itself cannot be excluded.

Nevertheless, whatever the mechanism of action, the results of this study showed that active immunization of ewes with a preparation of inhibin derived from bovine follicular fluid significantly increased ovulation rate. Thus, immunization with inhibin preparations may have some potential as a means of increasing fecundity in this species.

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## FSH influences follicle viability, oestradiol biosynthesis and ovulation rate in Romney ewes

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**Summary.** Injection of steroid-free bovine follicular fluid (bFF; 2 × 5 ml s.c. 12 h apart) into anoestrous ewes lowered plasma FSH concentrations by 70% and after 24 h had significantly ( $P < 0.01$ ) reduced the number of non-atretic follicles ( $\geq 1$  mm diam.) without influencing the total number of follicles ( $\geq 1$  mm diam.) compared to untreated controls. Hourly injections of FSH (10  $\mu$ g i.v. NIH-FSH-S12) for 24 h did not influence the number of non-atretic follicles but did negate the inhibitory effects of bFF on follicular viability. Hourly injections of FSH (50  $\mu$ g i.v., NIH-FSH-S12) + bFF treatment for 24 h significantly increased the total number of non-atretic follicles, and particularly the number of medium to large non-atretic follicles ( $\geq 3$  mm diam.) compared to the untreated controls (both  $P < 0.01$ ). The 10  $\mu$ g FSH regimen (without bFF) significantly increased aromatase activity in granulosa cells from large ( $\geq 5$  mm diam.;  $P < 0.01$ ) but not medium (3-4.5 mm diam.) or small (1-2.5 mm diam.) follicles compared to controls. The 10  $\mu$ g FSH + bFF regimen had no effect on granulosa-cell aromatase activity compared to the controls. However, the 50  $\mu$ g FSH plus bFF regimen increased the aromatase activity of granulosa cells from large, medium and small non-atretic follicles 2.6-, 8.3- and  $\geq 11$ -fold respectively compared to that in the control cells.

Ewes ( $N = 11$ ) that ovulated 2 follicles had significantly higher plasma FSH concentrations from 48 to 24 h and 24 to 0 h before the onset of a cloprostenol-induced follicular phase (both  $P < 0.01$ ) than in the ewes ( $N = 12$ ) that subsequently ovulated one follicle. Hourly FSH treatment (1.6  $\mu$ g i.v., NIAMDD-FSH-S15) for 24 h but not for any 6 h intervals between 48 and 24 h or 24 and 0 h before a cloprostenol-induced luteolysis also resulted in significant increases ( $P < 0.05$ ) in the number of ewes with 2 ovulations.

We conclude that (1) the number of non-atretic antral follicles in sheep ovaries is influenced by plasma FSH concentrations; (2) the level of follicular oestradiol biosynthesis can be enhanced by FSH treatment; and (3) sustained elevations of plasma FSH concentrations for 24 h but not 6 h within 48 h of the onset of luteolysis significantly enhances the ovulation rate in Romney ewes.

### Introduction

Follicle-stimulating hormone (FSH) is believed to act specifically on granulosa cells to influence the viability of ovarian follicles and ultimately their ability to ovulate (Hirshfield, 1979; Richards, 1980; Peters & McNatty, 1981; Monniaux, Chupin & Saumande, 1983). The viability of an antral follicle may be assessed from its morphological characteristics and/or the ability of its granulosa cells to synthesize oestradiol-17 $\beta$  (Byskov, 1978; McNatty, 1981; Tsafri & Braw, 1984; McNatty *et al.*, 1985). Pregnant mares' serum gonadotrophin (PMSG) is known to influence the proportions of

atretic and non-atretic follicles in rats and sheep without altering the total antral follicle population (Peters, Byskov, Himmelstein-Braw & Faber, 1975; Dott, Hay, Cran & Moor, 1979; Braw & Tsafiriri, 1980; McNatty *et al.*, 1982). However, it is not known whether the 'atresia-preventing' or 'atresia-reversing' effects of PMSG are due solely to its FSH-like properties or to those of both LH and FSH (Monniaux *et al.*, 1983). In rats, FSH is known to induce/activate oestrogen-synthetase (aromatase) activity in granulosa cells (Dorrington, Moon & Armstrong, 1975), but in sheep it is not known whether FSH influences granulosa-cell aromatase activity directly by modulating enzyme activity or indirectly by influencing follicle viability (McNatty, 1981).

FSH treatment of animals of various species increases the ovulation rate (Greenwald, 1962; Laster, 1973; Elsdon, Nelson & Seidel, 1978; Wright, Bondioli, Grammer, Kuzan & Menino, 1981; Armstrong & Evans, 1983), whereas low concentrations of plasma FSH resulting from a clinical disturbance, oestrogen implants or follicular fluid treatment leads to a short-term delay in ovulation, anovulation or inadequate luteal function (Ross *et al.*, 1970; Stouffer, Coensgen, di Zerega & Hodgen, 1981; Zeleznik, 1981; McNeilly, 1984). Ewes and cows that ovulate 2 or more follicles might therefore do so because they have higher FSH concentrations than do animals that ovulate only one follicle. However, in sheep, the evidence for a positive relationship between endogenous FSH concentrations and ovulation rate has been equivocal (Bindon, Blanc, Pelletier, Terqui & Thimonier, 1979; Scaramuzzi & Radford, 1983; Lahlou-Kassi, Schams & Glatzel, 1984).

The aim of this study on Romney ewes was to examine the temporal relationships between FSH concentrations in plasma and follicle viability, granulosa-cell aromatase activity and ovulation rate.

### Materials and Methods

The animals in this study were 2.5- to 3.5-year-old parous Romney ewes.

*Experiment 1* was designed to examine the effects of lowering or raising the plasma FSH concentrations on follicle viability and granulosa-cell aromatase activity. In Romney ewes, the levels of granulosa-cell aromatase activity do not differ for a given follicle size during the oestrous cycle or anoestrus (McNatty *et al.*, 1984). However, during anoestrus, the ovarian follicle population ( $\geq 1$  mm diam.) is approximately 2-fold higher than that during the oestrous cycle. We therefore reasoned that plasma FSH effects on ovarian follicle viability would more easily be observed during anoestrus. Accordingly, anoestrous ewes ( $N = 28$ ) were injected with saline (0.9% w/v NaCl;  $N = 8$ ), steroid-free bovine follicular fluid ( $N = 6$ ; WA batch V, bFF; 2 injections of 5 ml s.c. 12 h apart), ovine FSH ( $N = 6$ ; 10  $\mu\text{g}$  NIH-FSH-S12 (biopotency = 1.25 U/mg; 1 U = 1 mg NIH-FSH-S1) i.v. once per h for 24 h), ovine FSH ( $N = 4$ ; 10  $\mu\text{g}$  NIH-FSH-S12 injected as above) plus bFF (injected as above), or ovine FSH ( $N = 4$ ; 50  $\mu\text{g}$  NIH-FSH-S12 injected at hourly intervals as above) plus bFF injected at 12-h intervals (as above). The purpose of injecting bFF was to lower the FSH concentrations in blood (McNeilly, 1984). The method for removing steroids from bFF was identical to that described by Henderson & Franchimont (1981). The ovaries of all the above animals were excised for further study 24 h after the start of treatment. In an additional study to ascertain the influence of bFF (WA batch V; 2  $\times$  5 ml injections s.c. 12 h apart) on FSH concentrations, blood samples were taken hourly from 16 anoestrous ewes (8 controls, 8 treated animals, 2.5 ml blood/sample) for 6 h before the first bFF injection and every hour thereafter for 24 h.

*Experiment 2* was designed to compare plasma FSH concentrations in ewes with two and one ovulations before, and after, an injection of cloprostenol (125  $\mu\text{g}$ ; ICI Tasman Vaccine, Upper Hutt, N.Z.) on Days 8–10 of the oestrous cycle. On the day before blood sampling began, 44 ewes were penned indoors and each was fitted with an intrajugular cannula. When blood sampling began, the animals were bled (2.5 ml) through the jugular cannulae, once every hour for 120 consecutive hours. Cloprostenol was injected into all ewes after 72 h and oestrous activity was recorded in all ewes by using 2 vasectomized Romney rams fitted with marking harnesses which were

introduced to the ewes 102.5 h after blood sampling began. At the end of the intensive blood sampling schedule, all animals were sent out to pasture and bled once daily for 21 days. After 7 days at pasture all animals were subjected to laparoscopy to determine their ovulation rates. Eleven animals had 2 corpora lutea (CL), 30 had 1 CL and 3 had no visible CL. The blood samples from 12 of the animals with a single CL and all animals with 2 CL were retained for a study of FSH concentrations in blood. The use of only 12 of the animals with 1 CL was due to the limited amount of FSH antiserum.

*Experiment 3.* In Exp. 3, luteal phase (Days 7–10) ewes were injected with FSH (NIAMDD-FSH-S15 (biological potency 20 U/mg; 1 U = 1 mg NIH-FSH-S1), 1.6 µg i.v. once per h) for 6 or 24 h before or after an injection of cloprostenol (125 µg i.m.) to induce ovulation. The different FSH preparation in Exp. 3 from that used in Exp. 1 was determined by the availability of materials from the National Institute of Arthritis, Metabolism and Digestive Diseases, U.S.A. In the 6-h FSH injection regimen, there were 10 control and 10 treated ewes in each 6-h time frame from 72 h before to 24 h after cloprostenol treatment. In the 24-h FSH treatment regimen there were 15 control and 15 treated ewes covering the 24-h time frames –72 to –48, –48 to –24, –24 to 0 and 0 to +24 h from cloprostenol injection (given at time 0). All ewes were subjected to laparoscopy 6–12 days after cloprostenol injection. During the experimental period, the animals were penned indoors in the absence of a ram. To determine FSH concentrations before and after FSH treatment, 10 of the above animals injected with FSH hourly for 24 h were blood sampled by venepuncture before the first FSH injection, at 1-h after the first injection, and again at 10-min intervals until 1 h after the second injection.

The potency of the NIH-FSH-S15 preparation (oFSH) and that of a human FSH preparation (NIAMDD-FSH-2; hFSH) was assessed by measuring their ability to stimulate adenosine cyclic 3',5'-monophosphate (cAMP) synthesis from pools of granulosa cells recovered from non-atretic follicles ( $\geq 3$  mm diam.), atretic follicles ( $\geq 3$  mm diam.) and from a mixture of atretic and non-atretic follicles (1–2.5 mm diam.). The granulosa cells were recovered from 75 follicles ( $\geq 1$  mm diam.) of 2 ewes on Day 10 of the oestrous cycle.

All the heparinized blood samples from Exps 1, 2 and 3 were centrifuged (4000 g at 18–20°C for 20 min) within 15 min of collection and the plasma recovered and frozen to –20°C until hormone analysis.

*Ovarian studies.* Excised ovaries were weighed and their gross morphology recorded. All individual follicles ( $\geq 1$  mm diam.) were dissected free of extraneous tissue under a stereomicroscope and their diameters recorded to the nearest 0.5 mm. A small slit was made in the follicle wall to allow the antral fluid to escape into a Petri dish whence it was aspirated through a finely drawn-out Pasteur pipette, taking care not to remove clumps or sheets of granulosa cells. The fluid was then discarded. The granulosa cells were recovered and counted by haemocytometer. During the recovery of follicular fluid or granulosa cells, the oocyte was also isolated and subjectively assessed as healthy or degenerate as previously described (McNatty *et al.*, 1985). To help classify follicles as non-atretic or atretic, the presence or absence of thecal blood capillaries (at  $\times 10$  magnification) and of debris in follicular fluid was noted. In addition, after removal of the granulosa cells, the colour of the theca interna (i.e. red, pink or white) was recorded. For the purpose of this study a healthy follicle was defined as one with: (a) visible thecal blood capillaries, (b) follicular fluid devoid of debris, (c) an apparently healthy oocyte, (d) a pink- to red-coloured theca interna, and (e)  $> 25\%$  of the maximum number of granulosa cells for a given follicle size (McNatty *et al.*, 1985). Conversely, an atretic follicle was one to which any of these criteria did not apply.

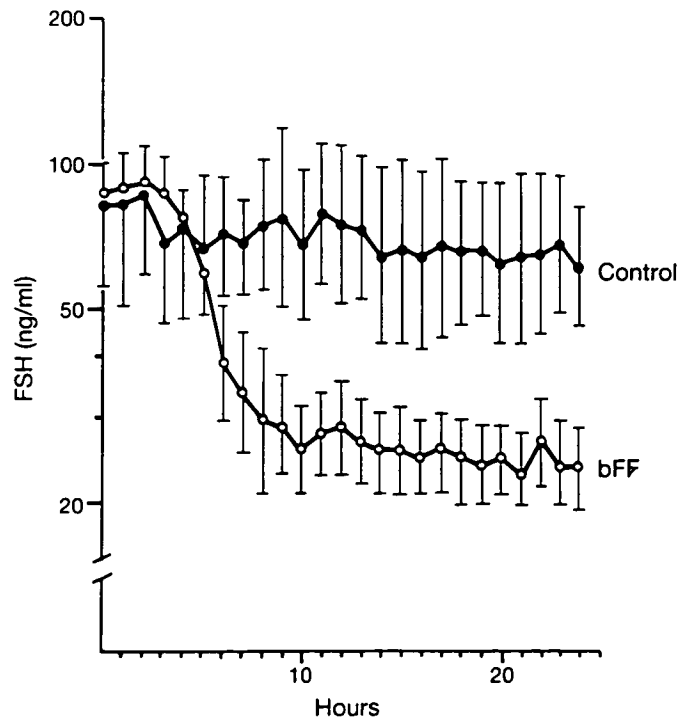
*Granulosa cell aromatase assay.* Pools of granulosa cells from follicles at similar stages of development (3 experiments) were collected into Medium 199 containing sodium bicarbonate (0.85 g/l), Earle's salts, L-glutamine (0.10 g/l), HEPES buffer (20 mM) and 1% BSA (w/v) (Medium A, pH = 7.4). They were washed and resuspended in Medium A so that the final cell concentration was  $6\text{--}60 \times 10^4$  granulosa cells/ml; 0.5 ml aliquants of these cell suspensions were placed in



10 mm × 75 mm plastic tubes containing 0.5 ml of a solution of 2000 ng testosterone/ml. The cell suspensions were gassed with 5% CO<sub>2</sub> in air, stoppered and then incubated for 3 h in a shaking water bath at 37°C. At the end of the incubation, the tubes containing medium plus cells were frozen at -20°C. Subsequently, the contents of the tubes were thawed, centrifuged and the supernatants assayed for oestradiol-17β.

*Granulosa cell incubations for cAMP measurements.* The granulosa cells from non-atretic or atretic follicles of ≥ 3 mm diameter or from a mixture of non-atretic and atretic follicles of 1–2.5 mm diameter in Medium A were centrifuged and the pellet resuspended in Dulbecco's phosphate-buffered saline solution (KC Biological Inc., Lenexa, Kansas, U.S.A.) plus 0.1% BSA (Medium B) to a concentration of 2–5 × 10<sup>5</sup> cells/ml. Aliquants (0.5 ml) of these cells were added to 10 mm × 75 mm test-tubes containing 0.5 ml Medium B with 0, 1, 10, 100, 1000 or 10 000 ng of ovine FSH (NIH-FSH-S15) or human FSH (NIAMDD-hFSH-2; biopotency 3926 i.u. FSH/mg, reference preparation WHO FSH/LH 70/45). The tubes were capped and then incubated for 1 h in a shaking water bath at 37°C. All assays were performed in triplicate with the appropriate controls. At the end of the incubation, the assay tubes were plunged into boiling water for 20 min and then snap frozen to -70°C. Subsequently, the contents of the tubes were thawed and assayed for cAMP.

*Assays.* The heterologous radioimmunoassay for ovine FSH was identical to that described by McNatty *et al.* (1984). Briefly, the FSH standard was NIH-FSH-S11 (1.15 U/mg), a Papkoff ovine FSH preparation G4-150C (Dr H. Papkoff, Hormone Research Laboratory, University of California, San Francisco, U.S.A.) was used as the iodinated tracer, and the antibody was a rabbit anti-human FSH (M-94) supplied by Dr W. Butt (Women's Hospital, Birmingham, U.K.). The mean within- and between-assay coefficients of variation were 5.6 and 8.5% respectively over the working assay range of 2–30 ng per tube.



**Text-fig. 1.** Effect of steroid-free bovine follicular fluid (bFF), 5 ml s.c. at time 0 and 12 h on plasma FSH concentrations in anoestrous Romney ewes. Results are expressed as geometric means and 95% confidence limits (i.e. vertical bars; 8 controls, 8 treated ewes).

Progesterone in plasma and oestradiol-17 $\beta$  in culture medium were measured by radioimmunoassay procedures described in detail elsewhere (McNatty *et al.*, 1982, 1984). For progesterone which was extracted from plasma with 2  $\times$  5 volumes of petroleum ether (boiling range 40–50 $^{\circ}$ ), the antiserum used was WA-26 (see McNatty *et al.*, 1984, for details concerning cross-reactivity) and the minimal detectable quantity in plasma was 200 pg/ml.

Oestradiol-17 $\beta$  in Medium A (the aromatase assay solution) was measured directly, without extraction. The antiserum used was WA-27 (McNatty *et al.*, 1984) and the minimum detectable quantity of oestradiol-17 $\beta$  in 0.1 ml Medium A was 5 pg.

The intra- and inter-assay coefficients of variation for the above steroids were < 10%.

The media (0.1 ml) were assayed directly, without extraction, for cAMP by radioimmunoassay using the New England Nuclear  $^{125}$ I-labelled cAMP radioimmunoassay kit; the acetylation step was included. The results were expressed as pmol cAMP/10 $^6$  granulosa cells. All samples were measured in the same assay; the intra-assay coefficient of variation was 8%.

*Analysis of results.* To compare FSH concentrations in animals that subsequently ovulated 1 or 2 follicles (i.e. Exp. 2), a log-transformed mean FSH concentration for each animal was determined for each of the following time frames: –72 to –48, –48 to –24, –24 to 0 and 0 to +24 h from cloprostenol injection. Thereafter, for each of the above time frames, the overall mean for animals that had 1 CL was compared with that for animals that had 2 CL by an unpaired two-tailed Student's *t* test.

The numbers of animals with a single or a double ovulation after exogenous FSH treatment (Exp. 3) were compared to the corresponding numbers in the untreated controls by  $\chi^2$  analysis; in these experiments none of the animals ovulated more than 2 follicles.

The effects of bFF and/or FSH inoculation in sheep on follicle number, follicle viability and aromatase activity were evaluated by analysis of variance.

## Results

### *Effect of FSH and/or steroid-free bovine follicular fluid (bFF) treatment on plasma FSH concentrations, follicle viability and granulosa-cell aromatase activity (Exp. 1)*

The FSH concentrations in anoestrous ewes injected with bFF or saline (2  $\times$  5 ml injections s.c. 12 h apart) are shown in Text-fig. 1. Treatment with bFF caused a progressive reduction in the geometric mean (95% confidence limits in parentheses) plasma FSH concentrations from 88 (76, 100) ng/ml at the start of treatment to 26 (21, 32) ng/ml some 10 h later ( $P < 0.01$ ); the latter concentrations once reached were maintained for at least 14 h.

The effects of bFF and/or FSH treatment on the number of non-atretic follicles and their distribution with respect to follicular diameter are shown in Table 1. There was no effect of any of the treatments on the total number of follicles present in each ewe. Treatment with bFF significantly reduced the number of medium to large ( $\geq 3$  mm diam.) non-atretic follicles. Treatment of ewes with the 10  $\mu$ g FSH regimen alone had no effect on the numbers of non-atretic follicles, but the 10  $\mu$ g FSH + bFF regimen neutralized the suppressive effects observed in ewes treated with bFF alone. Treatment of ewes with a 50  $\mu$ g FSH + bFF regimen significantly increased the number of non-atretic follicles and the number of medium to large non-atretic follicles ( $\geq 3$  mm diam.).

The effects of bFF and/or FSH inoculation on aromatase activity in granulosa cells are summarized in Table 2. The 10  $\mu$ g FSH regimen increased aromatase activity in cells from follicles of  $\geq 5$  mm diameter 4.7-fold compared to that in control ewes and 1.8-fold compared to that in ewes treated with bFF + 50  $\mu$ g FSH. In contrast, treatment with 10  $\mu$ g FSH had no stimulatory effect on aromatase activity in medium or small diameter follicles. Aromatase activity in cells from the ewes treated with bFF + 10  $\mu$ g FSH was not significantly different from that in the control animals, but activity in cells from the bFF + 50  $\mu$ g FSH-treated ewes was significantly different from that in the

**Table 1.** Effect of steroid-free bovine follicular fluid (bFF) and/or FSH treatment in sheep on the number of non-atretic follicles and their distribution with respect to follicular diameter

Treatment	No. of ewes	Total no. of follicles ( $\geq 1$ mm diam.) per ewe	No. of non-atretic follicles ( $\geq 1$ mm diam.) per ewe	No. of non-atretic follicles with respect to follicular diameter (mm)	
				$\geq 3$	1-2.5
Control	8	33.5 $\pm$ 3.6	7.3 $\pm$ 0.7	3.3 $\pm$ 0.5	4.0 $\pm$ 0.6
bFF	6	30.7 $\pm$ 3.9	*1.5 $\pm$ 0.5	*0.2 $\pm$ 0.2	1.3 $\pm$ 0.5
10 $\mu$ g FSH	6	29.0 $\pm$ 3.3	8.7 $\pm$ 0.7	2.2 $\pm$ 0.3	6.5 $\pm$ 0.6
10 $\mu$ g FSH + bFF	4	36.2 $\pm$ 4.7	7.7 $\pm$ 1.5	3.2 $\pm$ 0.8	4.5 $\pm$ 0.9
50 $\mu$ g FSH + bFF	4	39.0 $\pm$ 5.3	*23.8 $\pm$ 3.7	*18.3 $\pm$ 3.5	5.5 $\pm$ 1.1

Values are mean  $\pm$  s.e.m.

\*Values significantly different from that of control ( $P < 0.01$ ; ANOVA).

**Table 2.** Effect of steroid-free bovine follicular fluid (bFF) and/or FSH treatment in sheep on aromatase activity (ng oestradiol-17 $\beta$ /10<sup>6</sup> granulosa cells/3 h) in granulosa cells from non-atretic follicles

Treatment	Follicle diameter (mm)		
	$\geq 5$	3-4.5	1-2.5
Control	5.3 $\pm$ 0.3	2.9 $\pm$ 0.2	$\leq 0.3$
bFF	3.4 $\dagger$	—	$\leq 0.3$
bFF + 10 $\mu$ g FSH	5.6 $\pm$ 0.2	3.0 $\pm$ 0.3	$\leq 0.3$
bFF + 50 $\mu$ g FSH	14.0 $\pm$ 0.6*	24.2 $\pm$ 3.6*	3.3 $\pm$ 0.3*
10 $\mu$ g FSH	25.1 $\pm$ 0.6 $\ddagger$	2.6 $\pm$ 0.1	$\leq 0.3$

Values are mean  $\pm$  s.e.m. for 3 follicles.

\*Value significantly different from that of control ( $P < 0.01$ ; ANOVA).

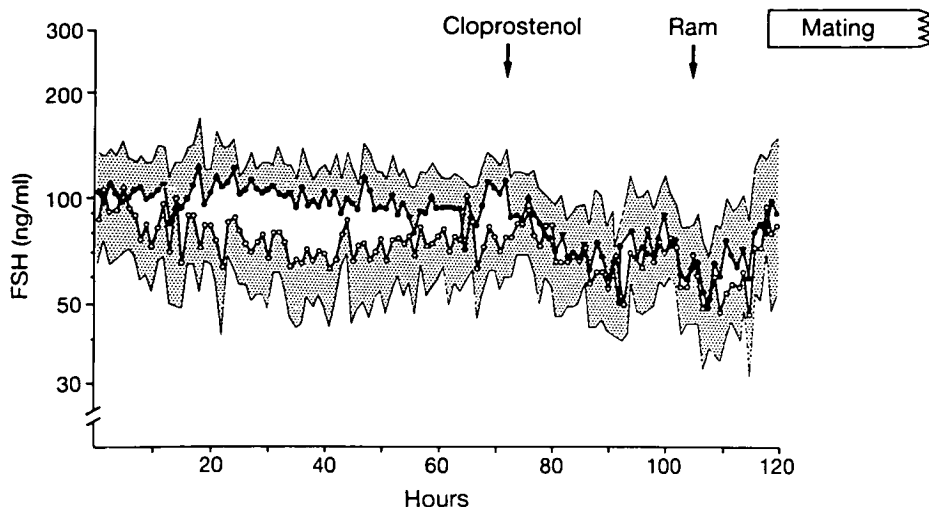
$\dagger$ Data from only one follicle and was excluded from the ANOVA.

$\ddagger$ Value significantly different from value of bFF + 50  $\mu$ g FSH ( $P < 0.01$ ; ANOVA).

control animals ( $P < 0.01$ ) over all follicle diameters. Indeed, the activity in follicles of 3-4.5 mm diameter was similar to that observed in cells from follicles of  $\geq 5$  mm diameter from ewes treated with 10  $\mu$ g FSH.

#### FSH concentrations in ewes with 1 or 2 ovulations (Exp. 2)

These data are summarized in Text-fig. 2. There were no significant differences in the geometric mean FSH concentrations between ewes that subsequently ovulated 1 or 2 follicles over the time frames -72 to -48 h, 0 to +24 h and +24 to +48 h from cloprostenol injection. However, over the time frames -48 to -24 h and -24 to 0 h from cloprostenol injection, the respective geometric mean FSH concentrations in the ewes that subsequently ovulated 2 follicles were significantly higher than in those that ovulated 1 follicle (both  $P < 0.01$ , unpaired Student's *t* test). The overall geometric mean (and 95% confidence limits) for the FSH concentrations at -48 to -24 h was 105 (102, 107) ng/ml for ewes that subsequently had 2 CL compared to 74 (72, 77) ng/ml for the ewes that subsequently had 1 CL, whereas at -24 to 0 h from cloprostenol injection the FSH concen-



**Text-fig. 2.** Geometric mean concentrations of plasma FSH each hour for Romney ewes from  $-72$  h before until  $48$  h after cloprostenol injection ( $125 \mu\text{g s.c.}$ ): ●—● represents the results for animals ( $N = 11$ ) that subsequently formed 2 corpora lutea whereas ○—○ represents the results for animals ( $N = 12$ ) that subsequently formed only 1 corpus luteum. The vasectomized rams were introduced to the flock at  $30.5$  h and the matings occurred from  $46$  to  $96$  h after cloprostenol injection. Shaded areas = 95% confidence limits. For the sake of clarity only the upper or lower limits are shown although these are equidistant each side of the mean.

trations were  $96$  ( $90, 102$ ) and  $79$  ( $76, 82$ ) ng/ml for the ewes that subsequently had 2 and 1 CL respectively.

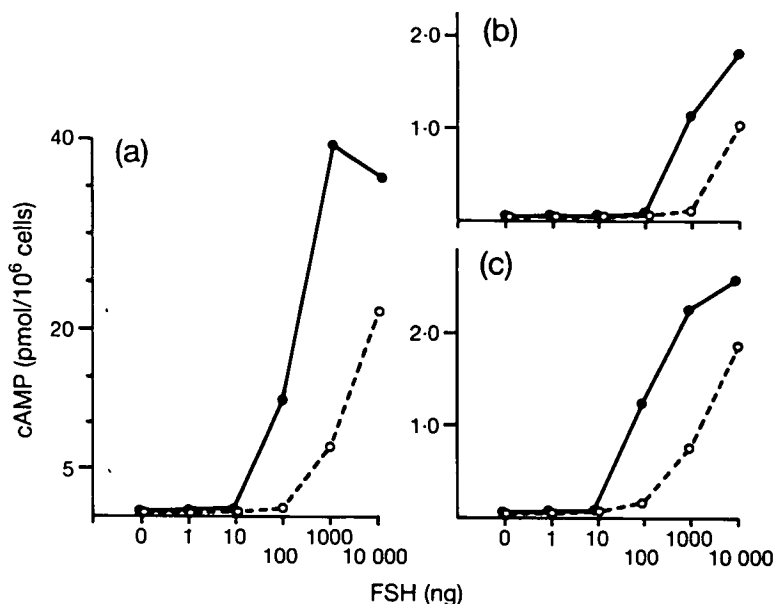
In the cycle after cloprostenol injection all ewes had plasma progesterone concentrations  $\geq 1$  ng/ml by 5 days after mating and all were adjudged to have had normal CL since the progesterone concentrations were  $\geq 1$  ng/ml for at least 8 days of the cycle. No significant differences were noted in mean daily concentrations of progesterone between those with 1 or 2 CL.

#### *Effect of FSH treatment on granulosa-cell cAMP synthesis and ovulation rate (Exp. 3)*

The effects of different doses of ovine FSH (NIAMDD-FSH-S15) and human FSH (NIAMDD-hFSH-2) on stimulation of granulosa cell cAMP synthesis are shown in Text-fig. 3. Doses of  $1$  and  $10 \mu\text{g}$  human FSH/ml and  $10 \mu\text{g}$  ovine FSH/ml stimulated granulosa-cell cAMP synthesis in all follicles. The  $10 \mu\text{g}$  dose of ovine FSH stimulated a cAMP response which was only 56–70% of that achieved with  $10 \mu\text{g}$  human FSH.

In the 10 ewes injected (i.v.) hourly with  $1.6 \mu\text{g}$  ovine FSH (NIH-FSH-S15) and blood sampled before and during treatment (see 'Materials and Methods'), the geometric mean (and 95% confidence limits) for the FSH concentrations before and during the first 2 h of treatment were  $81$  ( $59, 111$ ) and  $98$  ( $74, 128$ ) ng/ml respectively. The overall geometric mean FSH concentration during FSH supplementation was  $121 \pm 3\%$  of that before treatment.

There were no significant increases in the subsequent ovulation rates in treated compared to control animals when the treated ewes were injected with  $1.6 \mu\text{g}$  ovine FSH (NIAMDD-FSH-S15) once per h for 6 h over any of the consecutive 6-h time frames from  $72$  h before to  $24$  h after cloprostenol injection. However, when ewes were injected with  $1.5 \mu\text{g}$  ovine FSH (NIAMDD-FSH-S15) once per h for 24 h, the subsequent mean ovulation rates in the FSH and control ewes over the time frames  $-72$  to  $-48$  h,  $-48$  to  $-24$  h,  $-24$  to  $0$  h and  $0$  to  $+24$  h from cloprostenol injection were respectively:  $1.00$  and  $1.07$ ,  $1.40$  and  $1.07$ ;  $1.47$  and  $1.07$ ; and  $1.27$  and  $1.07$ . In this study,



**Text-fig. 3.** Effect of different doses of human FSH (●—●; NIAMDD-hFSH-2) and ovine FSH (○---○; NIAMDD-FSH-S15) on stimulation of cAMP synthesis by ovine granulosa cells from non-atretic (a) or atretic (b) follicles of  $\geq 3$  mm diameter or (c) a mixture of non-atretic and atretic follicles of 1–2.5 mm diameter. Results are those from a single experiment (each point in triplicate) on pools of cells recovered from two pairs of sheep ovaries.

the treated and control ewes had 1 or 2 CL; no animals were identified with 3 or more CL. When the numbers of ewes with 1 or 2 CL in the treated and control groups were compared by  $\chi^2$  analysis, there was a significant effect of FSH treatment over the 24 h time frames of  $-48$  to  $-24$  or  $-24$  to  $0$  h (both  $P < 0.05$ ) from PG injection but not for the other previously stated time frames.

### Discussion

The results of this study show that the proportions of non-atretic and atretic follicles ( $\geq 1$  mm diam.) in ovaries of anoestrous ewes are influenced markedly by the FSH concentrations in plasma. Compared to control ewes, treatment with bovine follicular fluid led to a 70% reduction in the plasma FSH concentrations and a significant reduction ( $P < 0.01$ ) in the number of non-atretic follicles (i.e. from 22 to 5%) without any alterations in the total number of antral follicles ( $\geq 1$  mm diam.; Table 1). The hourly administration of ovine FSH ( $10 \mu\text{g}$  i.v.; NIH-FSH-S12) for 24 h did not influence either the number of antral follicles ( $\geq 1$  mm diam.) or the number of non-atretic follicles compared to the controls. Nevertheless, the  $10 \mu\text{g}$  FSH regimen completely negated the effects of bovine follicular fluid when the two treatments were given together; the numbers of non-atretic follicles were similar to those found in the controls. This finding suggests that the effects of bovine follicular fluid were due to a reduction in the plasma concentrations of FSH. The  $50 \mu\text{g}$  + bFF regimen did not influence the total number of antral follicles ( $\geq 1$  mm diam.) but it caused a significant ( $P < 0.01$ ) increase in the number of non-atretic follicles ( $\geq 1$  mm diam.) compared to the untreated controls. This increase was due mainly to a 5.5-fold increase in the number

of non-atretic  $\geq 3$  mm diameter follicles. The FSH treatment dose was presumably in excess of that required to offset the inhibitory effects of bovine follicular fluid and so the ovaries were subjected to a sustained (i.e. 24 h) high level of FSH stimulation.

On the basis of the above results, it seems reasonable to suppose that follicle viability during the oestrous cycle is also influenced by the plasma concentrations of FSH. However, it cannot be assumed that follicle viability during the oestrous cycle would be influenced by FSH in precisely the same way as described in the present study on anoestrous Romney ewes. In this breed of ewe, there are significantly more ( $P < 0.01$ ) antral follicles ( $\geq 1$  mm diam.) and significantly higher ( $P < 0.05$ ) plasma FSH concentrations during anoestrus than during the luteal phase of the oestrous cycle (McNatty *et al.*, 1984).

The present experiments show that FSH stimulates aromatase enzyme activity in ovine granulosa cells from non-atretic follicles as well as influencing follicle viability. The 10  $\mu\text{g}$  FSH regimen caused a 5-fold increase in aromatase activity in large ( $\geq 5$  mm diam.) follicles compared to that from control ovaries. However, the 10  $\mu\text{g}$  FSH regimen had no effect on aromatase activity in medium (3–4.5 mm diam.) or small ( $\leq 2.5$  mm diam.) follicles. The 10  $\mu\text{g}$  FSH + bFF regimen resulted in granulosa cells having a level of aromatase activity identical to that in cells from control ovaries for all follicle diameters. In contrast, the 50  $\mu\text{g}$  FSH + bFF regimen caused a 2.6-fold, an 8.3-fold and a  $\geq 11$ -fold increase in granulosa-cell aromatase activity in cells from large, medium and small follicles, respectively, relative to that in cells from the corresponding controls. Presumably, the high levels of granulosa cell aromatase activity observed in this experiment was due to FSH treatment although some augmentation by effects of bovine follicular fluid cannot be ruled out (McLachlan, Colvin, Quigg, Burger & Lee, 1984).

These results raised the possibility that the FSH concentrations in Romney ewes are normally insufficient to stimulate maximum oestrogen synthesis in developing follicles. For example, the level of granulosa-cell aromatase activity in large follicles ( $\geq 5$  mm diam.) of anoestrous ewes (Table 2) was identical to that observed in preovulatory follicles during the breeding season (McNatty *et al.*, 1984). However, this level of activity (i.e. 5.3 ng oestradiol/ $10^6$  cells/3 h) was only 0.2 times that produced by cells exposed to additional amounts of FSH *in vivo* (Table 2).

A major finding in the present study was that ewes ovulating 2 follicles had significantly higher ( $P < 0.01$ ) plasma FSH concentrations from 48 to 24 h and 24 to 0 h before the onset of luteolysis than did ewes ovulating a single follicle. It seems that the 20–40% higher FSH concentrations before luteolysis in the sheep with twin ovulations (i.e. compared to those with a single ovulation) were causally related to the increase in ovulation rate. The hourly administration of ovine FSH (1.6  $\mu\text{g}$  i.v.; NIH-FSH-S15) increased the mean plasma FSH concentrations by  $\sim 20\%$  ( $P < 0.01$ ) and this FSH regimen over the 24-h time frames of 48 to 24 h or 24 to 0 h before luteolysis also led to a significant increase ( $P < 0.05$ ) in the number of Romney ewes with twin ovulations. This increased frequency of twin ovulations after supplementary ovine FSH treatment was achieved under a dose regimen which was probably submaximal at the level of the granulosa cells. For example, the response of granulosa cells to 1.6  $\mu\text{g}$  ovine FSH (NIH-FSH-S15) in terms of cAMP production *in vitro* was  $\leq 50\%$  of that attainable with human FSH (NIAMDD-hFSH-2). Also, the exogenous ovine FSH regimen was effective when administered for 24 h but not when administered for only 6 h. These findings suggest that during the 6 h treatment regimen the dose of ovine FSH may have been too low and/or that the granulosa cells were not exposed to the additional FSH for a sufficient period of time. The latter is a possibility because of the time required for proteins to traverse to the granulosa cells from the peripheral circulation (Cran, Moor & Hay, 1976). For example, the time delay between an elevation of immunoreactive FSH and/or LH in plasma and a corresponding increase in follicular fluid was about 4 h (McNatty, Dobson, Gibb, Kieboom & Thurley, 1981; Dieleman, Bevers, Poortman & van Tol, 1983).

The administration of PMSG (500 i.u.) simultaneously with cloprostenol is known to enhance ovulation rates in Romney ewes (Gibb, Thurley & McNatty, 1981; McNatty *et al.*, 1982). However, hourly injections of ovine FSH (1.6  $\mu\text{g}$  NIAMDD-FSH-S15 i.v.) or gonadotrophin-releasing

hormone (500 ng i.v.; data not shown) for 24 h from the onset of luteolysis failed to increase the ovulation rate in Romney ewes. Perhaps the difference in effectiveness between the three treatments was due to the longer half-life of PMSG than that of FSH and LH *in vivo* (Schams *et al.*, 1978), although differences in dose rates cannot be ruled out.

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## Use of bovine follicular fluid to increase ovulation rate or prevent ovulation in sheep

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**Summary.** Romney ewes were injected intramuscularly once or twice daily for 3 days with 0, 0.1, 0.5, 1 or 5 ml of bovine follicular fluid (bFF) treated with dextran-coated charcoal, starting immediately after injection of cloprostenol to initiate luteolysis on Day 10 of the oestrous cycle. There was a dose-related suppression of plasma concentrations of FSH, but not LH, during the treatment period. On stopping the bFF treatment, plasma FSH concentrations 'rebounded' to levels up to 3-fold higher than pretreatment values. The mean time to the onset of oestrus was also increased in a dose-related manner by up to 11 days. The mean ovulation rates of ewes receiving 1.0 ml bFF twice daily ( $1.9 \pm 0.2$  ovulations/ewe, mean  $\pm$  s.e.m. for  $N = 34$ ) or 5.0 ml once daily ( $2.0 \pm 0.2$  ovulations/ewe,  $N = 25$ ) were significantly higher than that of control ewes ( $1.4 \pm 0.1$  ovulations/ewe,  $N = 35$ ). Comparison of the ovaries of ewes treated with bFF for 24 or 48 h with the ovaries of control ewes revealed no differences in the number or size distribution of antral follicles. However, the large follicles ( $\geq 5$  mm diam.) of bFF-treated ewes had lower concentrations of oestradiol-17 $\beta$  in follicular fluid, contained fewer granulosa cells and the granulosa cells had a reduced capacity to aromatize testosterone to oestradiol-17 $\beta$  and produce cyclic AMP when challenged with FSH or LH. No significant effects of bFF treatment were observed in small (1-2.5 mm diam.) or medium (3-4.5 mm diam.) sized follicles. Ewes receiving 5 ml bFF once daily for 27 days, from the onset of luteolysis, were rendered infertile during this treatment period. Oestrus was not observed and ovulation did not occur. Median concentrations of plasma FSH fell to 20% of pretreatment values within 2 days. Thereafter they gradually rose over the next 8 days to reach 60% of pretreatment values where they remained for the rest of the 27-day treatment period. Median concentrations of plasma LH increased during the treatment period to levels up to 6-fold higher than pretreatment values. When bFF treatment was stopped, plasma concentrations of FSH and LH quickly returned to control levels, and oestrus was observed within 2 weeks. The ewes were mated at this first oestrus and each subsequently delivered a single lamb.

### Introduction

Follicular fluid is reported to contain a number of non-steroidal compounds including inhibin (Henderson, Franchimont, Charlet-Renard & McNatty, 1984), ovarian inhibitory protein (Kling *et al.*, 1984), oocyte maturation inhibitor (Channing *et al.*, 1982a), luteinization inhibitors (Ledwitz-Rigby *et al.*, 1977) and follicle-stimulating hormone receptor binding inhibitor (Sluss, Fletcher & Reichert, 1983), each of which may be important in regulating reproductive function. Administration of steroid-free bovine follicular fluid (bFF) to ewes specifically suppresses plasma concentrations of follicle-stimulating hormone (FSH), and delays the onset of oestrus if

administered during the preovulatory period (Miller, Critser, Rowe & Ginther, 1979; Cummins, O'Shea, Bindon, Lee & Findlay, 1983; McNeilly, 1984); these effects have been attributed to the inhibin content of follicular fluid. Although plasma FSH concentrations are suppressed during treatment of ewes with bFF, they 'rebound' to levels considerably higher than pretreatment values within 24–36 h of stopping the bFF treatment (Miller, Critser & Ginther, 1982; McNeilly, 1984). Since FSH, or FSH-like substances can be used to increase ovulation rate in sheep (Wright, Bondioli, Grammer, Kuzan & Menino, 1981; Boland, Crosby & Gordon, 1983; Kelly, Owens, Crosbie, McNatty & Hudson, 1983; McNatty *et al.*, 1985), it is possible that using bFF treatment to raise plasma concentrations of FSH might provide a means of increasing ovulation rate in sheep. This possibility was investigated in the present study which was undertaken to examine the effects of short-term (3 days) and long-term (27 days) bFF treatment on plasma gonadotrophin concentrations, follicular function and reproductive activity in Romney ewes.

### Materials and Methods

#### *Preparation of follicular fluid and plasma*

Ovaries were obtained at slaughter from cows at a local abattoir over a 6-month period. At each collection, all antral follicles visible on the surface of the ovaries were punctured with a 20-gauge needle, and the follicular fluid was aspirated under vacuum. Debris and red blood cells were removed from the pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and the supernatant was stored frozen at –20°C until later use. Once an adequate volume of fluid had been collected, the fluids were thawed, pooled and steroids removed as described previously (Henderson & Franchimont, 1981) by mixing for 16 h at 4°C with activated charcoal (Norit A, 1%, Fisher Scientific Co., Springfield, New Jersey, U.S.A.) pretreated with dextran T-70 (0.1% Pharmacia Fine Chemicals AB, Uppsala, Sweden) followed by centrifugation at 3000 g (twice) and filtration of the supernatant (Whatman No. 1). The filtrate was stored frozen at –20°C in volumes of 10–100 ml until required. The experiments in this study were performed using a single batch of bovine follicular fluid (bFF) in which dextran-coated charcoal treatment had reduced the concentrations of oestradiol-17 $\beta$ , androstenedione, testosterone and progesterone to <1% of their original values.

Plasma prepared from jugular venous blood taken from an ovariectomized Angus cow was pooled and treated with dextran-coated charcoal as described above for bFF. This was stored frozen in aliquants until required for treating control ewes.

#### *Sheep, treatments and blood sampling*

Parous New Zealand Romney ewes (aged 2.5–3.5 years and weighing about 50–60 kg) were used in this study. They were grazed on open pasture and run with a vasectomized ram fitted with a marking harness. The ewes were examined twice daily for signs of oestrus. The basic experimental procedure was as follows. On the 10th day after oestrus was observed (Day 0 of an experiment) jugular venous blood samples (~2 ml) were taken from ewes in the morning (08:30 h) and afternoon (16:30 h). The following morning (Day 1, 08:30 h), the ewes were bled again and given an intramuscular (i.m.) injection of the prostaglandin F-2 $\alpha$  analogue, cloprostenol (125  $\mu$ g: ICI Tasman Ltd, Upper Hutt, N.Z.) to initiate luteolysis. Immediately afterwards the ewes were injected (i.m.) with 0.1–5.0 ml bFF (treated ewes) or plasma from an ovariectomized cow (control ewes). Morning, or morning and afternoon, injections of follicular fluid or plasma were continued until the afternoon of Day 3 of the experiment. Twice daily blood samples were taken until the afternoon of Day 3, thereafter once daily blood samples were taken through to Day 11 of the experiment. Blood samples were taken immediately before any injections of bFF or plasma. The number of

ovulations (ovulation rate) was determined by laparoscopic examination of the ovaries of each ewe, 9–12 days after oestrus was observed, and counting the number of corpora lutea present. In modifications of this procedure, some ewes were treated with bFF or plasma and subjected to laparoscopy as above, but no blood samples were taken. In other ewes, ovariectomy was performed immediately following twice daily (08:30 h and 16:30 h) treatment with 1 ml or 5 ml bFF or control plasma for 24 or 48 h. In another group of ewes, the time to the onset of oestrus was determined after 3 days of once daily (08:30 h) treatment with 5 ml control plasma, 5 ml bFF, 5 ml bFF previously heated to 90°C for 45 min and the cooled, or 5 ml bFF plus 1000 i.u. PMSG (Folligon: Intervet International B.V., Boxmeer, The Netherlands) or 1000 i.u. hCG (Gonadotraphon L.H.: Paines & Byrne Ltd, Greenford, U.K.). In a final group of ewes, once daily treatment with 5 ml bFF or control plasma was continued for 27 days, laparoscopy being performed on the day of the last injection. In those ewes, blood samples (08:30 h and 16:30 h) were taken the day before the start of the injections (Day 0), once daily (08:30 h) for the next 3 days (Days 1–3) and thrice weekly thereafter until Day 49 of the experiment.

All blood samples collected were centrifuged at 1200 g for 15 min at 4°C. The plasma was removed and stored frozen (–20°C) until assayed for FSH, luteinizing hormone (LH) and progesterone by radioimmunoassay.

#### *Ovarian dissection and in-vitro studies*

Immediately after ovariectomy of bFF-treated and control ewes, the corpora lutea and all antral follicles  $\geq 1.0$  mm in diameter were individually dissected from each pair of ovaries into sterile Medium 199 with Earle's salts supplemented with HEPES buffer (20 mM), gentamicin (50 µg/ml: Gibco, Grand Island, New York, U.S.A.), sodium heparin (50 i.u./ml: Weddel Pharmaceuticals Ltd, London, U.K.) and 1.0% bovine serum albumin (Fraction V; Sigma Chemical Co, St Louis, MO, U.S.A.) (Medium A). After recording the follicular diameter and examining the thecal vasculature, each follicle was incised to release its contents. The released follicular fluid was examined for the presence or absence of debris, aspirated through a fine-bore capillary tube and stored frozen (–20°C) until assayed for oestradiol-17 $\beta$ . The internal face of the follicle wall was washed gently and repeatedly with 2 ml Medium A and the released clumps of granulosa cells were dispersed by pipetting several times through a finely drawn Pasteur pipette. The oocyte was isolated and assessed subjectively as being healthy or degenerate as previously described (McNatty *et al.*, 1983). The follicle wall was washed several times more, and the total number of granulosa cells in the pooled washings was counted using a haemocytometer. The colour of the theca interna was noted. On the basis of its morphological appearance, each follicle was classified as non-atretic or atretic (McNatty *et al.*, 1984). Follicles considered to be non-atretic were those with: visible thecal capillaries when viewed at  $\times 10$  magnification under a dissecting microscope, no debris in the follicular fluid, an oocyte of healthy appearance,  $\geq 26\%$  of the maximum number of recoverable granulosa cells for a follicle of a given size, and a pink to red theca interna. Follicles were considered to be atretic when one or more of these 5 criteria was not applicable.

The ability of granulosa cells to metabolize testosterone to oestradiol-17 $\beta$  (aromatase activity) and to produce adenosine 3',5'-monophosphate (cAMP) in response to challenge with LH or FSH was determined as described previously (Henderson, Kieboom, McNatty, Lun & Heath, 1985). To obtain sufficient cells from each pair of ovaries to perform replicate determinations it was often necessary to pool cells from follicles of a similar size and classification. Granulosa cells from follicles 1–2.5 mm in diameter were always pooled, cells from follicles 3–4.5 mm in diameter were occasionally pooled while cells from follicles  $\geq 5$  mm in diameter were never pooled. After collecting the granulosa cells, and pooling when appropriate, each sample of cells was split into 2 fractions. The cells were centrifuged at 200 g for 10 min and resuspended in Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin (DBS-BSA) for determining the cAMP response to challenge with LH or FSH, or in Medium A devoid of sodium heparin (Medium

B) for determination of aromatase activity. Aliquants of each were taken for determination of cell number by haemocytometer counts. To determine cAMP production in response to LH and FSH, aliquants of granulosa cells ( $150 \times 10^3$ ) in 0.5 ml DBS-BSA were dispensed into  $10 \times 75$  mm tubes containing 0.5 ml DBS-BSA or 0.5 ml of a solution of ovine FSH (NIADDK-oFSH-16, 200 ng/ml) or ovine LH (NIADDK-oLH-23, 200 ng/ml). The tubes were capped and incubated at 37°C for 1 h in a shaking water bath before being transferred to an 80°C water bath for 15 min. Some tubes were transferred directly to the 80°C bath so that the endogenous cAMP content of the cells at zero time could be determined. All the tubes were frozen ( $-20^\circ\text{C}$ ) until assayed for cAMP production by radioimmunoassay. Preliminary studies showed that cAMP production by granulosa cells incubated at 37°C was constant for 2 h.

For determination of aromatase activity, aliquants of cells ( $60\text{--}600 \times 10^3$ ) in 0.5 ml Medium B were pipetted into  $10 \times 75$  mm plastic tubes containing 0.5 ml of a solution of testosterone (2  $\mu\text{g}/\text{ml}$ ) in Medium B. The tubes were gassed with 5%  $\text{CO}_2$  in air, capped and incubated for 3 h at 37°C in a shaking water bath. At the end of the incubation, the tubes were snap frozen to  $-70^\circ\text{C}$ . Subsequently the tubes were thawed, centrifuged for 15 min at 1500  $g$  and the supernatants assayed for oestradiol-17 $\beta$ . Preliminary studies indicated that the aromatase reaction was constant over the 3 h period.

#### Radioimmunoassays

FSH, LH and progesterone were measured in plasma by specific radioimmunoassays described previously (Scaramuzzi, Caldwell & Moor, 1970; Salamonsen *et al.*, 1973; McNatty, Gibb, Dobson, Thurley & Findlay, 1981).

The standard FSH preparation was NIH-FSH-S11 and Papkoff preparation G4-150C was used as the iodinated tracer. The FSH antiserum was rabbit anti-human FSH (Butt, M94) and was used at an initial dilution of 1:8000. Its cross-reaction with ovine LH (NIH-LH-S21), ovine prolactin (NIH-P-S18), ovine growth hormone (NIH-GH-S11) and ovine thyroid stimulating hormone (NIH-TSH-S8) was  $<0.5\%$ . The sensitivity of the assay was 10 ng FSH/ml plasma. The intra- and inter-assay coefficients of variation were 6% and 9% respectively.

The LH antibody raised in a rabbit against NIH-LH-S11 was used at an initial dilution of 1:40 000. The LH antiserum exhibited low cross-reactions with other pituitary hormones: ovine prolactin (NIH-P-S12) 0.09%; ovine thyroid-stimulating hormone (NIH-TSH-S8) 2.4%; ovine growth hormone (NIH-GH-S11) 0.4%; ovine FSH (NIH-FSH-S10) 0.4%. The pituitary LH preparation for both the standard and the iodinated tracer was NIH-LH-S11. The sensitivity of the assay was 0.2 ng LH/ml plasma and the intra- and inter-assay coefficients of variation were 7% and 11% respectively.

Progesterone was extracted from plasma with petroleum ether and measured using an antiserum (WA-26) raised in an ovariectomized ewe against progesterone-11 $\alpha$ -hemisuccinate conjugated to bovine serum albumin. The extraction efficiency was monitored by the addition of tritiated progesterone, and the mean recovery was 82%. The antiserum was used at an initial dilution of 1:10 000 and major cross-reacting steroids were 11 $\alpha$ -hydroxyprogesterone (120%), 11 $\beta$ -hydroxyprogesterone (25%), 20 $\alpha$ -dihydroprogesterone (3.5%) and androstenedione (0.4%). The sensitivity of the assay was 0.2 ng/ml plasma, and the intra- and inter-assay coefficients of variation were  $<10\%$ .

Oestradiol-17 $\beta$  was measured directly in aliquants of follicular fluid and Medium B (the aromatase assay solution) by radioimmunoassay as described previously (McNatty *et al.*, 1981, 1984). The oestradiol-17 $\beta$  antiserum (WA-27) was raised in an ovariectomized ewe against oestradiol-6-(O-carboxymethyl) oxime conjugated to bovine serum albumin. The antiserum was used at an initial dilution of 1:16 000 and major cross-reacting steroids were oestrone (7%), oestriol (1%) and oestradiol-17 $\alpha$  (1%). The sensitivity of the assay (per tube) was 5 pg and the intra- and inter-assay coefficients of variation were  $<11\%$ .

Cyclic AMP was assayed directly in aliquants of DBS-BSA by radioimmunoassay as described previously (Harper & Brooker, 1975; Henderson *et al.*, 1985). The cAMP antibody was generated in a rabbit using O<sup>2</sup>-monosuccinyl-adenosine 3',5'-monophosphate coupled to bovine serum albumin as the antigen. The gamma globulin fraction was prepared from the serum and used in the radioimmunoassay at an initial dilution of 1:10 000. Relative cross-reactivity of the cAMP antibody with AMP, ADP, ATP, or cGMP was <0.0015%. The sensitivity of the assay (per tube) was 2.5 fmol and the intra- and inter-assay coefficients of variation were 7 and 14% respectively.

### Statistics

For analysis, plasma concentrations of FSH and LH in each ewe from the first day of treatment with bFF or plasma onwards were transformed to a percentage of the mean plasma value on the day before treatment (i.e. Day 0). To avoid making assumptions about the normality of the distribution of this transformation, the transformed data were analysed by non-parametric statistics using the Wilcoxon rank-sum test, or the Kruskal-Wallis test with multiple comparisons between treatments being made using a test based on differences between rank sums (Conover, 1980). The FSH and LH data have therefore been presented as medians and range. Other data were analysed by analysis of variance in conjunction with the Newman-Keuls test, Student's *t* test or  $\chi^2$  as appropriate. When heterogeneity of variance was indicated by Bartlett's test, the data were transformed to logarithms to equalize the variances before statistical analysis. In these instances the data have been presented as geometric means together with 95% confidence limits. The level of significance was set at  $P < 0.05$ .

## Results

### *Effect of 3 days treatment with bFF on plasma gonadotrophin concentrations, time to oestrus and ovulation rate*

Figure 1 shows the effect on plasma concentrations of LH, FSH and progesterone of administration of 5 ml bFF to ewes twice daily for 3 days, starting from the time of initiation of luteolysis with cloprostenol. In bFF-treated ewes, median concentrations of FSH, but not LH, fell rapidly and were significantly lower than those of the plasma treated control ewes from 8 h after the first injection to Day 4 of the experiment. Thereafter median levels of FSH in the bFF-treated ewes 'rebounded', and on Days 5-7, they were significantly higher than those of the control ewes. Median levels of LH were also significantly higher than those of control ewes on Days 4-8.

After the injection of cloprostenol, plasma progesterone concentrations declined in a similar fashion in control and bFF-treated ewes. In the controls, the plasma progesterone concentrations had risen markedly by Day 9 to levels indicative of the formation of new corpora lutea; in these ewes oestrus occurred  $2.2 \pm 0.2$  (mean  $\pm$  s.e.m.) days after cloprostenol treatment. In the bFF-treated ewes, the plasma progesterone concentrations were still at basal levels on Day 9; in these ewes oestrus did not occur until  $10.5 \pm 0.9$  days after cloprostenol treatment.

The above effects of bFF treatment on plasma gonadotrophin concentrations were dose-related (Fig. 2). The lowest median FSH values in response to bFF treatment on Days 1-3 occurred on Day 3, and the 'rebound' in FSH and rise in LH levels occurred during Days 5-8. Twice daily injection of 0.5 ml was the minimum dose of bFF which significantly suppressed plasma FSH concentrations, relative to values in control (0 ml bFF) ewes. The lowest median levels were achieved with 5.0 ml bFF administered twice daily. Twice daily injections of 1.0 ml bFF was the minimum dose causing a significant 'rebound' in FSH and rise in LH levels on Day 5, although by Day 7 the FSH concentrations had returned to control values. The rebound in FSH after injections of 5.0 ml bFF once or twice daily was comparable to that achieved with 1.0 ml bFF twice daily, but of longer

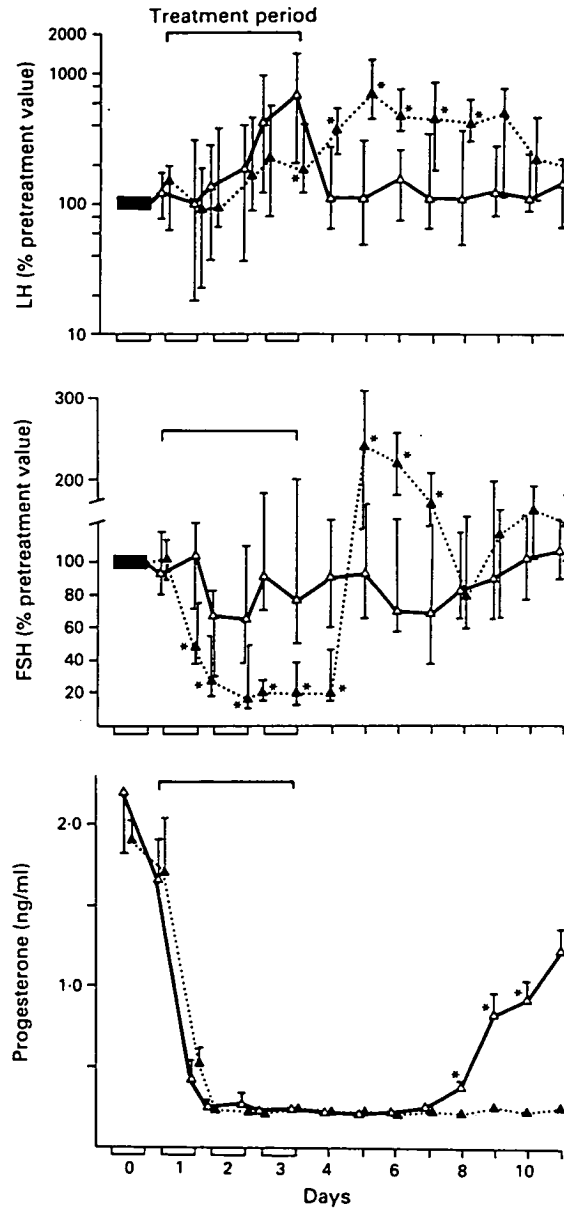


Fig. 1. Plasma concentrations of LH, FSH and progesterone in Romney ewes treated with bovine follicular fluid (5 ml twice daily,  $N = 4$ ,  $\blacktriangle \dots \blacktriangle$ ) or plasma ( $N = 8$ ,  $\triangle - \triangle$ ). LH and FSH values are medians with the range indicated by vertical lines. Progesterone values are means with vertical lines showing the s.e.m. if larger than the symbol for the mean value. Asterisks indicate significant differences ( $P < 0.05$ ) between the 2 groups on the same day.

duration, with elevated concentrations of FSH (relative to control ewes) still apparent on Day 7. By Day 9 there were no significant differences in plasma concentrations of FSH or LH between any of the groups.

The increase in time to oestrus after 3 days of bFF treatment was also dose-related (Table 1), 0.5 ml fluid injected twice daily being the lowest dose able to delay the onset of oestrus. The conse-

quent delay in formation of new corpora lutea was reflected in the plasma progesterone concentrations on Day 11 (Table 1). Between Days 0 and 7 there were no significant differences in plasma progesterone concentrations between any of the bFF doses (see Fig. 1 for mean progesterone values for 0 and 5.0 ml fluid twice daily). Therefore, the dose-related delay to oestrus was not a consequence of the bFF interfering with the cloprostenol-induced luteolysis.

Although none of these doses of bFF that were administered had a significant effect on ovulation rate (Table 1), the mean ovulation rates of ewes receiving 1.0 ml bFF twice daily, or 5.0 once daily, were 64% higher than that of control ewes (Table 1). When these 2 dose regimens were administered to a larger group of ewes, a significant increase in ovulation rate was observed (Table 2).

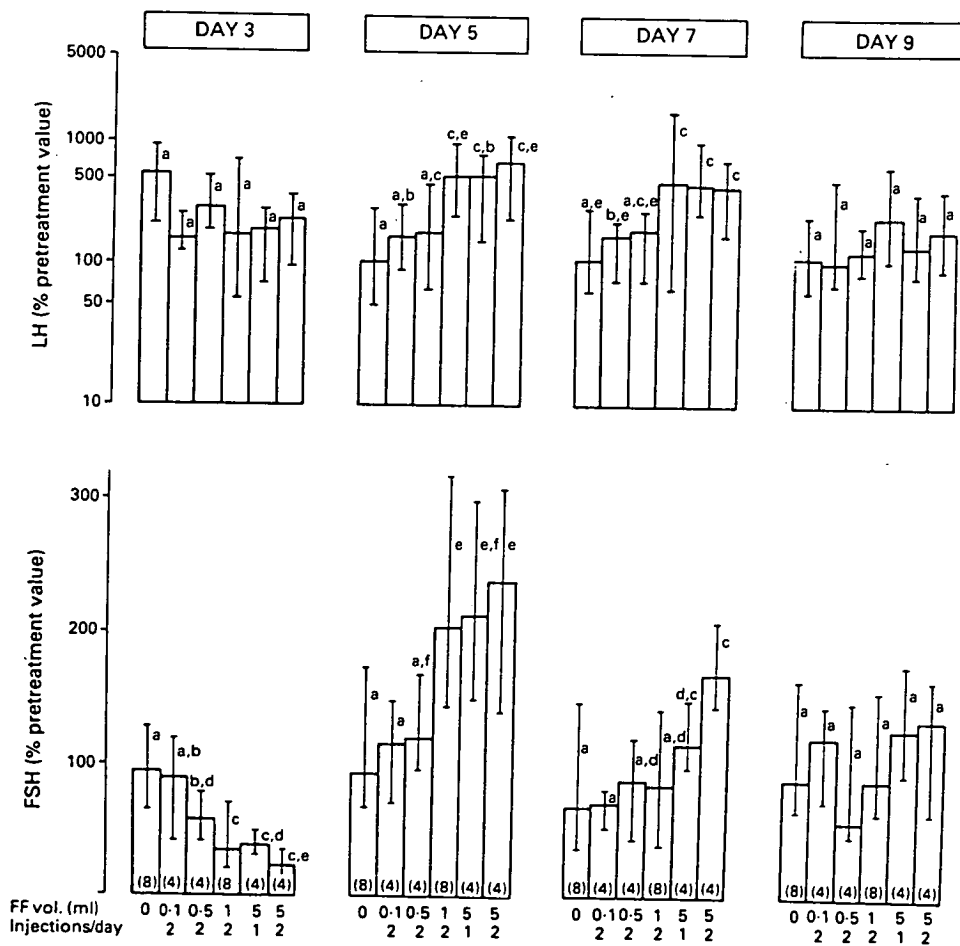


Fig. 2. Effect of different doses of bovine follicular fluid on plasma levels of LH and FSH. Values are medians of (N) sheep with the vertical lines showing the range. Median values with different letters within the same day are significantly different: ab, bc,  $P < 0.05$ ; ae,  $P < 0.02$ ; ac, de, dc,  $P < 0.01$ .

The pretreatment plasma concentrations of FSH and LH did not differ significantly between the groups. The values for ewes treated with 0, 0.1, 0.5, 1.0 or 5.0 ml bFF once daily and 5.0 ml bFF twice daily were  $130 \pm 17$ ,  $138 \pm 38$ ,  $161 \pm 34$ ,  $106 \pm 20$ ,  $107 \pm 22$  and  $130 \pm 21$  ng FSH/ml respectively (mean  $\pm$  s.e.m.) and  $0.7 \pm 0.1$ ,  $0.6 \pm 0.1$ ,  $0.6 \pm 0.1$ ,  $0.5 \pm 0.1$ ,  $0.7 \pm 0.2$  and  $0.6 \pm 0.1$  ng LH/ml respectively.



**Table 1.** Effect of treatment with bovine follicular fluid on time to oestrus, plasma progesterone concentrations and ovulation rate in Romney ewes

Dose of follicular fluid	No. of ewes	Days to oestrus after injection of cloprostenol	Plasma progesterone (ng/ml) on Day 11	Ovulation rate
None	8	2.2 ± 0.2 <sup>a</sup>	1.22 ± 0.12 <sup>a</sup>	1.4 ± 0.3
0.1 ml, twice daily	4	2.3 ± 0.2 <sup>a</sup>	1.20 ± 0.11 <sup>a</sup>	1.5 ± 0.3
0.5 ml, twice daily	4	4.8 ± 0.5 <sup>b</sup>	0.58 ± 0.16 <sup>b</sup>	1.5 ± 0.5
1.0 ml, twice daily	8	7.3 ± 0.3 <sup>c</sup>	0.27 ± 0.03 <sup>c</sup>	2.3 ± 0.4
5.0 ml, once daily	4	8.0 ± 0.4 <sup>c</sup>	0.34 ± 0.06 <sup>c</sup>	2.3 ± 0.3
5.0 ml, twice daily	4	10.5 ± 0.9 <sup>d</sup>	0.23 ± 0.02 <sup>c</sup>	1.5 ± 0.3

Values are mean ± s.e.m. Ewes were injected with bFF for 3 days beginning immediately after injection of cloprostenol on Day 10 of the oestrous cycle. Mean values with different superscripts in the same column are significantly different ( $P < 0.01$  for days to oestrus,  $P < 0.025$  for progesterone concentrations). Ovulation rates did not differ significantly between the treatments.

**Table 2.** Frequency table showing the effect of treatment with bovine follicular fluid on ovulation rates in Romney ewes

Dose of follicular fluid	Ovulation rate					Mean ovulation rate ± s.e.m.
	1	2	3	4	5	
None	23	10	2	0	0	1.4 ± 0.1
1 ml, twice daily	10	19	3	1	1	1.9 ± 0.2
5 ml, once daily	6	14	4	1	0	2.0 ± 0.2

Ewes were injected with bFF for 3 days starting immediately after administration of cloprostenol to initiate luteolysis on Day 10 of the oestrous cycle.  $\chi^2$  analysis indicates that both doses of bFF significantly increased ovulation rate ( $P < 0.005$ ). Ovulation rate frequencies did not differ significantly between the 2 doses of bFF.

The ability of 5 ml bFF to delay the onset of oestrus when given once daily for 3 days from the start of luteolysis ( $7.5 \pm 0.2$  vs  $2.2 \pm 0.2$  days to oestrus for treated and control ewes respectively; mean ± s.e.m.,  $N = 5$ ) was lost if the bFF had previously been heated to 90°C for 45 min ( $2.6 \pm 0.2$  days to oestrus,  $N = 5$ ) or if it was administered with 1000 i.u. PMSG ( $2.1 \pm 0.1$  days to oestrus,  $N = 5$ ). Simultaneous administration of 1000 i.u. hCG failed to overcome the effects of the bFF ( $6.8 \pm 0.5$  days to oestrus,  $N = 5$ ).

#### Follicular characteristics after treatment with bFF

The results of Fig. 3 show the consequence at the ovarian level of treatment with bFF. Ewes were injected twice daily (08:30 and 16:30 h), from the time they received cloprostenol, with 5 ml bFF for 24 or 48 h or 1 ml bFF for 48 h (4 ewes for each time and dose of bFF). The ewes were then ovariectomized. No significant differences were observed between the doses of bFF or the time of treatment and so the ovarian data obtained from the bFF-treated ewes have been pooled. The ovarian data obtained from the control ewes which received plasma from an ovariectomized cow for 24 or 48 h (4 ewes at each time) have also been pooled because no significant differences were observed between the 2 time periods. Treatment with bFF had no significant effect on the number of small (1–2.5 mm), medium (3–4.5 mm) or large ( $\geq 5$  mm) diameter ovarian antral follicles present (Fig. 3). Moreover, the mean diameter of the largest follicles  $\geq 5$  mm diameter in the bFF-treated ewes ( $6.6 \pm 0.2$  mm, mean ± s.e.m.,  $N = 10$ ) did not differ significantly from that in

the control ewes ( $6.9 \pm 0.3$  mm,  $N = 8$ ). In addition, bFF treatment had no significant effect on the mean number of follicles in each size grouping considered to be non-atretic by their morphological characteristics. The mean  $\pm$  s.e.m. numbers of small, medium and large non-atretic follicles were  $6 \pm 1$ ,  $1.1 \pm 0.4$  and  $1.1 \pm 0.2$  respectively in the control ewes ( $N = 8$ ) and  $7 \pm 2$ ,  $1.8 \pm 0.8$  and  $0.6 \pm 0.2$  respectively in the bFF-treated ewes ( $N = 12$ ). In large follicles, bFF treatment resulted in there being significantly fewer granulosa cells per follicle ( $P < 0.01$ ), a significantly lower concentration of oestradiol-17 $\beta$  in follicular fluid ( $P < 0.05$ ) and a significant reduction in the capacity of the granulosa cells to aromatize testosterone to oestradiol-17 $\beta$  ( $P < 0.05$ ) and to produce cAMP in response to challenge with LH or FSH ( $P < 0.001$ ) (Fig. 3). No significant effect of bFF on any of these characteristics was observed in small or medium-sized follicles.

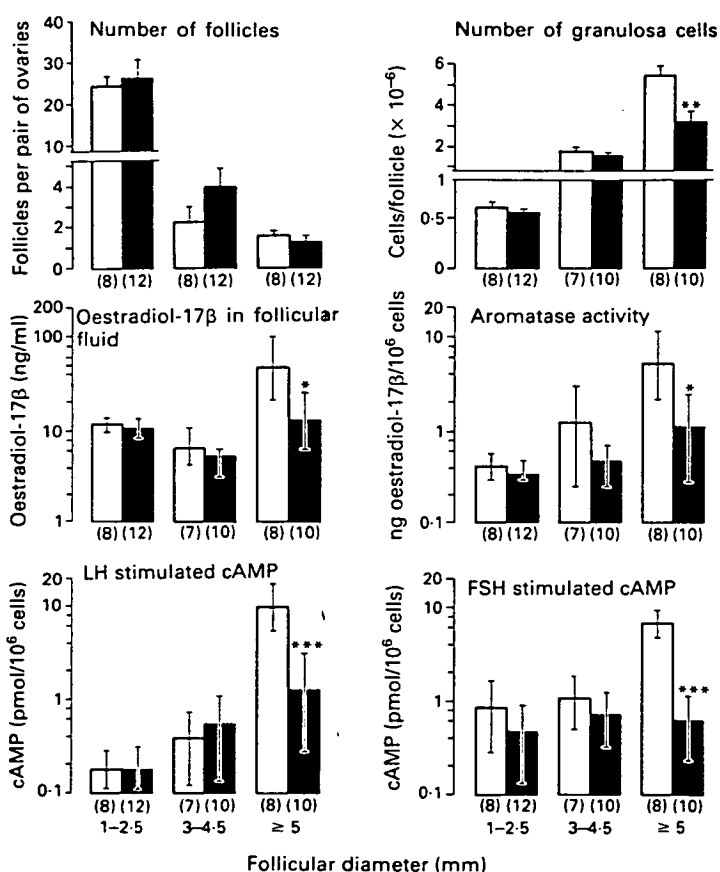


Fig. 3. Effect of bFF treatment on the number of ovarian follicles, the number of granulosa cells per follicle, oestradiol-17 $\beta$  concentrations in follicular fluid, the aromatase activity of granulosa cells and the ability of granulosa cells to produce cAMP in response to challenge with LH and FSH ( $\square$  control ewes,  $\blacksquare$  bFF-treated ewes).

Data showing follicle and granulosa cell numbers have been presented as means with vertical lines indicating the s.e.m. The remaining data have been presented as geometric means with 95% confidence limits indicated by the vertical lines. Numbers in parentheses refer to the number of sheep studied. Asterisks indicate significant differences between control and bFF-treated ewes for follicles  $\geq 5$  mm in diameter (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). The endogenous cAMP content of the granulosa cells was 0.19 pmol/10 $^6$  cells (geometric mean value) with 95% confidence limits at 0.16 and 0.22 pmol/10 $^6$  cells.

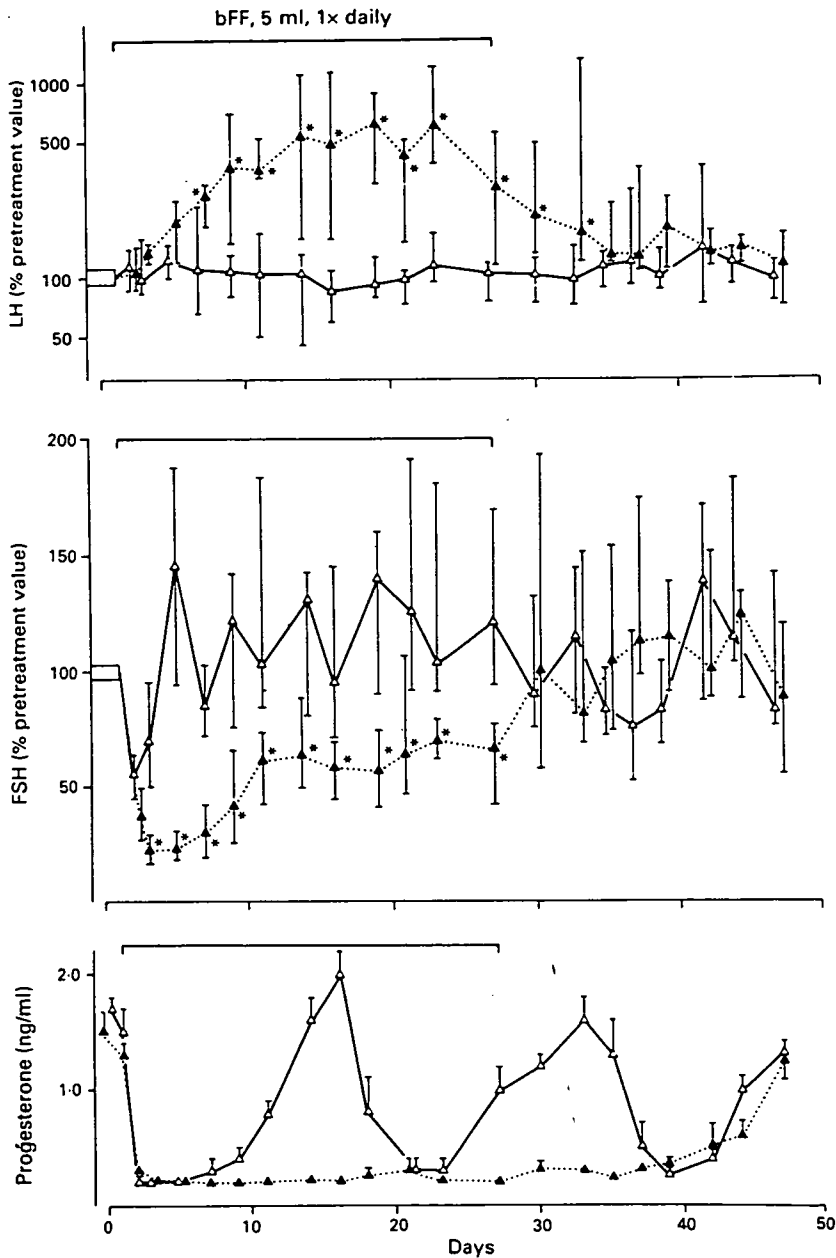


Fig. 4. Plasma concentrations of LH, FSH and progesterone in ewes treated daily for 27 days with 5 ml bFF ( $\blacktriangle \dots \blacktriangle$ ,  $N = 5$ ) or plasma from an ovariectomized cow (control ewes,  $\triangle - \triangle$ ,  $N = 5$ ). Treatment started immediately after injection of cloprostenol to initiate luteolysis on Day 10 of the oestrous cycle (Day 1 of the experiment). LH and FSH values are medians with the range shown by vertical lines. Asterisks indicate significant differences ( $P < 0.05$ ) between the 2 groups on the same day. Progesterone values are means with vertical lines showing the s.e.m. when larger than the symbol for the mean value. Pretreatment plasma gonadotrophin concentrations did not differ significantly between the 2 groups. Values (mean  $\pm$  s.e.m.) were  $0.5 \pm 0.1$  and  $0.4 \pm 0.1$  ng LH/ml and  $120 \pm 13$  and  $127 \pm 19$  ng FSH/ml for the control and bFF-treated ewes respectively.

*Effect of 27 days treatment with bFF on ovarian function*

Figure 4 shows the effect on plasma LH, FSH and progesterone concentrations of once daily injections of 5 ml bFF for 27 days, starting from the initiation of luteolysis with cloprostenol. By Day 3, median plasma FSH concentrations in the bFF-treated ewes had fallen to 20% of the pretreatment values. Thereafter, median values rose gradually to reach about 60% of pretreatment levels by Day 11 where they stayed for the remainder of the treatment period. Median plasma concentrations of LH, in contrast to those of FSH, gradually rose about 5-fold during the first 14 days of the treatment period and stayed at this level for most of the remainder of the treatment period. Oestrus was not observed in the bFF-treated ewes during the 27-day treatment period. There was also no evidence of ovulation occurring, as indicated by the absence of visible corpora lutea at laparoscopy at the end of the treatment period, and low concentrations of plasma progesterone throughout the treatment period. In contrast, control ewes displayed oestrus on Days 3 and Days 19–20, and their plasma progesterone concentrations were consistent with normal ovarian cyclicality. At the end of the treatment period, median plasma FSH concentrations in the bFF-treated ewes rose to control levels within 3 days, and median LH concentrations fell to control values within 8 days. Oestrus was observed in all 5 ewes between Days 36 and 41. Plasma progesterone concentrations also gradually rose to normal luteal values. All the control and bFF-treated ewes were successfully mated with a fertile ram at the first oestrus after the treatment period, and subsequently each ewe delivered a single lamb.

**Discussion**

Although the effects of treatment of ewes with bovine follicular fluid on plasma concentrations of gonadotrophins and oestrous cycle length have been studied before (Miller *et al.*, 1979; Miller, Critser & Ginther, 1982; Cummins *et al.*, 1983; McNeilly, 1984) the dose-dependency of observed effects or the consequence at the ovarian level of follicular fluid treatment have not been reported hitherto. The dose-related increase in time to oestrus after bFF administration was probably a consequence of the suppression of plasma FSH concentrations; the lower FSH concentrations were the longer oestrus was delayed. Moreover, simultaneous administration of PMSG, which is rich in FSH activity, with bovine follicular fluid prevented the increase in time to oestrus. Behavioural oestrus is an event dependent upon the large amounts of oestradiol-17 $\beta$  produced by a follicle as it attains ovulatory maturity. One would predict, therefore, that the increase in time to oestrus occurs as a consequence of the low plasma FSH concentrations in follicular fluid-treated ewes reducing follicular production of oestradiol-17 $\beta$ . This was confirmed by examination of the ovaries of treated ewes. Compared to control ewes, large follicles in these ewes had lower concentrations of oestradiol-17 $\beta$  in follicular fluid, fewer granulosa cells, and the granulosa cells had a reduced ability to aromatize androgen to oestradiol-17 $\beta$  (Fig. 3). In addition, the granulosa cells produced reduced amounts of cAMP when challenged with FSH or LH; a high production of cAMP in response to FSH is necessary for maximum follicular oestradiol-17 $\beta$  biosynthesis (Henderson *et al.*, 1985). Granulosa cell proliferation, follicular oestradiol biosynthesis and the development of granulosa cell responsiveness to FSH and LH (as measured by cAMP production in this study) are each FSH-dependent events (Henderson, 1979; Richards, 1979). Therefore, the effects on large follicles are consistent with their being exposed to inadequate levels of FSH due to the suppression of plasma FSH concentrations by follicular fluid treatment. It is, however, possible that bovine follicular fluid might also have directly inhibited FSH action at the ovary. Treatment with follicular fluid had no significant effects on small or medium-sized follicles (Fig. 3). Perhaps early follicular growth (from small to medium sizes) is less critically dependent upon FSH than later growth (from medium to large sizes).

Another consequence of the deleterious effects of follicular fluid treatment on large antral follicles, which are the major sources of follicular oestradiol-17 $\beta$  (McNatty *et al.*, 1984) and inhibin

(Tsonis *et al.*, 1983; Henderson *et al.*, 1984), would be a reduction in the negative feedback effects of oestradiol-17 $\beta$  and inhibin on pituitary gonadotrophin secretion. This could account for the elevation in plasma LH concentrations (Figs 1, 2 & 3) and for the rebound in FSH values which occurred when treatment was stopped (Figs 1 & 2). As normal follicular development resumed when treatment ceased, the increased output of follicular oestradiol-17 $\beta$  and inhibin would subsequently reduce pituitary gonadotrophin output once more to 'normal' physiological levels.

An important result from the 3-day treatments with bovine follicular fluid was the increased frequency of multiple ovulations observed with doses of 1 ml twice daily, or 5 ml once daily (Table 2). Wallace & McNeilly (1985) have also reported that treatment with bovine follicular fluid can increase ovulation rates in sheep. However, in that study, ewes of the prolific Damline breed were used, and relatively high doses of follicular fluid (10 ml intravenously twice daily) were given for 11 days during the luteal phase. In the present study, the increased ovulation rate followed treatment with relatively small amounts of follicular fluid for only 3 days after the start of luteolysis. Administration of FSH or substances with FSH-like activity will increase ovulation rate in sheep (Wright *et al.*, 1981; Boland *et al.*, 1983; Kelly *et al.*, 1983; McNatty *et al.*, 1985). Perhaps the elevated plasma concentrations of FSH and LH occurring on cessation of treatment allowed a greater than normal number of follicles to attain ovulatory maturity in synchrony in some ewes, hence the increased frequency of multiple ovulations.

Ewes were rendered temporarily infertile during 27 days of continuous, daily treatment with bovine follicular fluid; oestrus did not occur and ovulation was prevented. The low plasma concentrations of FSH occurring during the treatment period causing abnormal follicular development was probably the underlying cause of the infertility. After cessation of treatment, FSH and LH concentrations returned to pretreatment values and oestrus, mating and conception occurred; no adverse post-treatment effects on fertility were noted. The lowest median values of FSH (~20% of pretreatment levels) occurred 3–5 days after treatment began. Thereafter, median concentrations gradually rose to reach ~60% of pretreatment levels by Day 11 and remained at this value for the remainder of the treatment period. This partial loss in the FSH-suppressing capacity of bovine follicular fluid, which has been noted in monkeys (Channing, Tanabe, Turner & Hodgen, 1982b) and rats (Thomas & Nikitovitch-Winer, 1984), may represent the development of refractoriness to the treatment. Alternatively, the initial low levels of plasma FSH may be the result of pituitary FSH secretion being suppressed by a combination of the effect of bovine follicular fluid and negative feedback by follicular steroids and inhibin. As follicular development becomes impaired by the low levels of FSH, the ovarian negative-feedback effects would be lessened, thereby allowing a partial increase in pituitary FSH secretion.

The component(s) of follicular fluid responsible for the effects observed in this study is unknown, but inhibin would seem a likely candidate. Inhibin is a heat-labile product of granulosa cells which inhibits pituitary FSH production and is present in high concentrations in follicular fluid (Henderson & Franchimont, 1981, 1983; Tsonis *et al.*, 1983; Henderson *et al.*, 1984). However, the possibility of other components in follicular fluid being responsible, perhaps by acting directly on the ovary, cannot be excluded. In summary, the results of this study support the view that follicular fluid components may have potential usefulness as agents to increase fertility, through increasing ovulation rate, or to impair fertility, by preventing ovulation.

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## Effect on ovulation rate of increasing or decreasing ovarian exposure to follicle stimulating hormone during the preovulatory period in ewes

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### ABSTRACT

Ovine FSH was administered hourly to ewes for 48 h from the initiation of luteolysis. The mean ovulation rates of ewes receiving 2.5  $\mu\text{g}$  FSH/h ( $2.1 \pm 0.2$  ovulations/ewe, mean  $\pm$  s.e.m.,  $n = 8$ ) and 5.0  $\mu\text{g}$  FSH/h ( $6.2 \pm 2.0$  ovulations/ewe,  $n = 6$ ) were significantly higher ( $P < 0.01$ ) than that of control ewes ( $1.3 \pm 0.1$  ovulations/ewe,  $n = 16$ ). Mean plasma concentrations of FSH were also raised above normal by these doses of FSH. Plasma concentrations of FSH were reduced below normal by twice daily administration of steroid-free bovine follicular fluid (bFF) for 48 h from the initiation of luteolysis. As a consequence follicles failed to achieve ovulatory maturity. Large follicles ( $\geq 5$  mm diameter) from ewes treated with bFF for 48 h had reduced concentrations of oestradiol-17 $\beta$  in follicular fluid, fewer granulosa cells, and the cells had a reduced ability to metabolise testosterone to oestradiol-17 $\beta$  and to produce cyclic AMP when challenged with FSH or LH.

**Keywords** Follicle stimulating hormone; follicular fluid; ovulation rate; granulosa cells; sheep.

### INTRODUCTION

The pituitary gonadotrophins follicle stimulating hormone (FSH) and luteinising hormone (LH) are essential for follicles to develop to ovulatory maturity, and at ovulation, release an egg capable of fertilisation. Indeed, ovulation rates in several domestic species can be increased by administration of preparations rich in FSH and LH activity, such as pregnant mares serum gonadotrophin (PMSG). The relative role(s) of FSH and LH in regulating follicular development is, however, still ill-defined. In this study, some aspects of the role of FSH in regulating follicular development in ewes were examined by increasing or decreasing ovarian exposure to FSH during the preovulatory period. Ovarian exposure to FSH was increased by administration of highly purified ovine FSH. Ovarian exposure to FSH was decreased by administration of inhibin (in the form of steroid-free bovine follicular fluid) which specifically suppresses plasma concentrations of FSH (Henderson *et al.*, 1986).

### MATERIALS AND METHODS

#### Studies *in vivo*

Parous New Zealand Romney ewes (aged 2.5 to 3.5 years) were grazed on open pasture and run with a vasectomised ram fitted with a marking harness. The ewes were examined twice daily for signs of oestrus. On the 10th day after oestrus was observed, the ewes received an intramuscular injection of the prostaglandin F $_{2\alpha}$  analogue, cloprostenol (125  $\mu\text{g}$ ; ICI Tasman Ltd, Upper Hutt, New Zealand) to initiate luteolysis. Immediately after this injection, treatment with FSH or bovine follicular fluid (bFF) began.

The ovine FSH preparation used was NIADDK-oFSH-16 (biopotency of 20 U/mg; 1 U = 1 mg of

NIH-FSH-S1) and its contamination with LH and other pituitary hormones was negligible. Solutions containing 2.5 and 5  $\mu\text{g}$  FSH/ml were prepared in saline (0.9% NaCl w/v) containing 0.2% ovine serum albumin. One ml, once per hour for 48 h was administered to ewes through an intrajugular cannula using an infusion pump adjusted to deliver 1 ml/minute, once/hour. Control ewes received vehicle alone. For comparative purposes, some ewes received a cruder preparation of FSH (F.S.H.-P; Burns-Biotec, Nebraska, USA) at dosages of 0.1 and 0.5 mg/h for 48 h. Jugular venous blood samples were taken from all ewes at 1 to 4 h intervals starting the day before FSH treatment began, and finishing at the end of the treatment period. Ten days after the end of the treatments, the ovaries of each ewe were examined by laparoscopy and the number of ovulations (ovulation rate) was determined by counting the number of corpora lutea present.

Ewes given bFF were injected intramuscularly 0, 12, 24, 36 and 48 h after receiving cloprostenol with 5 ml bFF which had been treated with dextran-coated charcoal to remove steroids, as described previously (Henderson *et al.*, 1986). Control ewes received 5 ml dextran-coated charcoal treated plasma from an ovariectomised cow. Jugular venous blood samples were taken twice daily starting the day before bFF treatment began, through to the end of the treatment period. In some ewes, the ovaries were removed immediately after the final injection of bFF or 'ovariectomised' plasma. Other ewes were left to run with a vasectomised ram to determine the time to oestrus. In these ewes, daily blood sampling for progesterone determination was continued for a further 14 days after the last injection of bFF.

All blood samples collected were centrifuged at



2000 g for 15 min at room temperature. The plasma was removed and stored at  $-20^{\circ}\text{C}$  until assayed for FSH and/or progesterone. FSH was measured using a radioimmunoassay kit provided by NIADDK, National Institutes of Health, Bethesda, USA and progesterone was measured by radioimmunoassay as described previously (McNatty *et al.*, 1981).

#### Studies *in vitro*

Immediately after ovariectomy, all antral follicles  $\geq 1.0$  mm in diameter were individually dissected from each pair of ovaries. Details of the methods used to collect follicular fluid and measure its oestradiol- $17\beta$  content, to isolate and quantitate follicular granulosa cells, to measure the ability of granulosa cells to metabolise testosterone to oestradiol- $17\beta$  (aromatase activity), and to produce adenosine 3', 5'-monophosphate (cAMP) in response to challenge with FSH and LH have all been reported in full elsewhere (Henderson *et al.*, 1986).

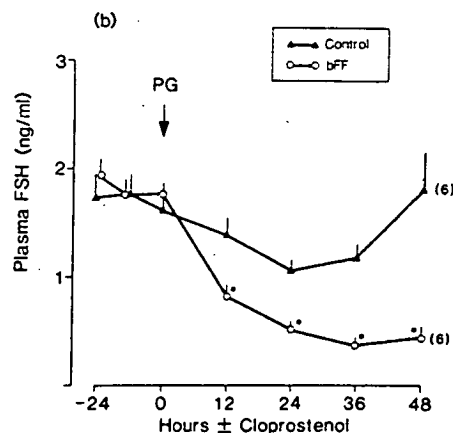
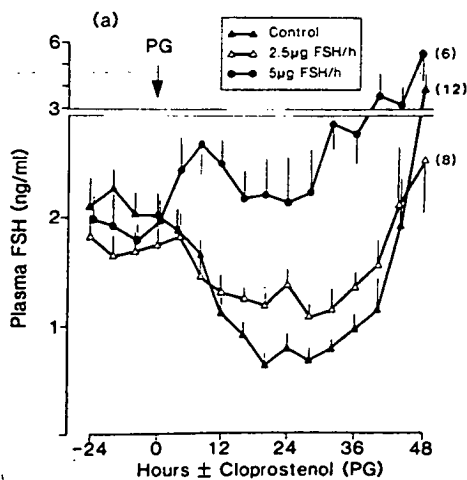


FIG. 1 Effect of administration of (a) ovine FSH or (b) follicular fluid (bFF) on mean plasma concentrations of FSH in ewes (numbers in parentheses). Bars indicate standard errors of the means. Asterisks indicate mean values for bFF treated ewes are significantly different from corresponding control mean values ( $P < 0.01$ ).

## RESULTS

### Effects of FSH

Figure 1(a) shows the effect of administration of ovine FSH on plasma concentrations of FSH, at 4-hourly intervals. In ewes receiving  $5 \mu\text{g}$  FSH/h, mean plasma concentrations of FSH were significantly higher than those of control ewes and ewes receiving  $2.5 \mu\text{g}$  FSH/h from 8 h to 40 h after injection of cloprostenol ( $P < 0.01$ , analysis of variance and Newman-Keuls test). In ewes receiving  $2.5 \mu\text{g}$  FSH/h, mean plasma concentrations of FSH were significantly higher than those of control ewes from 20 h to 28 h after injection of cloprostenol ( $P < 0.05$ ). Mean plasma concentrations of FSH did not differ significantly between the 3

groups before injection of cloprostenol or 4, 44 and 48 h after injection of cloprostenol. The mean ovulation rates of groups of ewes receiving each dose of ovine FSH or the 2 doses of F.S.H.-P tested were significantly higher than that of control ewes (Table 1).

### Effects of Treatment with Follicular Fluid

By 12 h after the first injection of bFF, the mean plasma concentration of FSH in bFF treated ewes was significantly lower than that of control ewes, and it remained significantly lower for the remainder of the treatment period (Fig. 1b). There was a significant increase ( $P < 0.001$ , Student's *t*-test) in the mean time to oestrus, after injection of cloprostenol, in the bFF treated ewes ( $9.3 \pm 0.5$  d, mean  $\pm$  s.e.m. for  $N = 6$ ) relative to control ewes ( $2.2 \pm 0.2$  d,  $N = 6$ ). In control ewes mean plasma concentrations of progesterone 8 days after the last injection of 'ovariectomised' plasma was  $1.4 \pm 0.1$  ng/ml ( $N = 6$ ), indicative of the formation of new corpora lutea. In contrast, in bFF treated ewes,

plasma progesterone concentrations were still at basal levels ( $0.2 \pm 0.02$  ng/ml,  $N = 6$ ).

Examination of the ovaries of ewes treated for 48 h with bFF showed that relative to control ewes there were no significant differences ( $P < 0.05$ , Student's *t*-test) in the mean numbers of small (1 to 2.5 mm), medium (3 to 4.5 mm) or large ( $\geq 5$  mm) diameter follicles. For control ( $N = 8$ ) v bFF treated ( $N = 12$ ) ewes there were  $24 \pm 3$  v  $27 \pm 4$  small follicles,  $2.3 \pm 0.7$  v  $4.0 \pm 0.9$  medium diameter follicles and  $1.6 \pm 0.2$  v  $1.3 \pm 0.3$  large follicles (values are mean  $\pm$  s.e.m.). However, relative to control ewes, large follicles from bFF treated ewes had lower concentrations of oestradiol- $17\beta$  in follicular fluid, contained fewer granulosa cells and the granulosa cells had a reduced ability to metabolise

**TABLE 1** Effect of treatment with ovine FSH or F.S.H.-P on ovulation rates in Romney ewes.

Treatment	Ovulation rate				Mean ovulation rate
	1	2	3	4 to 14	
Controls	10	6	0	0	1.3 (1.1-1.5)
2.5 µg/h FSH	1	5	2	0	2.0 (1.5-2.7)*
5 µg/h FSH	0	3	0	3	3.7 (1.8-12.6)**
0.1 mg/h F.S.H.-P	1	3	0	3	2.6 (1.5-5.3)**
0.5 mg/h F.S.H.-P	1	2	0	4	3.2 (1.6-9.6)**

Data were transformed to reciprocals for analysis and mean values are back transformed means. Ninety five % confidence limits are given in parentheses. Asterisks indicate that mean values are significantly different from control values. Ovulation rates did not differ significantly between the different FSH and F.S.H.-P treatments.

testosterone to oestradiol-17β (aromatase activity) and to produce cAMP in response to challenge with LH or FSH (Table 2). No significant effect of bFF on any of these characteristics was observed in small or medium sized follicles.

**DISCUSSION**

Both FSH and LH are essential for antral follicle development. Although plasma concentrations of LH in sheep increase during the later stages of follicular maturation (Baird and Scaramuzzi, 1976; Baird *et al.*, 1981) those of FSH decrease (Fig. 1; Baird *et al.*, 1981; Miller *et al.*, 1981). Granulosa cells are the target cells for FSH action in the ovary, and recent studies have shown that as follicles enlarge, the granulosa cells become more sensitive to FSH (Henderson *et al.*, 1985). This may be important in ensuring that final maturation of follicles can occur in spite of declining plasma FSH concentrations. Large ovulatory follicles(s) may be protected from the deleterious effects of low plasma FSH concentrations by the increased sensitivity to FSH of the granulosa cells, thereby permitting the follicle(s) to continue to respond to FSH, and so continue development. Follicles not having acquired this increased sensitivity to FSH, when plasma FSH levels decline, likely undergo atresia due to lack of FSH. By this reasoning, one may argue that additional

follicles could be brought to ovulatory maturity in synchrony by raising plasma concentrations of FSH during the preovulatory period, thereby increasing follicular exposure to FSH. This was found to be the case as hourly administration of ovine FSH at doses of 2.5 µg/h or 5.0 µg/h for 48 h from the initiation of luteolysis significantly increased mean ovulation rates (Table 1). Interestingly, F.S.H.-P (a crude FSH preparation having considerable contamination with LH-like activity), which is commonly used for super-ovulation, was also effective in increasing mean ovulation rates, but at much higher dosages than ovine FSH. The lowest dose of F.S.H.-P which significantly increased the mean ovulation rate was 0.1 mg/h, whereas just 2.5 µg/h of ovine FSH was effective.

While raising mean plasma concentrations of FSH during the preovulatory period increased mean ovulation rates (Table 1), administration of bFF to reduce mean plasma FSH concentrations below normal was deleterious to the proper development of large follicles. (Previous studies have shown that the effects of bFF treatment can be over-ridden by simultaneous administration of FSH or PMSG (Henderson *et al.*, 1986; McNatty *et al.*, 1985; McNeilly, 1985). Thus the bFF likely acts primarily through reducing plasma levels of FSH). Granulosa cell proliferation, follicular

**TABLE 2** Effect of bFF treatment on properties of large ovine follicles.

	No. of granulosa cells/follicle (x10 <sup>6</sup> )	Oestradiol-17β in follicular fluid (ng/ml)	Aromatase activity (ng oestradiol-17β/10 <sup>6</sup> cells)	Stimulation of granulosa cell cAMP production (pMol/10 <sup>6</sup> cells) by	
				LH	FSH
Controls N = 8	5.3 4.2-6.4	43 20-95	5.1 2.1-11.0	10.0 5.6-17.4	6.5 4.6-9.1
bFF treated N = 10	3.1** 2.0-4.2	12* 6-25	1.2* 0.2-3.9	1.3*** 0.3-3.1	0.6*** 0.2-1.1

Values are arithmetic means (granulosa cell numbers) or geometric means (others) with 95% confidence limits below. Asterisks indicate that mean values are significantly different from controls.

oestradiol-17 $\beta$  production and granulosa cell responsiveness to FSH and LH, are each FSH-dependent events necessary for proper follicular development, and all were reduced in large follicles of bFF treated ewes (Table 2). The observed ovarian consequences of bFF treatment are thus consistent with inadequate follicular exposure to FSH. The failure of bFF treated ewes to display oestrus at the expected time after cloprostenol treatment ( $\sim$ 48 h) is also consistent with impaired development of large follicles. Behavioural oestrus is an event dependent upon the large amounts of oestradiol-17 $\beta$  produced by a follicle as it attains ovulatory maturity.

The results of this study are consistent with the view that plasma concentrations of FSH during the preovulatory period may be critical in determining the number of follicles that can normally achieve ovulatory maturity in sheep. Raising plasma FSH concentrations during this period, thereby increasing ovarian exposure to FSH, allows an increased number of follicles to achieve ovulatory maturity in synchrony. In contrast, reducing plasma FSH concentrations during the pre-ovulatory period prevents follicles from attaining ovulatory maturity.

The normal decline in plasma FSH concentrations during the preovulatory period occurs as a consequence of the negative feedback effects of follicular oestradiol-17 $\beta$  and inhibin. One approach to increasing ovulation rates and hence fecundity in ewes might be to attempt to reduce the negative feedback effects of these 2 compounds, particularly that of inhibin thereby possibly raising plasma FSH concentrations.

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**Authors:** K.M. Henderson & K.P. McNatty

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## FACTORS INFLUENCING OVULATION RATE IN SHEEP

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**INTRODUCTION** The number of fertilizable ova shed at ovulation (i.e. ovulation rate) is a major determinant of prolificacy in sheep. An increase in ovulation rate requires that a greater than normal number of ovarian follicles must achieve ovulatory maturity at the same time. Our understanding of the factors which determine whether a follicle develops to ovulatory maturity or undergoes atresia is still incomplete. However, it is known that adequate exposure to the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) is essential for follicular maturation. Indeed the administration of compounds rich in FSH- and LH-like activity, such as pregnant mares serum gonadotrophin (PMSG), are commonly used to increase ovulation rate or superovulate sheep. In this paper we shall discuss how LH and FSH act together to bring follicles to ovulatory maturity, and how relatively minor changes in the plasma concentrations of these gonadotrophins can modify the ovulation rate in sheep.

**Follicular actions of LH and FSH** Specific plasma membrane receptors for LH are located on the theca interna and granulosa cells, while specific plasma membrane receptors for FSH are confined to the granulosa cells. Recent studies of ovine thecal LH receptors (by  $^{125}\text{I}$ -human chorionic gonadotrophin (hCG) binding studies) indicate that they are present on all antral follicles  $>1.0$  mm in diameter, and that follicle size and health have little influence on either the maximum binding capacity  $B(\text{max})$  or the equilibrium dissociation constant ( $K_d$ ) of the receptors (1). In contrast granulosa cell receptors for LH/hCG increase markedly with increasing follicular diameter and there is generally more LH/hCG bound to non-atretic than to atretic follicles (2,3).

Specific receptors for FSH are present on granulosa cells of all antral follicles  $>1.0$  mm diameter, irrespective of follicle size or health. While binding studies using iodinated FSH can demonstrate the presence of specific receptors for FSH on ovine granulosa cells, the proportion of  $^{125}\text{I}$ -FSH specifically bound to the cells is generally very low. Consequently, it has proven to be difficult to determine how the characteristics of the FSH receptor (i.e.  $B(\text{max})$  and  $K_m$ ) are influenced by follicle size and health. Both FSH and LH on interacting with their plasma membrane receptors stimulate the production of cyclic adenosine 3',5'-monophosphate (cAMP), which mediates the intracellular actions of these gonadotrophins. Thus the measurement of cAMP production by granulosa cells exposed to FSH or LH *in vitro* provides a convenient method of measuring the responsiveness of the cells to FSH and LH, or 'functional receptor' activity. Using this technique it can be shown that as follicles enlarge the granulosa cells become increasingly sensitive to FSH, i.e. the same amount of cAMP can be produced in response to smaller

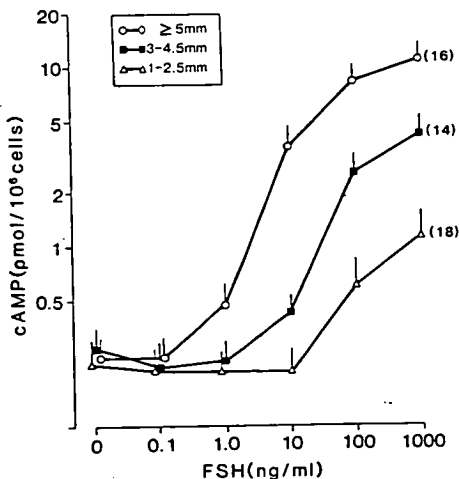


Figure 1. Cyclic AMP production in response to increasing doses of ovine FSH (NIADDK-oFSH-16) by granulosa cells from small, medium and large diameter ovine follicles. Values are mean + s.e.m. for (n) sheep.

amounts of FSH as follicle size increases (Fig. 1). Granulosa cells from atretic follicles are generally less responsive to FSH than cells from non-atretic follicles (4). Thus as follicles develop to ovulatory maturity, there is little change in the thecal sensitivity to LH, whereas the granulosa cells become increasingly sensitive to FSH and also develop receptors for LH.

Stimulation of cAMP production by LH interacting with its thecal receptors results in increased androgen production with androstenedione being the major androgen in the sheep. While most of the androstenedione is released into the bloodstream, some also reaches the granulosa cells, where it can be metabolized to oestradiol-17 $\beta$  by the aromatase enzyme complex. The activity of this enzyme complex, which is absent in theca cells, is influenced markedly in granulosa cells by the plasma concentrations of FSH. Infusing sheep with FSH to raise the plasma levels of FSH increases aromatase activity in cells from follicles of all sizes (5), while lowering FSH levels by administration of inhibin reduces granulosa cell aromatase activity (6). The intra-follicular actions of oestradiol, which together with FSH, include stimulating granulosa-cell proliferation and LH receptor formation, are essential in bringing

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**Plasma gonadotrophin concentrations and follicular development during the breeding season** In sheep, oestrus occurs within 48h of the initiation of luteolysis. As the plasma concentrations of progesterone fall, during corpus luteum regression, those of LH rise due to increases in the frequency of LH release from the pituitary (9,10). These rising concentrations of LH stimulate increased production of androgen and oestradiol-17 $\beta$  by the developing follicles(s). Ultimately the rising levels of oestradiol-17 $\beta$  induce oestrous behaviour and the preovulatory LH surge. Ovulation then ensues permitting ova to be released and, after a fertile mating, pregnancy to be established. While plasma LH concentrations rise in response to luteolysis, plasma FSH concentrations fall (Fig. 2) (10,11) due to the negative feedback effects of the increasing amounts of oestradiol-17 $\beta$  and inhibin produced by the developing follicles(s). As discussed above, granulosa cells become increasingly more sensitive to FSH as follicles enlarge. This may be critically important in ensuring that the final maturation of follicles can occur in the face of declining plasma levels of FSH during the periovulatory period. The increasing sensitivity to FSH of granulosa cells in large developing follicles is likely to protect them from the deleterious effects of low plasma FSH concentrations. Follicles less sensitive to FSH are likely to undergo atresia due to lack of FSH. By this reasoning, one may argue that additional follicles could be induced to ovulatory maturity by simply raising the plasma concentrations of FSH during the preovulatory period, thereby increasing follicular exposure to FSH. This is indeed the case. The hourly administration of low doses of highly purified ovine FSH (2.5  $\mu$ g or 5  $\mu$ g/h of NIADDK-oFSH-16) for 48h from the initiation of luteolysis raised the plasma concentrations of FSH (Fig. 2) and led to a significant increase in mean ovulation rates (Table 1). In contrast to the effects of FSH, the administration of ovine LH (NIADDK-oLH-25) lowered the mean ovulation rates relative to the controls (Table 1).

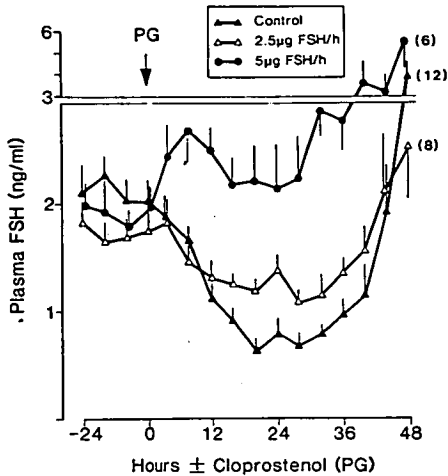


Figure 2. Effect on plasma FSH concentrations of administration of ovine FSH (once hourly for 48) from the onset of luteolysis which was initiated by injection of cloprostenol (125  $\mu$ g, i.m.) on day 10 of the oestrous cycle. Values are mean + or - s.e.m. for (n) sheep.

Table 1. Effect of treatment with ovine FSH or LH on ovulation rates in Romney ewes

Treatment	Ovulation rate					Geometric mean ovulation rate (95% confidence limits)
	0	1	2	3	4 to 14	
Controls	0	10	6	0	0	1.3 (1.1-1.6)
2.5 $\mu$ g/h FSH	0	1	6	3	0	2.1 (1.7-2.7)
5.0 $\mu$ g/h FSH	0	0	3	0	5	4.8 (2.4-8.8)
5.0 $\mu$ g/h LH	2	6	0	0	0	0.7 (0.3-1.2)

Geometric mean values differed significantly between all groups  $P < 0.05$

which can normally achieve ovulatory maturity in sheep. Raising plasma FSH concentrations during this period, thereby increasing ovarian exposure to FSH allows an increased number of follicles to attain ovulatory maturity at the same time. In contrast, reducing plasma FSH concentrations during the preovulatory period prevents follicles from maturing fully.

While ovulation rates can be increased by administration of FSH from the initiation of luteolysis, the percentage of double ovulations in Romney ewes (which normally have >70% single ovulations) can also be increased by giving FSH for 24h during the 48h before the initiation of luteolysis (5). This is likely the result of FSH advancing the maturation of a follicle which would normally have undergone atresia. By advancing its maturation and thereby increasing its sensitivity to FSH, this follicle, like the original follicle destined to ovulate, is equipped to survive the post-luteolytic decline in plasma FSH concentrations and so can attain ovulatory maturity. The plasma concentrations of FSH during the 48h

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period pre-luteolysis rather than post-luteolysis are likely to be the physiological determinant of single or double ovulations in Romney ewes. Comparisons of plasma FSH concentrations before and after the initiation of luteolysis between ewes subsequently having a single or double ovulation, indicate that significant differences occur in plasma FSH concentrations before but not after luteolysis (5). In the 48h before the initiation of luteolysis, the overall geometric mean (and 95% confidence limits) plasma FSH concentrations in ewes subsequently having a double ovulation were 2.2 (1.9, 2.5) ng/ml, compared to 1.6 (1.4, 1.8) ng/ml for ewes subsequently having a single ovulation (5).

The Booroola breed of sheep is one of the most prolific breeds known. These sheep are endowed with a so-called "fecundity" gene which is responsible for the very high ovulation rates; up to 10 or 11 ovulations being recorded for individual ewes. Recently, the plasma FSH concentrations have been compared between homozygous (FF) Booroola x Merino ewes (defined as having at least one ovulation rate recording of >5) and Booroola x Merino ewes not carrying the fecundity gene (++). These studies have shown that mean plasma FSH concentrations of FSH are higher in FF ewes than in ++ ewes during anoestrus, the luteal phase and the follicular phase (Table 2) (12). It seems reasonable to conclude that this continual exposure of the ovaries of FF ewes to elevated levels of FSH is responsible for their high ovulation rates.

Table 2. Plasma FSH concentrations (ng/ml) in Booroola x Merino ewes which were homozygous (FF) or non-carriers (++) of the fecundity (F) gene

Genotype	Anoestrus	Luteal phase	Follicular phase 12-36h after onset of luteolysis
FF	2.5±0.3	2.1±0.2	0.9±0.1
++	1.3±0.1	1.5±0.1	0.5±0.1

Values are mean ± s.e.m. for 12 sheep. Mean values for ++ ewes were each significantly less than corresponding FF mean values, P<0.05.

higher than mean levels in ++ ewes. Thus to successfully detect differences in plasma FSH concentrations between groups of ewes, it is essential that an intensive blood sampling regimen is performed over a prolonged period of time.

Although differences may exist between groups of ewes in mean ovulation rates and mean plasma FSH concentrations, the plasma FSH concentrations in individual ewes within a group can vary widely. It is not uncommon for a ewe with a high ovulation rate to have relatively low plasma FSH levels relative to a ewe with a low ovulation rate, and vice versa. The plasma levels of FSH necessary to support the attainment of ovulatory maturity of even a single follicle can vary widely between individual ewes, a phenomenon reported for women about a decade ago (14). The factors which govern the setting of this "threshold" level of FSH, however, remain obscure. However, it is likely that this variation in the threshold levels of FSH between sheep contributes, at least in part, to the unpredictability of the ovulatory response when gonadotrophin preparations such as FSH-P or PMSG are used to increase ovulation rate in sheep.

Plasma gonadotrophin concentrations and follicular development during the non-breeding season Most breeds of sheep enter a period of anoestrus during the summer months when no follicles attain ovulatory maturity. Measurement of plasma gonadotrophin concentrations during anoestrus shows that while the mean plasma concentrations of LH are comparable to luteal phase levels during the breeding season, they are lower than those found during the follicular phase (15). Although the mean concentrations of plasma LH, and LH peak frequency in anoestrous ewes are similar to luteal phase levels, anoestrous ewes have only about half as many high amplitude LH-peaks as luteal phase ewes. In contrast to LH, the mean plasma concentrations of FSH during anoestrus are either similar to or higher than those found during the breeding season (16). Thus during anoestrus, as in the luteal phase, follicles cannot attain ovulatory maturity because the ovary is exposed to inadequate levels of LH. Because LH is limiting, the thecal tissue cannot produce enough androgen precursor for the granulosa cells to make sufficient oestradiol-17 $\beta$  to promote follicular maturation, oestrus and the preovulatory LH surge. Oestradiol-17 $\beta$  and inhibin are the major follicular components which exert a negative feedback effect on pituitary FSH, and the production of the former are both androgen dependent (17). Reduced androgen production would therefore reduce the negative feedback influences on FSH production and could thereby account for the rise in plasma FSH concentrations during anoestrus.

As follicular development is restrained during anoestrus by insufficient LH, one might expect that this could be overcome by the exogenous administration of LH. Indeed the administration of LH or gonadotrophin releasing hormone (GnRH) during anoestrus will induce ovulatory cycles, and pregnancies can

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be achieved following mating (15,18). However, although ovulation occurs, the ovulation rate is no greater than that occurring normally during the breeding season. To increase the ovulation rate, FSH must also be administered because, once LH is no longer limiting, the actual number of follicles that achieve ovulatory maturity is limited by FSH (see above).

**Pharmacological manipulation of ovulation rate** From the above, it is clear that FSH plays a key role in determining ovulation rate in sheep. Indeed the most common means of increasing the ovulation rate or promoting superovulation is by the administration of crude forms of FSH, usually in the form of PMSG or FSH-P. These compounds are, however, notorious for the unpredictability of the ovulatory response which they induce. This is undoubtedly due, at least in part, to differences between individual ewes in the "threshold" requirements for FSH. In addition, these FSH preparations contain considerable LH-like activity. Excessive LH is likely to down-regulate thecal LH receptors and hence interfere with follicular development, e.g. Table 1. There is, therefore, a need among reproductive endocrinologists and veterinarians for inexpensive, highly pure forms of FSH that can increase ovulation rate in a more predictable fashion. One strategy to increase plasma FSH concentrations, and thereby ovulation rate, would be to reduce the negative feedback effects of oestradiol-17 $\beta$  and/or inhibin. Immunization against inhibin or oestradiol would be one way of achieving this. Inhibin research is still at a very early stage. Nevertheless, studies with semi-pure inhibin have shown that active immunization against inhibin will increase ovulation rates in sheep (19). Although the administration of crude inhibin reduces the plasma FSH concentrations, once the treatment is stopped the plasma FSH levels rise well above pre-treatment values; this "rebound" effect can also be used to increase ovulation rate (6). With the recent purification of ovarian inhibin to homogeneity (20,21), immunization procedures or the use of inhibin agonists or antagonists may all be useful methods of increasing ovulation rate. Immunization against oestradiol-17 $\beta$  will also raise plasma FSH concentrations, and increase ovulation rates in sheep (22). However, a high proportion of these ewes become infertile and so this technique cannot be used to increase prolificacy. Immunization against androstenedione increases the mean plasma FSH levels (23), increases ovulation rate, and also increases the number of lambs born per ewe mated (22). A commercial preparation of androstenedione immunogen (named Fecundin) is now available for increasing fecundity in ewes. Exactly how the immunization against androgen leads to an increase in plasma FSH remains unclear. It may be through interfering with androgen dependent inhibin production (17). Interference with some direct action of androgen at the level of the pituitary is also possible. At present our understanding of the regulation of FSH production, particularly at the level of the gonadotrope, is still limited. A fuller understanding of how FSH production is controlled at the cellular and mechanistic levels would likely lead to novel means of manipulating plasma FSH concentrations, and hence ovulation rates. Further research in this area should be encouraged.

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**Authors:** K.M. Henderson, L.C. Savage, R.E. Ellen, K. Ball & K.P. McNatty

**Title:** Consequences of increasing or decreasing plasma FSH concentrations during the preovulatory period in Romney ewes.

**Reference:** Journal of Reproduction and Fertility (1988) 84, 187-196

## Consequences of increasing or decreasing plasma FSH concentrations during the preovulatory period in Romney ewes

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**Summary.** Romney ewes were infused with ovine FSH (NIADDK-oFSH-16) for 48 h from the initiation of luteolysis with cloprostenol. Doses of 2.5 or 5 µg/h which partly or completely prevented the normal preovulatory decline in plasma FSH concentrations caused a significant increase in mean ovulation rates. Ovulation rates were not increased significantly if the FSH (5 µg/h) was infused for only 20 h starting from the initiation of luteolysis or 24 h later. Infusion of a less potent and relatively impure preparation of FSH (i.e. FSH-P) at 0.5 mg/h for 48 h after cloprostenol treatment also increased the mean ovulation rate significantly. However, if the FSH-P was given for only the first 24 h, or if the start of the infusion was delayed for more than 12 h, mean ovulation rates were not increased significantly. Infusion of LH (NIADDK-oLH-25, 5 µg/h) for 48 h from the initiation of luteolysis decreased the mean ovulation rate significantly.

Administration of bovine follicular fluid to suppress plasma FSH concentrations below normal during the first 24 h after cloprostenol injection did not delay oestrus. However, oestrus was delayed by ~2 days if plasma FSH concentrations were reduced by bovine follicular fluid 24 h after the initiation of luteolysis.

As ovulation rate increased, the mean weight of individual corpora lutea of each ewe decreased. In ewes with a single ovulation, most corpora lutea weighed > 600 mg, but as the ovulation rate increased the proportion of corpora lutea present weighing < 400 mg rose steadily. The mean concentrations of luteal tissue progesterone were not influenced significantly by ovulation rate.

**Keywords:** sheep; ovulation rate; FSH; follicular fluid

### Introduction

In sheep, as in other species, adequate exposure to the pituitary gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) is essential for follicles to mature and ovulate. After the onset of luteolysis, plasma concentrations of LH rise progressively as pituitary LH output is released from suppression by luteal progesterone. In contrast, plasma concentrations of FSH fall progressively due to the negative feedback effects on pituitary FSH production of the increasing amounts of oestradiol-17β (and possibly inhibin) secreted by the developing follicle(s) (Miller *et al.*, 1981; Baird, 1983). This decline in plasma FSH concentrations persists until positive steroidal feedback elicits the preovulatory surge of gonadotrophins. Despite the falling plasma FSH concentrations during the preovulatory period, sustained follicular development still requires some FSH, because reducing plasma FSH concentrations even further by administration of bovine follicular fluid (a rich source of inhibin) prevents follicles from attaining preovulatory maturity (Henderson *et al.*, 1986).

As follicle size increases, the sensitivity of sheep granulosa cells to FSH increases (Henderson *et al.*, 1985, 1987). This may be important in ensuring that final maturation of follicles can occur in the face of declining plasma FSH concentrations during the preovulatory period. The increasing sensitivity to FSH of granulosa cells in large developing follicles probably protects them from the deleterious effects of low plasma FSH concentrations. Follicles less sensitive to FSH are likely to undergo atresia due to lack of FSH. By this reasoning, one may argue that additional follicles could be induced to reach ovulatory maturity by raising plasma concentrations of FSH during the preovulatory period, thereby increasing follicular exposure to FSH. This possibility was examined in the present study. Highly purified ovine FSH (NIADDK-oFSH-16) was infused to raise plasma FSH concentrations, while administration of bovine follicular fluid was used specifically to suppress plasma FSH concentrations (McNeilly, 1985; Henderson *et al.*, 1986). For comparative purposes, the effects of infusion of FSH-P were also studied. FSH-P is commonly used to superovulate cows and goats, and less frequently sheep (Armstrong & Evans, 1983; Monniaux *et al.*, 1983). However, compared to NIADDK-oFSH-16, it is a relatively impure FSH preparation, and generally has more LH than FSH activity (Monniaux *et al.*, 1983; Lindsell *et al.*, 1986).

### Materials and Methods

**Gonadotrophins.** Ovine FSH (NIADDK-oFSH-16, FSH activity  $20 \times$  NIH-FSH-S1 U/mg, LH activity  $0.04 \times$  NIH-LH-S1 U/mg) and ovine LH (NIADDK-oLH-25; LH activity  $2.3 \times$  NIH-LH-S1 U/mg, FSH contamination  $<0.5\%$  by weight) were provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, U.S.A. FSH-P was obtained from Burns-Biotec Laboratories Inc., Omaha, Nebraska, U.S.A. Solutions of these gonadotrophins were prepared in saline (0.9% NaCl w/v) containing 0.2% ovine serum albumin immediately before their infusion into sheep.

**Follicular fluid.** A single batch of bovine follicular fluid (bFF) was used for the experiments in this study. Ovaries were collected from cows slaughtered at a local abattoir. Antral follicles on the surface of the ovaries were punctured with a 20-gauge needle, and the bFF aspirated under vacuum. Debris and red blood cells were removed from the pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and the supernatant was stored frozen at  $-20^\circ\text{C}$ . Several collections of bFF were subsequently thawed, and steroids removed by treatment with dextran T-70 (0.1%, Pharmacia Fine Chemicals AB, Uppsala, Sweden) coated charcoal (Norit A, 1%, Fisher Scientific Co., Springfield, New Jersey, U.S.A.) as described previously (Henderson *et al.*, 1986). The bFF was then stored at  $-20^\circ\text{C}$  until needed.

**Sheep and their treatments.** Parous New Zealand Romney ewes (aged 2.5–3.5 years, and weighing 50–60 kg) were used. These flock ewes were grazed on pasture and run with a vasectomized ram fitted with a marking harness. The ewes were examined daily for signs of oestrus.

Ewes between Days 8 and 11 of the oestrous cycle (Day 0 = day of oestrus) were penned indoors. Those to be treated with bFF were fitted with one intrajugular cannula, for blood sampling. Those to receive gonadotrophins were fitted with two contralateral intrajugular cannulae, one for collecting blood samples and the other for infusing gonadotrophins. The next day luteolysis was initiated with an intramuscular (i.m.) injection of cloprostenol (125 µg, Coopers Animal Health Ltd, Upper Hutt, New Zealand). Infusions of FSH, FSH-P, LH or vehicle alone began immediately thereafter. The solutions were delivered by an infusion pump adjusted to deliver 1 ml of solution in 1 minute, once per hour for up to 48 h. The infusate reservoir was packed in ice. Ewes receiving bFF were injected intramuscularly with 5 ml bFF at selected times after receiving cloprostenol. Blood samples (2–3 ml) were taken from all ewes at selected times before injection of cloprostenol, and at 4-h intervals thereafter for up to 72 h. At the end of this period the cannulae were removed, and the ewes were injected intramuscularly with antibiotic (5 ml Streptopen: Glaxo New Zealand Ltd, Palmerston North, New Zealand), and returned to pasture. Ewes which had received bFF, together with their controls, were bled daily for a further 12 days. A vasectomized ram fitted with a marking harness was present with these sheep at all times, and the ewes were examined at least twice daily for signs of oestrus.

At 13 days after receiving cloprostenol, the ewes which had been subjected to infusion were slaughtered and their ovaries removed. The number of ovulations (ovulation rate) was determined by counting the number of corpora lutea present.

All blood samples were centrifuged at 1200 g for 15 min at 4°C. The plasma was removed and stored frozen ( $-20^\circ\text{C}$ ) until assayed for FSH, LH and progesterone by radioimmunoassay.

**Luteal tissue.** Corpora lutea were dissected from the ovaries, trimmed free of adherent connective tissue, and weighed individually. Each corpus luteum was homogenized in 10 ml chloroform:methanol (2:1, v/v) and left to stand overnight at room temperature. The chloroform:methanol was then removed and stored at  $-20^\circ\text{C}$  until assayed for progesterone. A single homogenization in 10 ml chloroform:methanol was found to be sufficient to extract  $>99\%$

of luteal progesterone, as assessed by the additional amount of progesterone extracted following a second homogenization in a further 10 ml of extractant.

**Radioimmunoassays.** Plasma concentrations of FSH were measured using a double-antibody sheep FSH radioimmunoassay kit and procedures provided by the National Hormone and Pituitary Program, NIADDK, Bethesda, Maryland, U.S.A. The FSH reference preparation was NIAMDD-oFSH-RP-1 and the iodinated tracer was NIAMDD-oFSH-I-1. The FSH antiserum (rabbit) was NIAMDD-anti-oFSH-1. The sensitivity of the assay was 0.05–0.1 ng per assay tube (0.2–0.4 ng/ml plasma) and the intra- and inter-assay coefficients of variation were <10%. Plasma concentrations of LH were measured by radioimmunoassay as described previously (McNatty *et al.*, 1981), using an antibody raised in a rabbit against NIH-LH-S11. The LH antiserum exhibited low cross-reactions with other sheep pituitary hormones: prolactin (NIH-P-S12) 0.09%; thyroid-stimulating hormone (NIH-TSH-S8) 2.4%; growth hormone (NIH-GH-S11) 0.4%; FSH (NIH-FSH-S10) 0.4%. The pituitary LH preparation for both the standard and the iodinated tracer was NIH-LH-S11. The sensitivity of the assay was 0.04 ng per assay tube (0.2 ng/ml plasma), and the intra- and inter-assay coefficients of variation were 7% and 12% respectively. Progesterone was extracted from plasma with petroleum ether. The extraction efficiency was monitored by the addition of tritiated progesterone, and the mean recovery was 82%. Aliquants of the organic extracts of plasma and luteal tissue were evaporated under a stream of nitrogen, and reconstituted in 0.1 M-phosphate buffered saline (pH 7) containing 0.1% gelatin and 0.1% sodium azide. Progesterone was measured in these aqueous samples by a specific radioimmunoassay described previously (McNatty *et al.*, 1981). Steroids cross-reacting with the progesterone antiserum were 11 $\alpha$ -hydroxyprogesterone (120%), 11 $\beta$ -hydroxyprogesterone (25%), 20 $\alpha$ -dihydroprogesterone (3.5%) and androstenedione (0.4%). The limit of sensitivity of the assay (per tube) was 25 pg (125 pg/ml), and the intra- and inter-assay coefficients of variation were <10%.

**Statistics.** The data were analysed statistically using analysis of variance in conjunction with Newman–Keuls multiple range test, or Student's *t* test as appropriate. When heterogeneity of variance was indicated by Bartlett's test, the variances were equalized by transforming the data to logarithms ( $\ln(x + 1)$ ). In these instances the data have been presented as geometric means together with 95% confidence limits (Tables 1–3, Fig. 4). The level of significance was set at  $P < 0.05$ .

## Results

### *Effects of infusion of ovine FSH (NIADDK-oFSH-16) and LH (NIADDK-oLH-25)*

Figure 1 shows the effect on plasma FSH concentrations of infusion of FSH (0, 2.5 and 5  $\mu$ g/h) for 48 h from the initiation of luteolysis. Blood samples were taken 24, 16, 8 and 0 h before injection of cloprostenol, and at 4-h intervals thereafter for 48 h. From 8 to 40 h after injection of cloprostenol, mean plasma concentrations of FSH were significantly higher in ewes receiving 5  $\mu$ g FSH/h than in ewes receiving 0 (control ewes) or 2.5  $\mu$ g FSH/h ( $P < 0.01$ ). In ewes receiving 2.5  $\mu$ g FSH/h, mean plasma concentrations of FSH were significantly higher than those of control ewes from 16 to 36 h after injection of cloprostenol ( $P < 0.05$ ). Infusion of 2.5 and 5  $\mu$ g FSH/h for the 48-h period also caused a dose-dependent increase in mean ovulation rates (Table 1). Control ewes had ovulation rates of 1 or 2. In ewes receiving 2.5  $\mu$ g FSH/h, most ewes had ovulation rates of 2 or 3, but no higher, whereas in ewes receiving 5  $\mu$ g FSH/h ovulation rates of 4, 6, 8, 9 and 14 were recorded in 5 ewes.

In contrast to the effects of FSH, infusion of LH for the 48-h period resulted in a significant decrease in mean ovulation rate, relative to control ewes. Plasma LH concentrations were raised substantially during the infusion of LH, relative to control ewes ( $2.5 \pm 0.2$  vs  $0.6 \pm 0.1$  ng LH/ml, overall mean values  $\pm$  s.e.m. for 8 ewes/group,  $P < 0.001$ ). However, mean FSH concentrations did not differ significantly from those of the control ewes ( $1.0 \pm 0.2$  vs  $0.9 \pm 0.1$  ng FSH/ml for LH-infused and control ewes respectively, overall mean values  $\pm$  s.e.m. for 8 ewes/group,  $P > 0.05$ ). Figure 2 shows the effect on plasma FSH concentrations of infusion of FSH (5  $\mu$ g/h) for 20 h beginning from the start or 24 h after the start of luteolysis. Blood samples were taken 24, 12 and 0 h before administration of cloprostenol and at 4-h intervals thereafter for 48 h. In ewes receiving FSH from the start of luteolysis (Fig. 2a), the mean plasma FSH concentrations were significantly higher than in control ewes (infused with vehicle alone) from 8 to 20 h after injection of cloprostenol. In addition, in those FSH-infused ewes, the mean plasma FSH concentrations were only  $\leq 1$  ng/ml for a 12-h period from 24 to 36 h after cloprostenol, whereas in control ewes mean values were  $\leq 1$  ng/ml for 28 h from 12 to 40 h after cloprostenol. In ewes infused with FSH for 20 h,

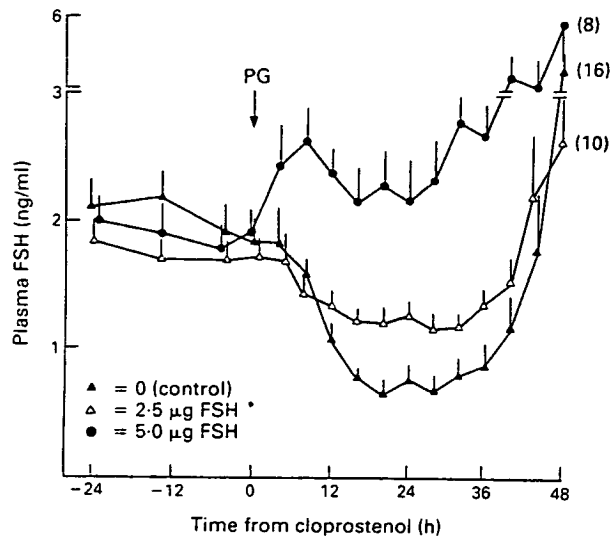


Fig. 1. Plasma FSH concentrations of ewes infused with 0, 2.5 or 5 µg FSH (NIADDK-oFSH-16)/h for 48 h from the start of luteolysis with cloprostenol (PG). Values are means of (N) ewes. Vertical lines show the s.e.m.

Table 1. Frequency of ovulation rates and mean ovulation rates after infusion of ovine FSH (NIADDK-oFSH-16) or LH (NIADDK-oLH-25) for 48 h from the start of luteolysis

Treatment	Ovulation rate					Geometric mean ovulation rate (95% confidence limits)
	0	1	2	3	4-14	
Vehicle alone	0	10	6	0	0	1.3 (1.1-1.6)
2.5 µg FSH/h	0	1	6	3	0	2.1 (1.7-2.7)
5.0 µg FSH/h	0	0	3	0	5	4.8 (2.4-8.8)
5.0 µg LH/h	2	6	0	0	0	0.7 (0.3-1.2)

Geometric mean values differ significantly between all groups,  $P < 0.05$ .

starting 24 h after injection of cloprostenol (Fig. 2b), the mean plasma concentrations of FSH were only significantly higher than those in the control ewes in the blood sampling at 36 h. However, mean plasma FSH concentrations rose above 1 ng/ml from 32 h after cloprostenol in the FSH-infused ewes, whereas in the control ewes mean FSH values did not rise consistently above 1 ng/ml until 40 h after cloprostenol. Infusion of FSH for these 20-h periods did not significantly influence mean ovulation rates. In ewes infused with FSH for 20 h from the time of injection of cloprostenol, the mean ovulation rate was  $1.6 \pm 0.2$  (s.e.m.), compared to  $1.4 \pm 0.2$  for control ewes ( $N = 8$ ,  $P > 0.05$ ). In ewes in which the start of the 20 h infusion was delayed until 24 h after injection of cloprostenol, the mean ovulation rate was  $1.4 \pm 0.2$  and in the control ewes it was  $1.5 \pm 0.2$  ( $N = 8$ ,  $P > 0.05$ ).

Infusion of FSH for the 20-h periods or for 48 h had no significant effect on plasma progesterone concentrations, relative to control ewes. In all groups, mean plasma concentrations of progesterone were  $\sim 2$  ng/ml immediately before injection of cloprostenol. Mean values then fell to about 0.5 ng/ml within 24 h and remained at this level for the remainder of the 48-h post-injection sampling period.

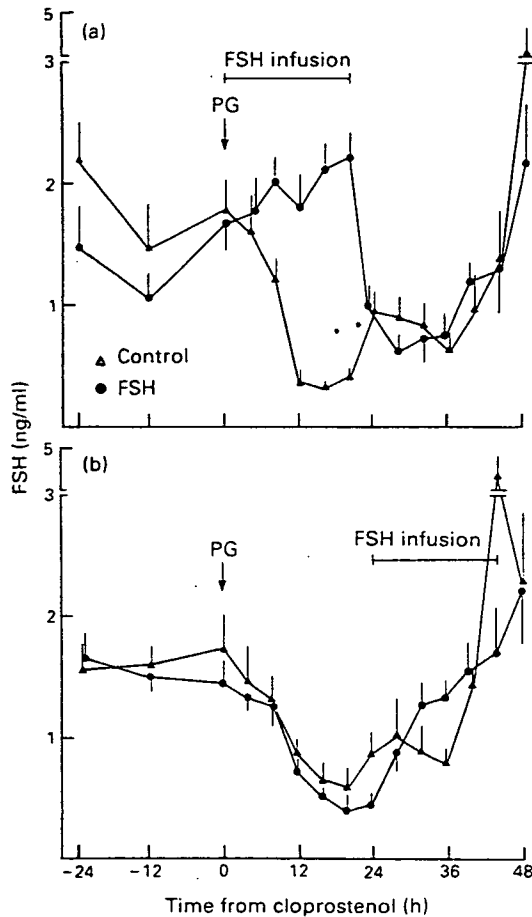
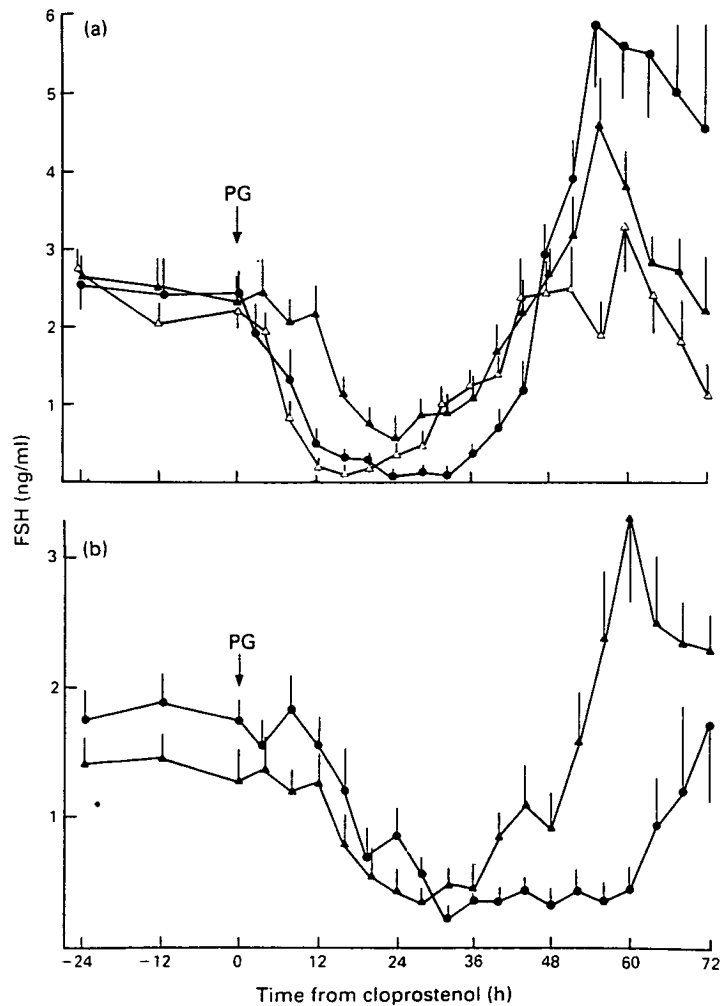


Fig. 2. Plasma FSH concentrations of ewes infused with 5  $\mu$ g NIADDK-oFSH-16/h for 20 h starting (a) at the time of cloprostenol injection (PG) and (b) 24 h after injection of cloprostenol. Values are means of 8 ewes with vertical lines showing the s.e.m.

*Reduction of plasma FSH concentrations with bFF*

A single intramuscular injection of 5 ml bFF at the time of cloprostenol treatment caused a marked fall in mean plasma FSH concentrations (Fig. 3a). Within 8–12 h of injection, the mean FSH concentrations were significantly below the control values, but had risen to control values by 24 h. A second intramuscular injection of 5 ml bFF, 12 h after the first, kept mean FSH concentrations significantly below control values for a further 28 h. In ewes given the single injection of bFF, the mean time to oestrus from injection of cloprostenol was  $2.5 \pm 0.2$  (s.e.m.) days, which was not significantly different from that for control ewes ( $2.3 \pm 0.2$  days,  $N = 5$ ,  $P > 0.05$ ). However, in ewes given 2 injections of bFF, the mean time to oestrus was lengthened significantly to  $4.8 \pm 0.4$  days ( $P < 0.05$ ,  $N = 5$ ). Administration of 2 intramuscular injections of 5 ml bFF 24 and 36 h after cloprostenol injection also caused a significant delay in the time to oestrus ( $4.2 \pm 0.5$  vs  $2.4 \pm 0.1$  days, mean  $\pm$  s.e.m. for  $N = 5$ ,  $P < 0.01$ ). Plasma FSH concentrations did not differ between this group and its control group for the first 36 h after cloprostenol injection (Fig. 3b). Thereafter, the mean FSH concentrations in control ewes rose steadily while those in the bFF-treated ewes remained at basal values until 60 h after cloprostenol treatment. The increase in the time to oestrus

after injection of bFF, and the consequent delay in formation of new corpora lutea was also reflected in plasma progesterone concentrations. In all groups, mean plasma concentrations fell rapidly from  $\sim 2$  to  $0.5$  ng/ml within 24 h of administration of cloprostenol. In control ewes and in ewes receiving a single injection of bFF at the time of cloprostenol administration, mean plasma concentrations of progesterone had risen above  $1$  ng/ml again, indicative of the formation of new corpora lutea, by 9 days after receiving cloprostenol. However, in ewes receiving bFF, 0 and 12 h or 24 and 36 h after cloprostenol, it was 10 and 11 days respectively after cloprostenol injection before mean plasma progesterone concentrations were again  $> 1$  ng/ml.



**Fig. 3.** Effect of administration of bovine follicular fluid on plasma FSH concentrations in sheep. Values are means of 5 ewes with vertical lines showing the s.e.m. (a) ▲—▲ cloprostenol alone (controls); △—△ 5 ml bFF i.m. at time of cloprostenol injection; ●—● 5 ml bFF i.m. 0 and 12 h after cloprostenol injection (PG). (b) ▲—▲ cloprostenol alone (controls); ●—● 5 ml bFF i.m. 24 and 36 h after cloprostenol injection (PG).

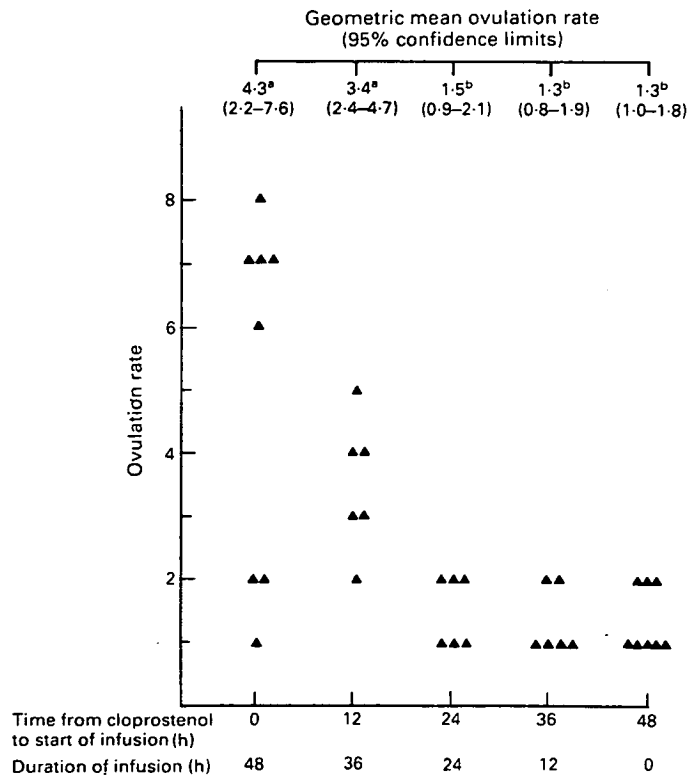
Effects of infusions of FSH-P

Table 2 shows that infusion of FSH-P at 0.5 mg/h for 48 h from the start of luteolysis significantly increased the geometric mean ovulation rate, relative to control ewes (infused with vehicle alone). The mean ovulation rates were not increased significantly if this dose of FSH-P was infused for only the first 24 h (Table 2), or if the start of the infusion was delayed for more than 12 h after injection of cloprostenol (Fig. 4).

**Table 2.** Frequency of ovulation rates and mean ovulation rates (8 ewes/group) after infusion of FSH-P from the start of luteolysis

Dose of FSH-P	Ovulation rate				Geometric mean ovulation rate (95% confidence limits)
	1	2	3	4-8	
0	5	3	0	0	1.3 <sup>a</sup> (1.0-1.8)
0.1 mg/h for 48 h	2	2	0	4	3.1 <sup>a,b</sup> (1.5-5.7)
0.5 mg/h for 48 h	1	2	0	5	4.3 <sup>b</sup> (2.2-7.6)
0.5 mg/h for 24 h	3	3	1	1	2.0 <sup>a,b</sup> (1.1-3.3)

Geometric mean values with different superscripts are significantly different ( $P < 0.025$ ).



**Fig. 4.** Effect on ovulation rate (▲ for individual ewes) of delaying the start of infusion of FSH-P (0.5 mg/h) after initiation of luteolysis with cloprostenol. Geometric mean ovulation rate values with different superscripts are significantly different ( $P < 0.025$ ).



*Corpus luteum weights and progesterone concentrations*

The corpus luteum data were grouped together irrespective of ewe treatment because, for any given ovulation rate, there was no significant effect of treatment on the weights or progesterone concentrations of the corpora lutea. As ovulation rate increased, the geometric mean weight of the individual corpora lutea per ewe fell significantly (Table 3). Examination of the individual corpus luteum weights showed that, when the ovulation rate was 1, 94% of corpora lutea weighed > 600 mg. However,  $\leq 30\%$  of CL weighed > 600 mg in ewes with ovulation rates  $\geq 2$ . As ovulation rate increased, the proportion of corpora lutea weighing < 400 mg increased progressively. The geometric mean concentrations of luteal progesterone were not significantly influenced by ovulation rate.

**Table 3.** Influence of ovulation rate on the distribution of corpus luteum weights, geometric mean luteal weights, and geometric mean luteal progesterone concentrations

Ovulation rate	Wt of corpora lutea (mg)						Geometric mean luteal wt (mg) per ewe (95% confidence limits)	Geometric mean luteal progesterone conc. ( $\mu\text{g/g}$ ) per ewe (95% confidence limits)	No. of ewes (N)
	100–399	400–499	500–599	600–699	700–799	>800			
1	0	0	3	19	16	14	722 <sup>a</sup> (694–752)	7.4 (6.7–8.2)	52
2	4	27	33	20	4	4	549 <sup>b</sup> (526–573)	7.5 (7.0–8.1)	46
3	8	5	3	2	0	0	435 <sup>c</sup> (381–497)	7.7 (6.5–9.2)	6
4, 5, 6	28	12	5	0	0	0	349 <sup>d</sup> (322–377)	7.1 (6.1–8.3)	9
7, 8, 9, 14	54	16	2	2	0	0	339 <sup>d</sup> (301–382)	6.6 (5.1–8.5)	9

The number of ewes with ovulation rates of 4, 5, 6, 7, 8, 9 and 14 were 4, 1, 4, 5, 2, 1 and 1, respectively. Geometric mean values with different superscripts in the same column are significantly different ( $P < 0.05$ ).

### Discussion

The present study demonstrates, for sheep, that relatively small changes in plasma FSH concentrations during the preovulatory period have a marked effect on follicular development and subsequent ovulation rate. Raising plasma FSH concentrations, and thereby increasing ovarian exposure to FSH, enhances follicular development and increases ovulation rates. In contrast, decreasing plasma FSH concentrations below normal impairs follicular development, as indicated by a lengthened time to oestrus.

Partial or complete prevention of the normal preovulatory decline in plasma FSH concentrations (Fig. 1) was associated with a subsequent increase in mean ovulation rates (Table 1). This is consistent with the notion that the normally low preovulatory plasma FSH concentrations may restrict the number of follicles which can normally attain preovulatory maturity. Only those follicles in which the granulosa cells (the target cells for FSH action) have an increased sensitivity to FSH (Henderson *et al.*, 1985, 1987) may be able to continue their development in the face of such low plasma FSH concentrations. By infusing FSH and thereby raising plasma FSH concentrations, additional follicles can be brought to ovulatory maturity and hence ovulation rates increased. In normal circumstances these additional follicles would probably have undergone atresia due to their

relative insensitivity to FSH. Raising mean plasma FSH concentrations for 20 h but then letting the concentrations drop back to normal values again (Fig. 2a) did not result in an increase in the mean ovulation rate. Continual exposure to elevated plasma FSH concentrations therefore seems necessary to bring these additional follicles to preovulatory maturity. Similarly, commencing infusion of FSH 24 h after initiating luteolysis (Fig. 2b) also failed to increase the mean ovulation rate. Thus, follicles that had previously been exposed to low FSH concentrations, and so likely to be already undergoing atresia, could not be 'rescued' and brought to ovulatory maturity in synchrony with the presumptive preovulatory follicle(s). An increase in the frequency of double ovulations can be achieved by infusing FSH for a 24-h period in the 48 h before initiating luteolysis (McNatty *et al.*, 1985). Presumably this treatment is advancing the maturation of a follicle (which would normally have undergone atresia), thereby allowing it to 'survive' the preovulatory fall in plasma FSH concentrations and attain ovulatory maturity.

In contrast to FSH, infusion of LH did not increase the mean ovulation rate (Table 1). During the preovulatory period, plasma LH concentrations are increasing (Baird, 1983). Presumably, therefore, it is the availability of FSH, rather than LH, which is the major factor which limits the number of follicles maturing to ovulation. However, it is still possible that the administration of LH together with FSH may allow more follicles to attain ovulatory maturity in synchrony than by giving FSH alone.

Specific suppression of preovulatory plasma FSH concentrations by administration of bFF has a deleterious effect on granulosa cell proliferation, follicular oestradiol-17 $\beta$  biosynthesis, and the development of cellular responsiveness to FSH and LH (Henderson *et al.*, 1986). Consequently, oestrus is delayed until bFF treatment is stopped and preovulatory maturation is restored (Henderson *et al.*, 1986). In the present study, bFF was used to reduce plasma FSH concentrations below normal at different times throughout the preovulatory period. If the normal fall in plasma FSH concentrations occurring after the initiation of luteolysis was accelerated (Fig. 3a), oestrus was not delayed, providing that plasma FSH concentrations returned to normal for the second 24 h period (Fig. 3a). However, if plasma FSH concentrations were suppressed for longer (Fig. 3a), or if bFF was given 24 h after the initiation of luteolysis (Fig. 3b), then oestrus was delayed. Adequate exposure to FSH therefore appears to be required for follicular development even during the final stages of maturation. This is consistent with the findings of Zeleznik & Kubik (1986) for the monkey. It is unlikely that the lengthened time to oestrus, resulting from bFF treatment, was due to a direct action of bFF on the ovary, rather than as a consequence of reduced plasma FSH concentrations. Previous studies have shown that this effect of bFF treatment can be prevented by simultaneous treatment with FSH (McNeilly, 1985) or pregnant mares' serum gonadotrophin (Henderson *et al.*, 1986).

Repeated injection of FSH-P, starting before the onset of luteolysis and continuing throughout the follicular period, have previously been found to increase ovulation rates in sheep (Wright *et al.*, 1981; Armstrong & Evans, 1983). Infusion of FSH-P for 48 h from the start of luteolysis also increased mean ovulation rate, although at a higher dosage than with the ovine FSH (Table 2). However, as with the ovine FSH, infusion of FSH-P for only the first 24 h also failed to increase the mean ovulation rate (Table 2). Similarly, if the start of the infusion was delayed for longer than 12 h after the initiation of luteolysis, ovulation rates  $>2$  were not observed (Fig. 4). Interestingly, the mean ovulation rate of ewes infused for 48 h with 0.5 mg FSH-P/h ( $5.0 \pm 1.0$ ) was similar to that obtained in Romney ewes given a similar total dosage as multiple injections over a 96-h period, starting 48 h before the removal of vaginal progestagen sponges (Armstrong & Evans, 1983; K. P. McNatty, unpublished observations). Perhaps the development of devices whereby low amounts of FSH can be given continuously (e.g. by slow release) may provide a more efficient means of inducing superovulation than by the administration of FSH preparations as repeated injections.

As ovulation rate increased, the mean corpus luteum weight decreased (Table 3), suggesting that an increasing number of follicles were ovulating at a smaller size than usual. The actual growth (i.e. increase in size) of a follicle may be independent of its acquisition of characteristics associated

with preovulatory maturity. Thus, raising plasma FSH concentrations above the normal threshold concentrations, thereby increasing ovarian exposure to FSH, may allow follicles to attain preovulatory maturity at a smaller size than normal. In highly prolific Booroola sheep, which naturally have high ovulation rates, mean corpus luteum weights are also inversely related to ovulation rate (McNatty *et al.*, 1986). In this breed, the presence of a fecundity gene is thought to result in more follicles ovulating at smaller diameters as a consequence of increased ovarian exposure to FSH (McNatty *et al.*, 1986, 1987).

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## Studies of the effectiveness of gonadotrophin-releasing hormone, steroids and follicular fluid in modulating ovine gonadotrophin output *in vivo* and *in vitro*

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**Summary.** Gonadotrophin-releasing hormone (GnRH) readily stimulated LH output by sheep pituitary cells *in vitro*, and raised plasma LH concentrations *in vivo* in sheep, in a dose-dependent fashion. However, increases in FSH levels were only marginal by comparison. Dose-dependent decreases in sheep pituitary cell FSH output and in plasma FSH concentrations were caused by sheep follicular fluid and oestradiol-17 $\beta$  *in vitro*, and by bovine follicular fluid and oestradiol benzoate *in vivo*. In contrast, LH concentrations were only reduced slightly at the higher doses of these reagents. Cumulative suppressive effects of follicular fluid and oestradiol-17 $\beta$  (oestradiol benzoate) on FSH levels were observed both *in vitro* and *in vivo*. The transient positive feedback effect of oestradiol benzoate on FSH output negated the suppressive effect of bovine follicular fluid on plasma FSH concentrations. Progestagens, androgens and catechol oestrogens also suppressed mean FSH output *in vitro*, though not as effectively as oestradiol-17 $\beta$ . While only 1–5 pg/ml of oestradiol-17 $\beta$  was needed to suppress significantly mean FSH output *in vitro*, > 500 pg/ml of the other steroids was required. Seminal plasma inhibin-like peptide failed to suppress mean FSH output by cultured sheep pituitary cells at doses from 1 pg/ml to 500 ng/ml. At higher doses, both FSH and LH output was suppressed and this was accompanied by morphological deterioration of the cells. It is suggested that, to raise plasma FSH concentrations with a view to increasing ovulation rates in sheep, the development of means to reduce the negative feedback effects of steroids, notably oestradiol-17 $\beta$ , and inhibin on FSH secretion may be a more appropriate pharmacological strategy than increasing pituitary exposure to GnRH.

**Keywords:** pituitary; gonadotrophins; follicular fluid; steroids; GnRH; sheep

### Introduction

The number of fertilizable ova shed at ovulation (i.e. ovulation rate) is a major determinant of prolificacy in sheep. Adequate exposure to both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) is essential for follicular maturation. Recent studies suggest that the low plasma FSH concentrations normally occurring during the preovulatory period may restrict the number of follicles which can attain ovulatory maturity in synchrony, and hence limit ovulation rate (Henderson & McNatty, 1987; Henderson *et al.*, 1988). Raising plasma FSH concentrations by infusion of low doses of purified FSH for 24 h in the 48 h before the onset of luteolysis, or for 48 h after the onset of luteolysis increases ovulation rates (McNatty *et al.*, 1985; Henderson *et al.*, 1988). In contrast ovulation rates are not increased above normal by raising plasma LH concentrations, either by infusion of purified LH (Henderson *et al.*, 1988), or by infusion of GnRH (McNatty *et al.*, 1981a). Ovulation rates can, however, be increased by injection of pharmacological amounts of

human chorionic gonadotrophin, though this may be a consequence of contamination of the preparation with FSH-like activity (Radford *et al.*, 1984). Gonadotrophin output is regulated by gonadotrophin-releasing hormone (GnRH), steroids and possibly inhibin. Several studies have focussed on the effects of these agents on sheep pituitary secretion of FSH and LH (Martin, 1984; Martin *et al.*, 1986, 1988). The purpose of this study was to gain additional information in sheep on the relative effectiveness of these agents in modulating gonadotrophin output, particularly that of FSH in view of its importance in influencing ovulation rate.

## Materials and Methods

### *Animals and reagents*

**Sheep.** Parous New Zealand Romney ewes (aged 2.5–3.5 years and weighing 50–60 kg) were used for this study. They were grazed on open pasture, and run with a vasectomized ram fitted with a marking harness to detect oestrous activity. The ewes were examined daily for signs of oestrus. In instances when ovariectomized ewes were required, ovaries were surgically removed at least 4 weeks, and no more than 9 weeks, before any experimental procedure (e.g. pituitary removal, in-vivo study) was performed. The minimum 4-week delay was to ensure completion of the major rise in plasma gonadotrophin concentrations which occurs after ovariectomy, and is indicative of the removal of ovarian feedback effects on the pituitary (Montgomery *et al.*, 1987; McNatty *et al.*, 1989).

**Reagents.** GnRH and seminal plasma inhibin-like peptide were obtained from Peninsula Laboratories Inc., Belmont, CA, USA. Oestradiol-17 $\beta$ ,  $\beta$ -oestradiol-3-benzoate, 2-hydroxy oestradiol, 4-hydroxy oestradiol, progesterone, testosterone, 5 $\alpha$ -dihydrotestosterone, sodium pyruvate, sodium bicarbonate, glutamine, gentamicin, collagenase (type IV), deoxyribonuclease I and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co., St Louis, MO, USA. Bovine serum albumin (BSA) was obtained from Immuno Chemical Products Ltd, Auckland, New Zealand; dispase from Boehringer Mannheim GmbH, Mannheim, W. Germany; amphotericin B from E. R. Squibb & Son Inc., Princeton, NJ, USA; penicillin from Glaxo Laboratories Ltd, Greenford, UK, and Dulbecco's Modified Eagle Medium and non-essential amino acids from Gibco Laboratories, Grand Island, NY, USA. Hanks' balanced salt solution and calcium- and magnesium-free Dulbecco's phosphate buffered saline (DPBS) were prepared in our laboratory using salts of tissue culture grade. The 3 $\alpha$ -hydroxy and 3 $\beta$ -hydroxy-4-pregnen-20-one were a gift from Dr J. Wiebe, University of Western Ontario, London, Canada. The sheep FSH radioimmunoassay kit and sheep LH preparations (NIDDK-oLH-I-3 and NIDDK-oLH-24) were gifts from the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, University of Maryland, USA.

Follicular fluid is rich in inhibin activity and obtained relatively easily, and so provides a convenient crude inhibin preparation. Single batches of bovine (b) and ovine (o) follicular fluid (FF) were prepared for this study. The oFF was used to investigate inhibin activity in pituitary cultures *in vitro*, and the bFF was used to investigate inhibin activity in the in-vivo studies. (The large amounts of FF required for the in-vivo studies made it impractical to use oFF.) Ovaries were obtained from cows and sheep slaughtered at a local abattoir. Antral follicles on the surface of the ovaries were punctured with a 20-gauge needle, and the FF aspirated. Debris and red blood cells were removed from pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and the supernatant was stored frozen at –20°C. Several collections of FF were subsequently thawed, and the steroids removed by treatment with dextran T-70 (0.1%, Pharmacia Fine Chemicals AB, Uppsala, Sweden) -coated charcoal (Norit A, 1%, Fisher Scientific Co., Springfield, New Jersey, USA) as described previously (Henderson *et al.*, 1986). The FF was then stored in aliquants at –20°C until needed.

A single batch of serum for use in the pituitary cell culture medium was obtained from ovariectomized ewes. Four ewes which had been ovariectomized 1 month previously were exsanguinated under anaesthesia through a catheter inserted into the jugular vein. The blood was allowed to clot and the serum collected and stored frozen at –20°C. After checking that none of the 4 sera were toxic to pituitary cells in culture, the sera were pooled and treated with dextran T-70 (0.01%) -coated charcoal (0.1%) as for the FF samples. The pooled sera were then stored at –20°C in 100 ml samples until needed.

### *Pituitary cell cultures*

Pituitaries were recovered from sheep (usually 2 sheep at a time) immediately after their slaughter at Wallaceville, and placed in chilled Hanks' Balanced Salt Solution containing 1% bovine serum albumin, gentamicin (50  $\mu$ g/ml), amphotericin B (2.5  $\mu$ g/ml) and penicillin (100 units/ml), pH 7.4 (HBSS). The pituitaries were washed several times with HBSS, and the posterior pituitary and any extraneous tissue was dissected off and discarded. Remaining anterior pituitary was thinly sliced and placed into 50 ml of pre-warmed (37°C) HBSS containing 25 mg collagenase, 50 mg dispase and 50  $\mu$ g deoxyribonuclease I, pH 7.4, and incubated with shaking for 45 min at 37°C. The enzyme solution was then replaced with 50 ml DPBS with added BSA (1%) and 2 mM-EDTA, pH 7.4, and the incubation continued for a further 10 min at 37°C with shaking. The tissue fragments were washed 5 times with DPBS and then dispersed by repeatedly drawing gently through the tip of a Pasteur pipette, and a series of needles (18–20 gauge) attached to a 1 or

2 ml syringe. As the cells were released they were transferred into HBSS. The collected cells were centrifuged at 200 *g* for 10 min, washed with HBSS and centrifuged again at 200 *g* for 10 min. The cells were finally resuspended in Dulbecco's Modified Eagle Medium containing 10% dextran-coated charcoal-treated ovariectomized sheep serum, glutamine (1%), non-essential amino acids, sodium pyruvate (1 mM), sodium bicarbonate (3.7 g/l), gentamicin (50 µg/ml), amphotericin B (2.5 µg/ml) and penicillin (100 units/ml), pH 7.4 (DCM). The cells were counted using a haemocytometer, and the volume of DCM was adjusted to give  $0.5-1 \times 10^6$  cells/ml. Samples of 1 ml were pipetted into individual round wells (16 mm diam.) of multi-welled tissue-culture dishes (Nunc, Kamstrup, Denmark) and incubated for 24 h at 37°C in an humidified atmosphere of 5% CO<sub>2</sub> in air. During this time the cells attached to the bottom of the wells. The medium was then replaced with fresh DCM (1 ml) with or without added GnRH, FF, inhibin-like peptide or steroids. Four replicated cultures for each dose of each treatment were normally established, and some treatments were repeated on separate occasions. The cells were incubated for a further 72 h with the medium being renewed every 24 h. The spent media were stored frozen at -20°C. At the end of the culture period, the cells were washed thoroughly with saline and stored frozen until assayed for protein by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard.

### Studies in vivo

**Treatment with GnRH.** On the day before treatment with GnRH 16 ovariectomized ewes, and on a separate occasion, 2 months later, 16 intact ewes at Day 9 of the oestrous cycle (Day 0 = day of oestrus), were penned indoors and each fitted with an intrajugular cannula for subsequent blood sampling and administration of GnRH. The next day, 2-3-ml blood samples were taken from each ewe at 30-min intervals for 4 h. GnRH was then administered intravenously in 1 ml saline at doses of 0, 0.5, 5 and 50 µg (4 ewes/group). Blood samples were taken every 10 min for 1 h, every 30 min for the next 2 h and hourly for the next 5 h. The cannulae were then removed and the ewes returned to pasture.

**Treatment with bFF and oestradiol benzoate.** Ovariectomized ewes were penned indoors: 20 ewes received 5 intramuscular injections at 12-h intervals (starting at 0 h) of bFF (5 or 10 ml), oestradiol benzoate (50 or 500 µg given in 1 ml vegetable oil) or 50 µg oestradiol benzoate plus 5 ml bFF (4 ewes/group), and the remaining 4 ewes served as controls. Blood samples (2-3 ml) were taken by venepuncture from all ewes at -8, -4, 0, 12, 16, 20, 36, 40, 44 and 60 h.

### Radioimmunoassays

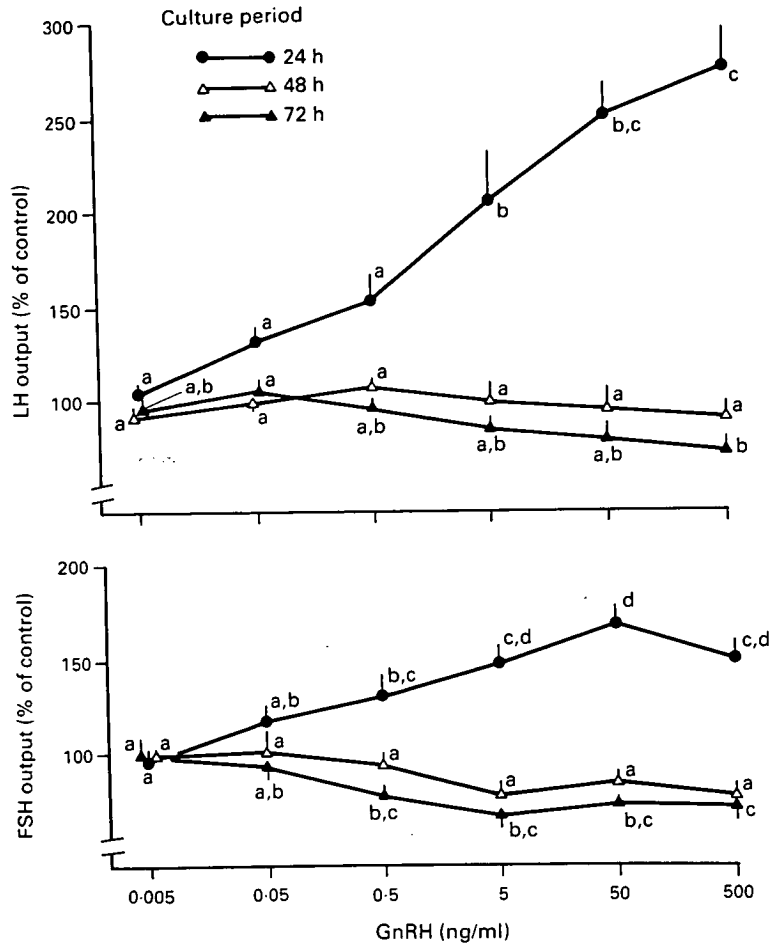
Gonadotrophin concentrations in plasma and culture medium were measured by specific radioimmunoassays. FSH was measured using the sheep FSH radioimmunoassay kit and protocol provided by the National Hormone and Pituitary Program, NIDDK, University of Maryland, USA (Technical Report No. 141). The FSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113) and it was used at a final tube dilution of 1:80 000 in a total assay incubation volume of 0.5 ml. The sheep FSH for iodination was NIAMDD-oFSH-1-1 (AFP-5679C) and the reference preparation was NIAMDD-oFSH-RP-1 (biopotency  $75 \times$  NIH-FSH-S1). The sensitivity of the assay was 0.05-0.1 ng per assay tube and the intra- and inter-assay coefficients of variation were < 10%. LH was measured using the specific radioimmunoassay described previously (McNatty *et al.*, 1981b). The antiserum used was generated in a rabbit against NIH-LH-S11 and was used at an initial dilution of 1:40 000. This LH antiserum exhibited low cross-reactions with other pituitary hormones: sheep prolactin (NIH-P-S12) 0.09%; sheep thyroid stimulating hormone (NIH-TSH-S8) 2.4%; sheep growth hormone (NIH-GH-S11) 0.4% and sheep FSH (NIH-FSH-S10) 0.4%. The pituitary LH reference preparation was NIADDK-oLH-24 and the iodinated tracer was NIADDK-oLH-1-3. The sensitivity of the assay was 0.025-0.05 ng per assay tube, and the intra- and inter-assay coefficients of variation were < 11%.

### Data analysis

Gonadotrophin output by all cell cultures (except for those of Fig. 7) was initially standardized with respect to the protein content of the pituitary cells at the end of the culture period. There was, however, considerable variation (up to 6-fold) in gonadotrophin output by equivalent pituitary cultures established on separate occasions. To compensate for this variation, when treatments were repeated on separate occasions (as for data in Figs 1 and 3), mean gonadotrophin output by treated cultures on each occasion was expressed as a percentage of the mean output by the appropriate control (zero dose) cultures also established on each occasion. Mean percentage values for each treatment were then calculated.

There was also considerable variation between individual sheep in plasma gonadotrophin concentrations before any treatment. To compensate for this between-sheep variation, plasma gonadotrophin concentrations for each ewe following the start of a treatment were expressed as a percentage of the mean pretreatment value. Mean percentage values for each treatment group of ewes were then calculated.

Analysis of variance in conjunction with the Newman-Keuls multiple range test was used to test for statistically significant differences between mean values. The level of significance was set at  $P < 0.05$ .



**Fig. 1.** Effect of GnRH on LH and FSH output by pituitary cells from ovariectomized ewes. The results were derived from 4 replicate experiments. In each experiment mean gonadotrophin output by GnRH-treated cultures was expressed as a percentage of the mean output by control (zero dose) cultures. FSH and LH output by control cultures ranged from 370 to 1185 ng FSH/mg protein and from 1 to 6  $\mu$ g LH/mg protein. Mean percentage values for the 4 replicate experiments were calculated, and these are presented with vertical lines showing the s.e.m. Mean values not sharing a common letter superscript for the same culture period are significantly different ( $P < 0.05$ ).

## Results

### *Effect of GnRH on gonadotrophin output*

Figure 1 shows the effect of increasing concentrations of GnRH on LH and FSH output *in vitro* by pituitary cells from ovariectomized ewes. A dose-dependent increase in mean LH and FSH output occurred during the first 24 h of culture. However, while mean LH output was increased by up to about 3-fold, mean FSH output was only increased about 1.5-fold. During the 2nd and 3rd days of culture, mean gonadotrophin output generally remained at control (zero dose) levels or in some instances, at the higher concentrations of GnRH, fell significantly. The relative insensitivity of FSH output to stimulation by GnRH was not peculiar to cells from ovariectomized ewes.



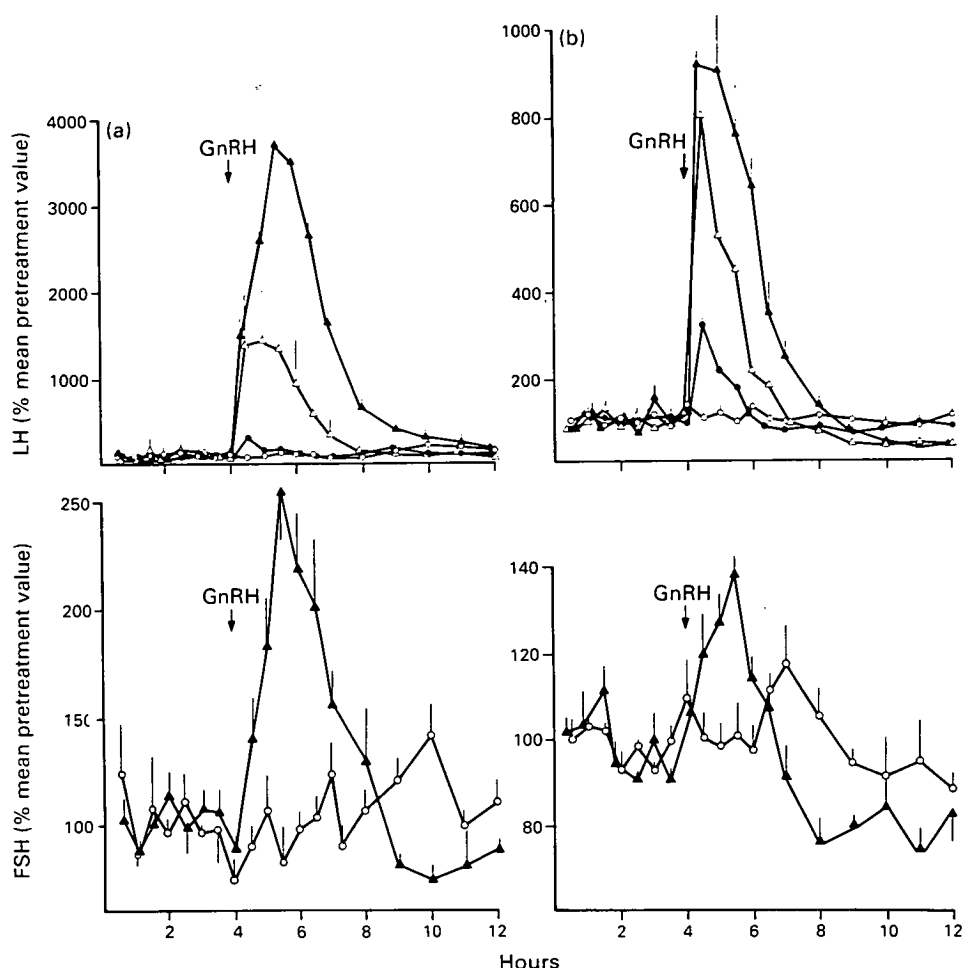
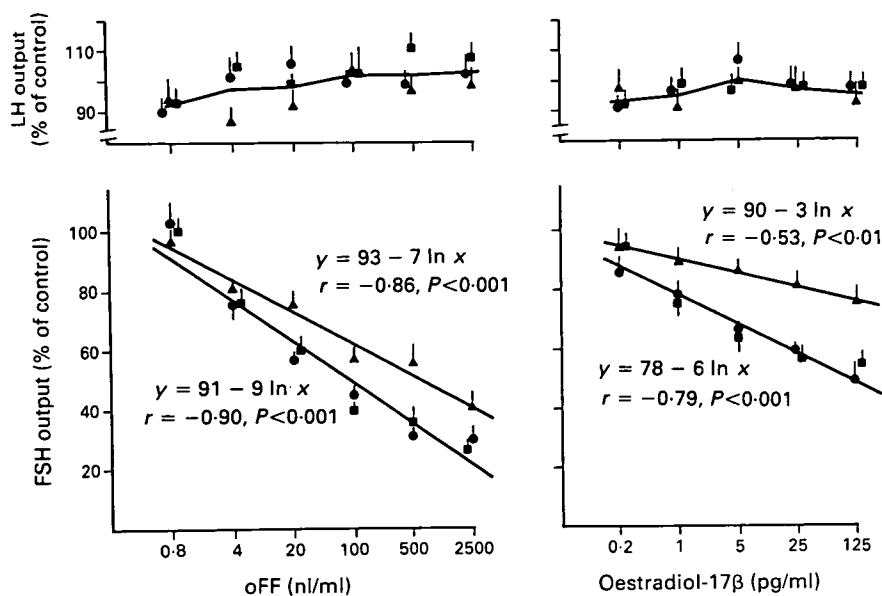


Fig. 2. Effect of GnRH on plasma gonadotrophin concentrations in (a) intact and (b) ovariectomized ewes. For each ewe, plasma gonadotrophin concentrations were expressed as a percentage of the mean concentration before injection of GnRH. (These mean concentrations ranged from 0.7 to 2.3 ng FSH/ml and 0.3 to 0.8 ng LH/ml for intact ewes, and from 8 to 20 ng FSH/ml and 3.6 to 10.5 ng LH/ml for ovariectomized ewes.) Mean percentage values for each treatment group of 4 ewes were then calculated. These mean values have been presented with vertical lines showing the s.e.m. when larger than the size of the symbol.  $\circ$ ,  $\bullet$ ,  $\triangle$ , and  $\blacktriangle$  refer to groups of ewes receiving 0, 0.5, 5 and 50  $\mu\text{g}$  GnRH, respectively. FSH data for ewes receiving 0.5 and 5  $\mu\text{g}$  GnRH are not shown because the mean FSH values did not differ significantly from those of ewes receiving 0  $\mu\text{g}$  GnRH.

Similar findings were also obtained in a single culture experiment with cells from an intact ewe at Day 10 of the oestrous cycle. During the first 24 h of culture, mean LH output increased in a dose-dependent fashion up to 4.8-fold above control (zero dose) cultures in response to 50 ng GnRH; mean FSH output only increased 1.2-fold. By Day 3 of culture, mean LH and FSH output in response to 50 ng GnRH was only 85 and 72% respectively of that of the control (zero dose) cultures. Similar differences in LH and FSH output in response to GnRH were also apparent *in vivo*. Figure 2 shows the effect of a single injection of several doses of GnRH on plasma gonadotrophin concentrations (expressed as a percentage of pretreatment values) in intact (Day 10) and



**Fig. 3.** Effect of increasing doses of oFF and oestradiol-17 $\beta$  on gonadotrophin output by pituitary cells from ovariectomized ewes during 3 days of culture.  $\blacktriangle$ ,  $\bullet$  and  $\blacksquare$  refer to Days 1, 2 and 3 of culture respectively. The results were derived from 7 replicate experiments. For each experiment, mean gonadotrophin output by treated cultures was expressed as a percentage of the mean output by the appropriate control (zero dose) cultures. FSH and LH output by the control cultures ranged from 220 to 1400 ng FSH/mg protein and from 0.9 to 11  $\mu$ g LH/mg protein. Mean percentage values for the 7 replicate experiments were calculated and these are presented with vertical lines showing the s.e.m. Linear regression lines have been fitted to the FSH data for Day 1, and the pooled FSH data for Days 2 and 3. The associated regression equations are also shown, together with the correlation coefficients ( $r$ ) and significance. The mean LH percentage values for the 3 days of culture at each dose of oFF and oestradiol-17 $\beta$  have been joined by straight lines.

ovariectomized ewes. GnRH increased mean plasma LH values in a dose-dependent fashion in both intact and ovariectomized ewes. However, mean FSH levels were only increased at the highest dose of GnRH (50  $\mu$ g). In intact ewes, 50  $\mu$ g GnRH increased mean peak LH concentrations by about 40-fold (from  $0.6 \pm 0.2$  to  $23.3 \pm 3.7$  ng/ml, mean  $\pm$  s.e.m.), but mean peak FSH concentrations by only about 2.5-fold (from  $1.5 \pm 0.2$  to  $3.8 \pm 0.5$  ng/ml). Similarly, in ovariectomized ewes, 50  $\mu$ g GnRH increased mean peak LH concentrations by about 10-fold (from  $4.9 \pm 0.7$  to  $45.1 \pm 8.5$  ng/ml), but mean peak FSH concentrations by only about 1.3-fold (from  $11.8 \pm 2.0$  to  $15.8 \pm 2.3$  ng/ml).

#### *Effect of oFF and oestradiol-17 $\beta$ on gonadotrophin output in vitro*

Ovine FF and oestradiol-17 $\beta$  caused a dose-dependent decrease in mean FSH output by cultured pituitary cells from ovariectomized ewes on each day of culture (Fig. 3). In contrast, mean LH output changed little, and fluctuated around 90–110% of that produced by control (zero dose) cultures, over the whole culture period.

Figure 4 shows FSH output by cells from ovariectomized ewes after 1 and 2 days of culture with oestradiol-17 $\beta$  (0, 5 and 125 pg/ml) and oFF (0, 0.1 and 2.5  $\mu$ l/ml) either alone or in combination. On both days of culture, oFF and oestradiol-17 $\beta$  significantly suppressed mean FSH output on their own. On Day 1, oestradiol-17 $\beta$  and oFF in combination suppressed mean FSH output to

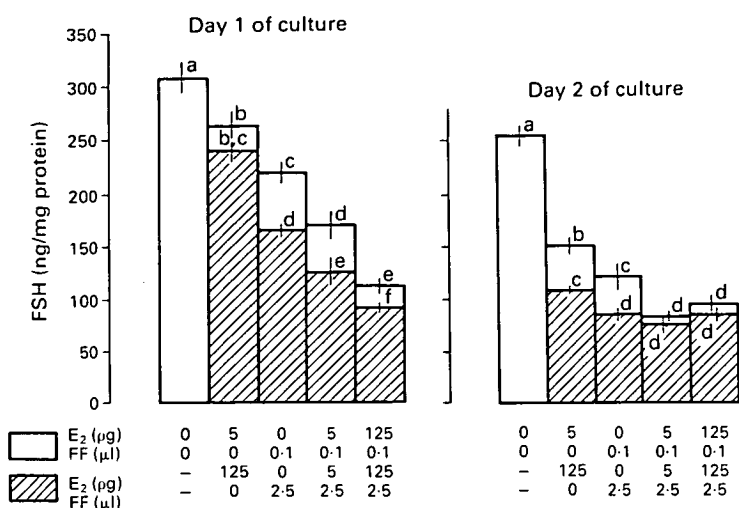


Fig. 4. Effect of oFF (0.1 and 2.5  $\mu$ l/ml) and oestradiol-17 $\beta$  (E<sub>2</sub>, 5 and 125 pg/ml) alone and together on FSH output by pituitary cells from ovariectomized ewes. Values are means of 5 replicate cultures with vertical lines showing the s.e.m. Mean values with different letter superscripts on the same day are significantly different ( $P < 0.05$ ).

levels significantly lower than when either was given alone. On Day 2, mean FSH output by cells cultured with 0.1  $\mu$ l oFF plus 5 or 125 pg oestradiol-17 $\beta$  was reduced to the levels produced by cells receiving 2.5  $\mu$ l oFF alone. However, no further significant reduction in mean FSH output was obtained by culturing with 2.5  $\mu$ l oFF plus 5 or 125 pg oestradiol-17 $\beta$ .

#### Effect of bFF and oestradiol benzoate on plasma concentrations of FSH and LH

Ovariectomized ewes were injected with bFF (0, 5 or 10 ml), oestradiol benzoate (0, 50 or 500  $\mu$ g) or bFF and oestradiol benzoate (5 ml and 50  $\mu$ g) at 12-h intervals for 48 h. Figure 5 shows plasma concentrations (expressed as a percentage of pretreatment values) of FSH and LH 12, 20, 40 and 60 h after the first injection. Both bFF and oestradiol benzoate caused a dose-dependent decrease in FSH concentrations. The mean  $\pm$  s.e.m. plasma FSH concentrations at 60 h following treatment with the higher doses of bFF and oestradiol benzoate were  $1.3 \pm 0.3$  and  $1.9 \pm 0.4$  ng/ml respectively, which were similar to the mean plasma FSH values before ovariectomy of the ewes ( $1.4 \pm 0.3$  ng/ml). At 12, 40 and 60 h, mean FSH concentrations in ewes receiving bFF (5 ml) and oestradiol benzoate (50  $\mu$ g) together were significantly lower than in ewes receiving either dosage alone, but were generally similar to those of ewes receiving injections of 10 ml bFF or 500  $\mu$ g oestradiol benzoate (Fig. 5). Although mean FSH concentrations were significantly reduced at 20 h after treatment with bFF alone, this reduction was negated in ewes receiving bFF plus oestradiol benzoate, the values being similar to those of ewes receiving oestradiol benzoate alone. A positive feedback effect of oestradiol benzoate resulting in increased plasma LH concentrations was also apparent at 20 h. At the other times, LH concentrations were unaffected by bFF or oestradiol benzoate.

#### Effect of progestagens, androgens and catechol oestrogens on FSH output in vitro

Androstenedione, 5 $\alpha$ -dihydrotestosterone, progesterone, 3 $\alpha$ -hydroxy-4-pregnen-20-one, 2-hydroxy oestradiol and 4-hydroxy oestradiol each significantly suppressed mean FSH output by

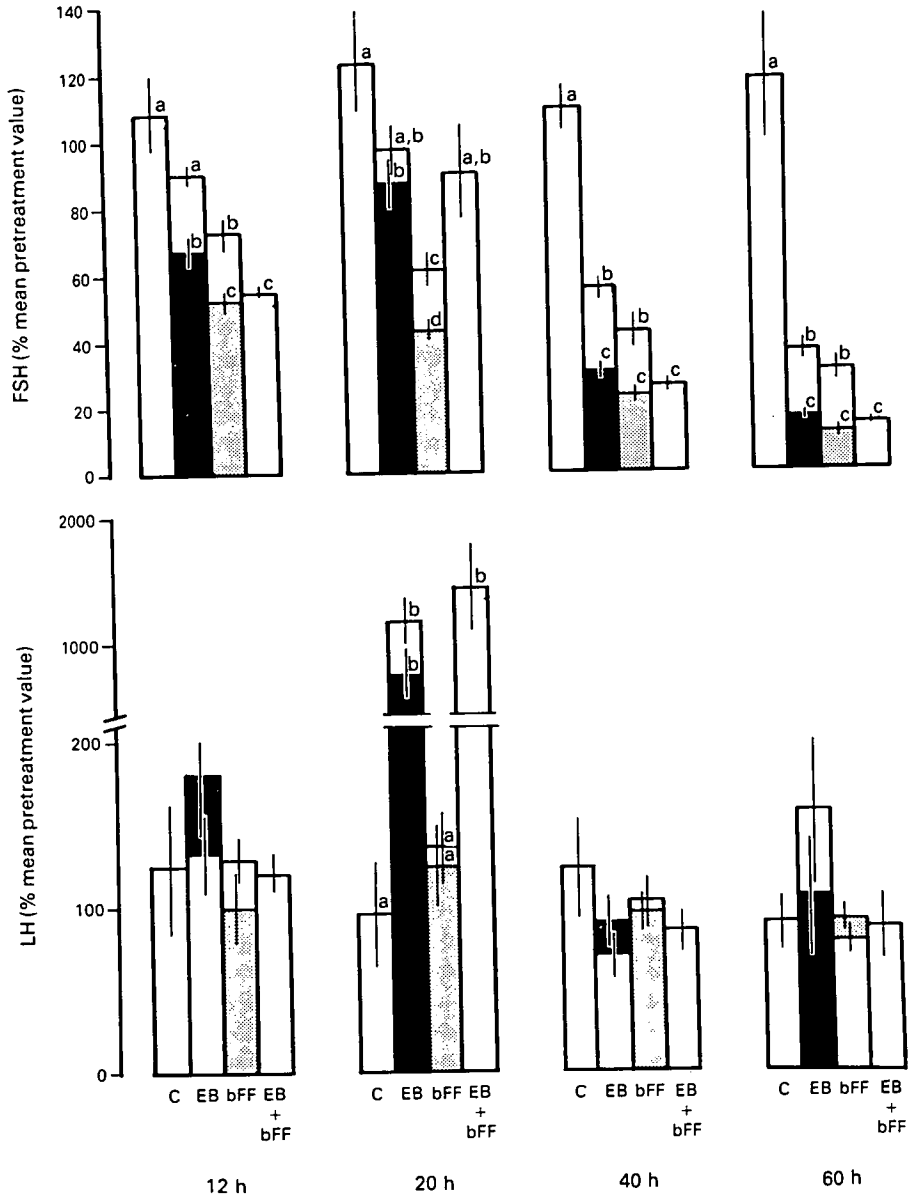
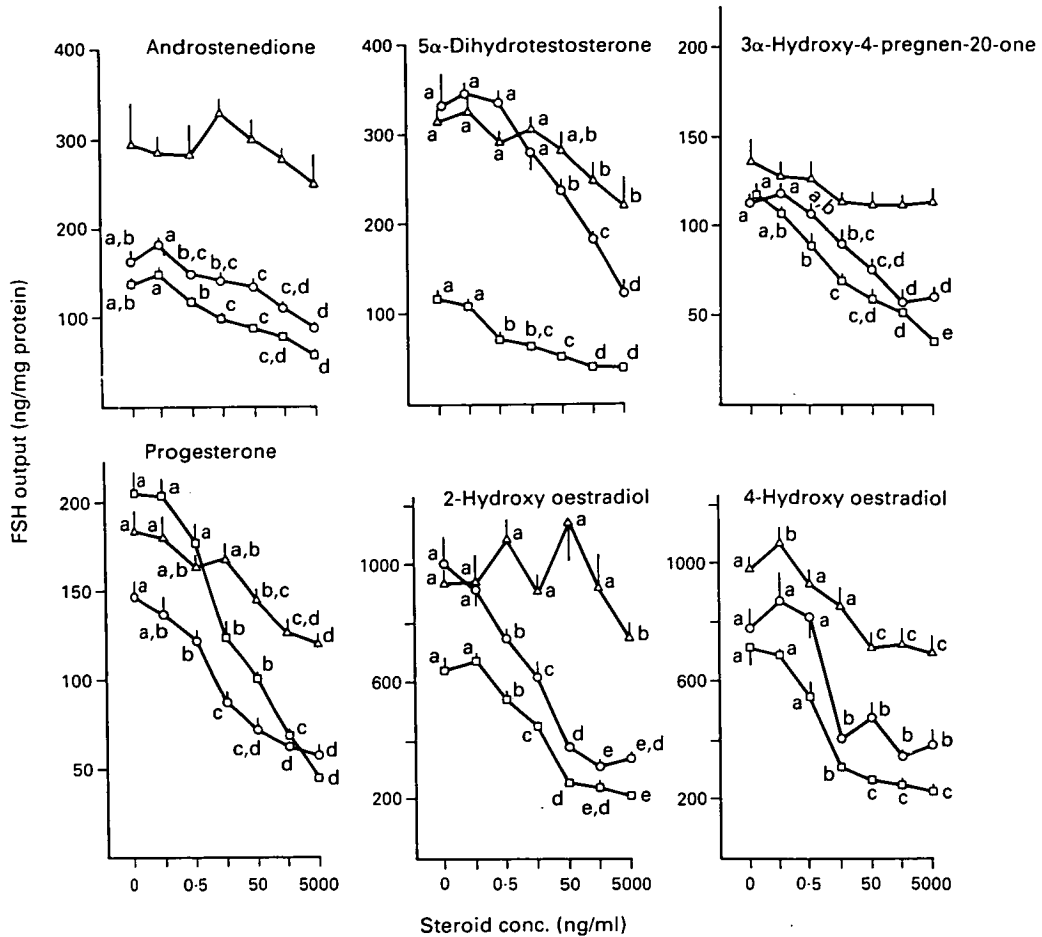


Fig. 5. Effect of bFF and oestradiol benzoate (EB) alone and in combination on plasma gonadotrophin concentrations in ovariectomized ewes. C, control (zero dose); EB, □ 50 µg, ■ 500 µg; bFF, □ 5 ml, ▨ 10 ml; 5 ml bFF + 50 µg EB, □. For each ewe, plasma gonadotrophin concentrations were expressed as a percentage of the mean pretreatment value, and these ranged from 11.2 to 16.5 ng FSH/ml and 3.4 to 4.7 ng LH/ml. Mean percentage values for each group of 4 ewes were calculated and these are presented with vertical lines showing the s.e.m. Mean values not sharing a common letter superscript within the same time period differ significantly ( $P < 0.05$ ). Groups of mean values without superscripts are not significantly different.



**Fig. 6.** Effect of steroids on FSH output by cultured pituitary cells from ovariectomized ewes. Values are means of 4 replicate cultures with vertical lines showing the s.e.m. when larger than the size of the symbol. Mean values not sharing a common letter superscript over the same time period are significantly different ( $P < 0.05$ ). Groups of mean values without superscripts are not significantly different.  $\Delta$ ,  $\circ$  and  $\square$  refer to Days 1, 2 and 3 of culture with steroid respectively.

cells from ovariectomized ewes in a dose-dependent manner (Fig. 6). Inhibition was observed from Day 1 of treatment with all of the steroids except for androstenedione and 3 $\alpha$ -hydroxy-4-pregnen-20-one with which significant inhibition was observed only on Days 2 and 3. Effects similar to those of 5 $\alpha$ -dihydrotestosterone and 3 $\alpha$ -hydroxy-4-pregnen-20-one were also observed with testosterone and 3 $\beta$ -hydroxy-4-pregnen-20-one respectively (data not shown). However, in contrast to the inhibitory effects of oestradiol-17 $\beta$  on mean FSH output, for which inhibition was observed at doses as low as 1–5 pg/ml (Fig. 3), significant suppression of FSH by the above-mentioned steroids was not observed at doses of <500 pg/ml. It is unlikely that the suppression of FSH output by these high doses of steroids was a consequence of their contamination with oestradiol-17 $\beta$ ; no such contamination was evident on their analysis by high-pressure liquid chromatography (Waters & Bondapak C18 column; acetonitrile:water (60:40 v/v) as mobile phase).

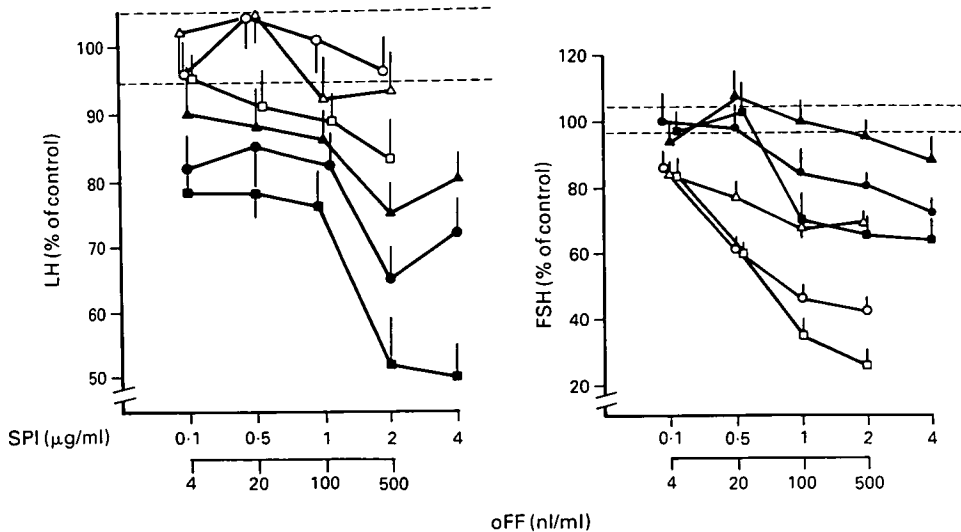


Fig. 7. Effect of seminal plasma inhibin-like peptide (SPI) and oFF on gonadotrophin output by pituitary cells from ovariectomized ewes during 3 days of culture. Gonadotrophin output (initially calculated as ng/culture) by treated cultures has been expressed as a percentage of the mean secretion of control (zero dose) cultures which ranged from 30 to 50 ng FSH/culture, and 250 to 340 ng LH/culture. Values are the mean percentages of 5 replicate cultures with vertical lines showing the s.e.m. The horizontal dotted line shows the s.e.m. associated with the control (zero dose) cultures.  $\blacktriangle$ ,  $\bullet$ ,  $\blacksquare$  refer to Days 1, 2 and 3 of culture respectively for SPI treatments and  $\triangle$ ,  $\circ$ ,  $\square$  refer to Days 1, 2 and 3 of culture respectively for oFF treatments.

#### *Effect of seminal plasma inhibin-like peptide (SPI) on FSH and LH output in vitro*

Figure 7 shows a comparison of the effects of SPI and oFF on FSH and LH output by pituitary cells from ovariectomized ewes. Ovine FF caused a marked dose-dependent suppression of mean FSH output, but had comparatively little effect on mean LH output. SPI had no effect on mean FSH output at doses from 1  $\mu$ g/ml to 500 ng/ml. At doses of  $\geq 1$   $\mu$ g/ml SPI did suppress mean FSH output, although less effectively than oFF, but it also had a marked suppressive effect on mean LH output. The inhibitory effect of 2 and 4  $\mu$ g SPI/ml on gonadotrophin output was also accompanied by a marked deterioration in the morphological appearance of the cells which shrank and detached from the culture wells. This toxic effect was also reflected in the cellular protein determined at the end of the culture period. Control (zero dose) cultures had mean  $\pm$  s.e.m. protein values of  $139 \pm 2$   $\mu$ g, whereas the values for cultures treated with 2 and 4  $\mu$ g SPI/ml were  $83 \pm 2$  and  $63 \pm 3$   $\mu$ g protein respectively. None of the doses of oFF used, or indeed of any of the above steroids studied, had any significant effect on the mean amount of cellular protein determined at the end of the culture period, relative to their control cultures.

#### Discussion

The present study demonstrates that there are marked differences in the way in which FSH and LH output by the sheep pituitary can be manipulated. While LH output can be readily increased with GnRH, FSH output can only be increased marginally by comparison. FSH output is, however, readily susceptible to suppression by FF (most likely reflecting its inhibin activity) and oestradiol-17 $\beta$ , whereas LH output is more resistant to the suppressive actions of these agents. These findings are consistent with the view that LH output is normally acutely controlled by hypothalamic GnRH

stimulation, whereas ovarian negative feedback effects normally control FSH output (Martin *et al.*, 1986, 1988).

The observation that GnRH more effectively increased LH than FSH output by cultured sheep pituitary cells is similar to the findings of Huang & Miller (1980). Stimulation of gonadotrophin output occurred during the first 24-h culture period, but not thereafter. This could be a consequence of effects on the GnRH receptor population of the dissociation procedure used to prepare the cells. Alternatively, it may be the result of 'desensitization' to GnRH, or depletion of 'releasable' pools of gonadotrophin. Similar effects can also occur *in vivo*. Although a single injection of GnRH will increase mean plasma concentrations of LH and marginally increase mean FSH concentrations (Fig. 2), repeated frequent injections or continuous infusion of GnRH can lead to a decline in plasma gonadotrophin concentrations after an initial stimulation (Fraser *et al.*, 1981; Crowder *et al.*, 1986).

Studies of the suppressive effects of oestradiol-17 $\beta$  and FF on gonadotrophin output were restricted to ewes which had previously been ovariectomized in order to release the pituitary from ovarian negative feedback effects. Oestradiol-17 $\beta$  (or oestradiol benzoate) and FF were very effective individually in suppressing FSH output *in vitro* and plasma FSH concentrations *in vivo*, and cumulative suppressive effects were apparent when they were given together (Figs 3–5). After ovariectomy, pituitary gonadotrophin composition appears to be altered, as reflected by an increased half-life for FSH (Fry *et al.*, 1987). It is therefore possible that the effects of oestradiol-17 $\beta$  (oestradiol benzoate) and FF on gonadotrophin output in ovariectomized ewes may differ to some extent from their effects in intact ewes. However, suppression of FSH output by oestradiol-17 $\beta$  and FF has also been observed previously in intact ewes both *in vitro* and *in vivo* (Reeves *et al.*, 1974; Pant, 1977; Henderson *et al.*, 1986, 1988; Tsonis *et al.*, 1986).

The relative contributions of oestradiol-17 $\beta$  and inhibin to the physiological regulation of FSH output in sheep remain unresolved. Although both oestradiol benzoate and FF could individually reduce plasma FSH concentrations in ovariectomized sheep to pre-ovariectomy levels, very high concentrations of each were required (Fig. 5). FSH output in sheep is therefore more likely to be regulated through the observed cumulative effects of oestradiol-17 $\beta$  and inhibin, as has been suggested by the studies of Martin *et al.* (1988). Although combined treatment with oestradiol benzoate and bFF generally had a cumulative effect in reducing plasma FSH concentrations (Fig. 5), this was not apparent 20 h after the initial injections when oestradiol benzoate was exerting a positive feedback effect on gonadotrophin output. At this time, combined treatment with oestradiol benzoate negated the reduction in plasma FSH brought about by bFF. This is consistent with the findings of Medhamurthy *et al.* (1987) for sheep: a bovine follicular fluid inhibin preparation failed to suppress the preovulatory FSH surge, which is initiated by the increasing amounts of oestradiol-17 $\beta$  secreted by the ovary. Previous studies in sheep have demonstrated a transient fall in plasma LH values within the first 5–12 h after administration of oestradiol-17 $\beta$  or oestradiol benzoate (Pant, 1977; Fraser *et al.*, 1981; Clarke *et al.*, 1982). The failure to observe suppression of plasma LH with oestradiol benzoate in the present study was probably due to the timing and infrequency of the blood sampling. No effect of oestradiol-17 $\beta$  was observed on LH output *in vitro*. However, because LH is normally released in a pulsatile fashion *in vivo* and is regulated through changes in pulse frequency and amplitude, such modulatory effects may not be readily discernible in a static culture system.

Although oestradiol-17 $\beta$  was effective in suppressing mean FSH output *in vitro* at doses as low as 1–5 pg/ml, other steroids examined could also suppress FSH output if a sufficiently high dose ( $\geq 500$  pg/ml) was used. Whether the suppression of FSH output found with such high doses reflects a physiological role of the steroids in FSH regulation, or is merely a pharmacological effect is uncertain. The inhibitory effect of progesterone on sheep FSH output *in vitro* has been demonstrated previously (Batra & Miller, 1985), although these authors found that androgens had only a minor (< 10% inhibition) or no effect on FSH output.

In rats, 3 $\alpha$ -hydroxy-4-pregnen-20-one has been shown to be extremely potent in selectively suppressing FSH output both *in vivo* and *in vitro*; doses of 10 pg/ml significantly suppress FSH output by rat pituitary cells *in vitro* (Wiebe & Wood, 1987). It was therefore felt that this steroid might be potentially useful for manipulating FSH output in sheep. However, in sheep pituitary cell cultures, doses of  $\geq 500$  pg/ml were required to suppress FSH output and suppression was only observed during the 2nd and 3rd days of culture (Fig. 6). Such species variation in the action of steroids on pituitary gonadotrophin output has been observed previously (Miller & Wu, 1981).

Inhibin preparations can be used to manipulate plasma FSH concentrations and increase ovulation rates in sheep, e.g. by repeated injections of bFF (Henderson *et al.*, 1986), or by active immunization with a partly pure inhibin preparation derived from bFF (Henderson *et al.*, 1984; Cummins *et al.*, 1986). Commercial application of such techniques might be encouraged by the availability of large amounts of inexpensive preparations of inhibin. The availability of a synthetic 31 amino acid peptide (seminal plasma inhibin-like peptide) reported to have inhibin activity offered some potential in this regard (Yamashiro *et al.*, 1984; Sairam *et al.*, 1987). However, while 2–6 ng of this peptide reportedly suppressed by 50% FSH output by mouse pituitaries *in vitro*, doses from 1 pg/ml to 500 ng/ml in the present study had no effect on FSH output by cultured sheep pituitary cells. At higher doses, both LH and FSH output were reduced, but this was accompanied by morphological deterioration of the cells, indicative of a toxic effect. It seems unlikely that this peptide will be useful for specifically manipulating FSH output in sheep. Other studies in rats with this peptide have also failed to demonstrate FSH-suppressing activity either *in vivo* or *in vitro* (Liu *et al.*, 1985).

The stimulatory effects of endogenous GnRH in combination with the suppressive effects of endogenous oestradiol-17 $\beta$  and inhibin are probably the major factors which determine the pituitary output of FSH, and hence circulating plasma FSH concentrations. Increases in plasma FSH concentrations could, therefore, be achieved by either increasing the stimulatory effect of GnRH and/or reducing the suppressive effects of oestradiol-17 $\beta$  and/or inhibin. This study demonstrates that exogenous GnRH has comparatively little stimulatory effect on pituitary FSH output compared to its effect on LH output. In contrast, exogenous oestradiol-17 $\beta$  and FF have marked suppressive effects on FSH output. Therefore, the development of pharmacological means to reduce the negative feedback effects of endogenous oestradiol-17 $\beta$  and/or inhibin on FSH output, rather than increasing pituitary exposure to GnRH, might be the more appropriate strategy to raise plasma FSH concentrations selectively, and hence increase ovulation rates in sheep.

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## Induction of twin ovulations in red deer hinds with steroid-free bovine follicular fluid

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### ABSTRACT

The manipulation of ovulation rate in the red deer hind was investigated by administration of steroid-free bovine follicular fluid (bFF).

Twenty-four adult hinds, treated with intravaginal progesterone (CIDR), received a daily intramuscular injection of either 0, 4, 7.5 or 11 mls of dextran-charcoal treated bFF (n = 6 per group) on days -1, 0 and +1 from the time of CIDR withdrawal.

After CIDR removal, plasma progesterone concentrations declined in all animals. In control hinds, oestrus occurred 2-3 days later and mean plasma progesterone concentrations began to increase thereafter. In bFF-treated hinds, oestrus was delayed by 3-5 days, and plasma progesterone concentrations remained low for at least 7 days after CIDR withdrawal. Plasma LH concentrations were 2-3 fold higher in the bFF-treated groups, relative to the control hinds, for at least 6 days after the end of bFF treatment. Twin ovulations were recorded in 1/6 control and 5/17 treated animals. At pregnancy diagnosis, 10/23 hinds were pregnant having conceived at the treated oestrus (3/6 control and 7/18 treated), but none had more than one foetus. There was no evidence of a dose-response to bFF treatment.

These results indicate that in the hind, bFF can be used to manipulate the timing of oestrus and ovulation rate. A component(s) of bFF may thus have the potential to regulate fecundity in deer.

**Keywords** Follicular fluid; red deer; oestrus; progesterone; LH; ovulation rate

### INTRODUCTION

Although twinning has long been suspected in red deer since there are observations in the wild of hinds accompanied by more than one calf (eg, MacNally, 1977), it is apparently very rare. In Scotland, Lowe (1969) shot over 800 female deer of all ages over an eight-year period and found only one set of twin foetuses although one of the pair had apparently died before the hind was shot. Similarly, Mitchell (1973) examined over 1500 uteri from shot hinds finding no twin embryos. There are few authentic reports of the birth of twin red deer calves (Guinness and Fletcher, 1971; MacNally, 1982) and although twinning in farmed red deer has been reported (eg, Lindeman, 1987), there are no estimates of the incidence.

Studies in other species indicate that the pituitary gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH), are the main regulators of follicle development and ovulation and that by increasing circulating FSH

concentrations in particular, ovulation rates can be increased (Hammond, 1949; McNatty *et al.*, 1985).

Inhibin, a complex glycoprotein produced by the granulosa cells of the ovary (Henderson and Franchimont, 1981) has been implicated in the regulation of FSH secretion (see Findlay and Clarke, 1987) in some species. Active immunisation against a partially purified preparation of inhibin increased ovulation rate in the ewe (Henderson *et al.*, 1984; Cummins *et al.*, 1986). Similarly, treatment of ewes with steroid-free bovine follicular fluid (bFF), an inhibin-rich material, during the luteal phase of the oestrous cycle (Wallace and McNeilly, 1985) or at the time of induced luteolysis (Henderson *et al.*, 1986) delayed oestrus and increased ovulation rate.

The present study was designed to investigate the effect of administration of bFF on ovulation rate in the red deer hind.

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## MATERIALS AND METHODS

Twenty-four adult red deer (*Cervus elaphus*) hinds (live weight 81-113 kg, mean 96.7 kg) were synchronised for oestrus with 15 days of intravaginal progesterone (9% w/w CIDR-S containing 340 mg progesterone, AHI Plastic Moulding Co, Hamilton, New Zealand) commencing on 2 April, 1986. Hinds were treated with a daily i.m. injection of either 0, 4, 7.5 or 11 mls of bFF (obtained and prepared as described by Henderson *et al.*, 1986) on days -1, 0 and +1 after CIDR withdrawal.

Mating was detected by running the hinds in 1 of 2 single-sire mating groups each with a colour-marked adult stag which was removed 12 days later. Ovulation rate was determined by laparoscopy and pregnancy by ultrasound scan under general anaesthesia (Fisher *et al.*, 1989) at 13 and 113 days respectively after stag introduction. In addition, all hinds were blood sampled daily by jugular venepuncture for 10 days beginning the day before the first injection of bFF (day -2). Plasma was removed and stored frozen until analysed.

Plasma progesterone concentrations were determined by solid phase <sup>125</sup>I radioimmunoassay (Diagnostic Products Corporation, Los Angeles, USA) and plasma LH by a heterologous double antibody radioimmunoassay which used rabbit antiserum to ovine LH and ovine LH for iodination and as assay standards. Parallelism was demonstrated by serial dilutions of hind plasma in phosphate buffered saline. LH concentrations > 5 ng/ml were assumed to be preovulatory LH surges and were excluded from statistical analyses. The log transformed LH data were grouped as either before, during or after bFF treatment and examined by analysis of variance, and Student's t-test.

## RESULTS

There were no differences between each of the mating groups so the data have been combined. Oestrus occurred in the control hinds 2 to 3 days after CIDR withdrawal but was delayed in the treated hinds, occurring 5-8 days after withdrawal.

Oestrus and progesterone

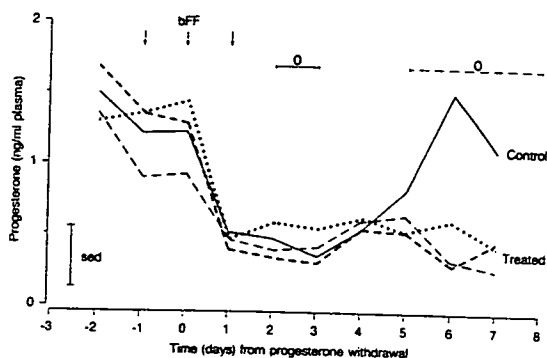


FIG. 1 Mean plasma progesterone concentrations around the time of progesterone withdrawal and oestrus (O) in untreated and bFF-treated (— 0 mls bFF; --- 4 mls bFF; - - - 7.5 mls bFF; ●●● 11 mls bFF) hinds.

Plasma progesterone concentrations (Fig. 1) averaged 1.30 ng/ml over the last 3 days of CIDR insertion then concentrations declined following device withdrawal. They then began to increase after oestrus (Fig. 1) in control hinds and averaged 1.15 ng/ml over the period 2 - 5 days after oestrus. In contrast, oestrus in treated hinds was delayed by 3-5 days and plasma progesterone concentrations remained low (mean 0.47 ng/ml) until at least day 7.

Mean plasma LH concentrations prior to bFF treatment (Table 1) ranged from 0-0.81 ng/ml

TABLE 1 Mean LH concentrations (ng/ml plasma) in untreated and bFF-treated hinds. Asterisks indicate the level of significant differences between control (0 mls bFF) and treated (4, 7.5 and 11 mls bFF) groups.

Group (mls bFF)	LH concentration		
	Before <sup>1</sup> treatment	During <sup>2</sup> treatment	After <sup>3</sup> treatment
0	0.19	0.35	0.34
4	0.29	1.07***	1.06***
7.5	0.32	0.66**	0.83***
11	0.26	0.73**	1.12***
SED	0.06	0.12	0.15

<sup>1</sup> Days 1 & 2 (days -3 and -2 from progesterone withdrawal)

<sup>2</sup> Days 3 - 5 (days -1, 0 and 1 from progesterone withdrawal)

<sup>3</sup> Days 6 - 10 (days 2-6 from progesterone withdrawal)

(mean 0.27 ng/ml). During treatment with bFF, LH concentrations were elevated significantly, independently of dose-rate in all groups, and this 2-3 fold increase was maintained in the period following treatment at least until sampling ceased.

Twin ovulations were observed in 1/6 untreated and 5/18 treated hinds (Table 2). At pregnancy diagnosis 10/23 (43%) hinds were pregnant having conceived at the induced oestrus (3/6 control and 7/17 treated), none with more than one foetus. Of the 6 hinds that had twin ovulations 5 were pregnant while 5/17 with single ovulations were pregnant.

TABLE 2 Ovarian and pregnancy data obtained from untreated and bFF-treated hinds.

Group (mls bFF)	Number of ovulations	Number of hinds pregnant
0	5 single	3
	1 double	0
4	1 anovulatory	-
	3 single	2
	2 double	2
7.5	5 single	0
	1 double	1
11	4 single	0
	2 double	2

There was no evidence of a dose response to bFF in LH concentrations, ovulation rate or pregnancy rate. There were no significant relationships between progesterone or LH concentrations and ovulation or pregnancy rates.

### DISCUSSION

The observed effects of bFF treatment on the timing of oestrus, and plasma concentrations of progesterone and LH in deer were similar to the effects found in sheep where there is also a substantial fall in plasma FSH concentrations during bFF treatment. Although, FSH has yet to be measured on the samples collected in the present experiment (a cervine FSH radioimmunoassay is currently being developed), it is likely that plasma FSH concentrations were suppressed during treatment. A decline in plasma

FSH concentrations would impair follicular development which would explain the delay in oestrus and subsequent luteal development. It would also reduce follicular oestradiol production so that there would be less negative feedback by oestradiol on the pituitary resulting in enhanced LH secretion.

In sheep (Henderson *et al.*, 1986) cessation of bFF treatment causes plasma FSH concentrations to "rebound" to levels considerably higher than pre-treatment values. Elevated FSH levels persist until follicular development returns to normal, and with it the normal negative feedback effects of oestradiol on pituitary gonadotrophin secretion. The temporary elevation in FSH and LH concentrations are thought to be responsible for the increased ovulation rate observed following treatment with bFF. Similar changes in gonadotrophin secretion may be responsible for the small increase in the number of hinds with twin ovulations following bFF treatment since hinds have been shown to respond to FSH-rich preparations with an increase in ovulation rate (Fisher and Fennessy, 1985).

Although twin births have been noted in red deer they are relatively rare as are natural twin ovulations (1/42 in untreated hinds examined by laparoscopy at Invermay; M.W. Fisher and G.H. Davis, pers. comm.). Thus the twin ovulations induced in the present experiment probably represent a significant effect of treatment. Despite the number of hinds with twin ovulations, none supported twin pregnancies; a finding consistent with the difficulty experienced in inducing viable multiple births in red deer (Kelly *et al.*, 1982; Adam *et al.*, 1985) except prior to the normal breeding season (Moore, 1987).

The reason for the low overall pregnancy rate in the present experiment is unknown particularly since control and treated hinds were similar. It is probably not an effect of synchronisation with exogenous progesterone (CIDR) since these same hinds had a 63% conception rate to a similar synchronisation treatment in the previous breeding season (M.W. Fisher and P.F. Fennessy, pers. comm.). Perhaps the yarding, handling and blood sampling regime imposed around the time of mating and conception had some deleterious

effect on the establishment of pregnancy. Interestingly, 5/7 treated hinds with twin ovulations were pregnant compared with only 2/12 of those with single ovulations. This suggests that multiple ovulation favoured conception and/or embryo survival in these animals perhaps because of enhanced progesterone secretion during pregnancy when multiple corpora lutea are present (Kelly *et al.*, 1982).

It is likely that inhibin is the factor in bFF responsible for the effects observed in this study, although other, as yet unknown, components can not be excluded. Inhibin and/or other factors in bFF may thus have the potential to regulate fecundity in deer. Taken collectively with other studies they also suggest that FSH may be important in preovulatory follicular growth in the red deer hind as in other species.

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**Title:** Effect of active immunization with follicular fluid on ovulation rates in Romney ewes.

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## Effect of active immunisation with follicular fluid on ovulation rates in Romney ewes

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### ABSTRACT

Active immunisation of Romney ewes with bovine follicular fluid (bFF) at monthly intervals for 4 months in the breeding season increased monthly mean ovulation rates by 8 to 100%, relative to non-immunised ewes, by increasing the frequency of double ovulations. The increases in ovulation rate were independent of the dose of bFF (from 1 to 10 ml) and whether an aqueous- or oil-based adjuvant was used. Immunisation with porcine FF (1 or 5 ml) did not increase ovulation rates significantly. Active immunisation with an androstenedione-based immunogen (Androvax), increased the mean monthly ovulation rates 31 to 63% by increasing the frequency of double and triple ovulations. Immunisation with bFF and Androvax together did not increase ovulation rates any more than immunisation with Androvax alone.

These results show that active immunisation with bFF can increase ovulation rates in sheep, though generally less effectively than by immunisation with an androstenedione-based immunogen.

**Keywords** Follicular fluid; androstenedione; immunisation; ovulation rate; sheep.

### INTRODUCTION

Inhibin is a complex glycoprotein produced by granulosa cells in ovarian follicles under the regulation of follicle stimulating hormone (FSH) and androgens (Henderson and Franchimont, 1983; Henderson *et al.*, 1984a). Inhibin acts on the pituitary to selectively suppress the secretion of FSH by reducing pituitary levels of mRNA coding for the b-subunit of FSH (Mercer *et al.*, 1987). In sheep, FSH has a major role in determining ovulation rate, which can be increased by raising endogenous FSH concentrations (Henderson *et al.*, 1988). Interfering with the normal regulation of FSH secretion can therefore provide a means of manipulating ovulation rate.

Active immunisation of ewes with partially purified inhibin increases the frequency of double and triple ovulations (Henderson *et al.*, 1984b). This is most likely a consequence of interference with the suppression of FSH secretion by endogenous inhibin. Inhibin therefore has the potential to be a useful agent for increasing twinning frequencies in sheep, and possibly other species such as the cow (Price *et al.*, 1987). Unfortunately, because of its complexity, inhibin is difficult and expensive to purify, even partially.

Thus to be commercially useful, a more convenient form of inhibin is required. Ovarian follicular fluid (FF) is rich in inhibin activity (Henderson *et al.*, 1984a), and is readily obtained in large amounts from slaughterhouse waste products. Recent studies have demonstrated that ovulation rates in sheep can be increased by repeated injections of FF (Henderson *et al.*, 1986). For practical purposes, however, an immunisation procedure would be more useful. The purpose of this study was to determine if active immunisation with FF could increase ovulation rates in sheep. In addition, the effectiveness of combined immunisation with FF and an androstenedione immunogen (known to be capable of increasing ovulation rate) was also studied.

### MATERIALS AND METHODS

#### Preparation of Immunogens

Ovaries were obtained from cows and pigs slaughtered at a local abattoir. Antral follicles on the surface of the ovaries were punctured with a 20-gauge needle, and the FF aspirated. Debris and red blood cells were removed from pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and



the supernatant was stored frozen at  $-20^{\circ}\text{C}$ . Several collections of FF were subsequently thawed, and the steroids removed by treatment with dextran T-70 (0.1%, Pharmacia Fine Chemicals AB, Uppsala, Sweden) coated charcoal (Norit A, 1% Fisher Scientific Co., Springfield, New Jersey, USA) as described previously (Henderson *et al.*, 1986). The FF was then lyophilised and stored at  $4^{\circ}\text{C}$  until needed.

Androvax (i.e., 6-hydroxyandrost-4-ene-3,17-dione-6 hemisuccinyl alacen), an androstenedione-based vaccine for increasing ovulation rates in sheep (McNatty *et al.*, 1988), was prepared at Wallaceville.

On the day before each immunisation, the FF and Androvax were formulated in a water-based adjuvant (aqueous DEAE-dextran, Pharmacia Fine Chemicals AB, Uppsala, Sweden) or in an oil-based adjuvant composed of Span 85 (sorbitan triolate; ICI Americas Inc, Washington, USA): Tween 85 (Polyoxyethylene 20 sorbitan triolate; Sigma Chemical Co., St Louis, Mo, USA): Marcol 52 mineral oil (Exxon Co., Houston, Texas, USA) in the ratio 1:1:8 v/v/v (STM) (Bokhout *et al.*, 1981).

FF in DEAE-dextran was prepared by dissolving lyophilised FF powder in 5% aqueous DEAE-dextran (w/v) to yield the equivalent of 1 to 10 ml FF in 1 to 4 ml of DEAE-dextran solution. FF in STM was prepared by dissolving lyophilised FF powder in distilled water and emulsifying with STM in the ratio 1:1.25 (aqueous:STM, v/v) to yield the equivalent of 5 ml FF in 4.5 ml emulsion. Androvax was prepared only in 5% aqueous DEAE-dextran (w/v) at a concentration of 5 mg/ml. All the formulations were stored at  $4^{\circ}\text{C}$  until administered to sheep the

next day.

### Sheep and their Treatment

Parous New Zealand Romney ewes aged 2.5 to 3.5 years and weighing an average of 51 kg were used in this study, which was undertaken over 2 consecutive breeding seasons. Different flocks of ewes, grazed on open pasture, were used in each of the 2 breeding seasons. The sheep were immunised at monthly intervals (March to June) during the breeding season. The FF (1.4 ml of DEAE-dextran solution or 4.5 ml STM emulsion) and Androvax (5 mg in 1 ml DEAE-dextran solution) immunisations were given subcutaneously into sites in the neck region or into the gracilar or axillary region. The number of ovulations (ovulation rate) was determined 1 month after each immunisation (April to July) by laparoscopic examination of the ovaries of each ewe, and counting the number of corpora lutea present.

### Data Analysis

Significant differences between the ovulation rates of non-immunised ewes, FF and Androvax immunised ewes were tested for by comparing the frequency of non-multiple (0 or 1) and multiple ( $\geq 2$ ) ovulations between the treatment groups, using either the Fisher exact test or  $\chi^2$  analysis. Frequency analysis was performed on each monthly set of data and on the combined data for each treatment group collected over each breeding season. The level of significance was set at  $P < 0.05$ .

TABLE 1 Effect of monthly immunisation with bovine (b) or porcine (p) FF on ovulation rate in Romney ewes.

Treatment	N	April	Ovulation rates in:		
			May	June	July
Non-immunised	18	0.8 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	0.9 $\pm$ 0.1
5 ml bFF	12	1.4 $\pm$ 0.2**	1.5 $\pm$ 0.2	1.7 $\pm$ 0.1	1.8 $\pm$ 0.4**
5 ml pFF	12	1.3 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.2	1.2 $\pm$ 0.2

Values are mean  $\pm$  s.e.m. for N ewes.

\*\* indicates value differs from corresponding non-immunised value (Fisher exact test).

**RESULTS**

**TABLE 2** Frequency table showing overall effect on ovulation rate of immunisation with bFF or pFF.

Treatment	Ovulation rate (OR)					Mean OR ± s.e.m.
	0	1	2	3	5	
Non-immunised	5	53	14	0	0	1.1±0.1
5 ml bFF	2	19	26	0	1	1.6±0.1***
5 ml pFF	1	31	12	4	0	1.4±0.1

\*\*\* Indicates value differs significantly from corresponding non-immunised value ( $\chi^2$  analysis).

Table 1 shows the effect of immunisation with 5 ml bovine (b) or 5 ml porcine (p) FF (given in DEAE-dextran) on monthly mean ovulation rates. A frequency table showing the overall distribution of ovulation rates is shown in Table 2. Immunisation with bFF increased mean ovulation rates significantly in April and July, when mean ovulation rates in the control ewes were at their lowest, and over all months there was a significant increase in the frequency of multiple ovulations. Immunisation with pFF also increased mean ovulation rates each month, but this failed to reach significance on any occasion, and overall there was no significant increase in the frequency of multiple ovulations. The effects of immunisation with bFF or pFF were independent of dose of FF. No significant differences in the mean ovulation rates each month, or in the overall frequency of multiple ovulations were obtained using 1, 5 or 10 ml bFF

**TABLE 3** Effect of monthly immunisation with 5 ml bFF and/or Androvax on ovulation rates in Romney ewes.

Treatment	Ovulation rates in:			
	April	May	June	July
Non-immunised	0.8±0.2	1.3±0.1	1.3±0.1	1.2±0.1
bFF (STM)	1.4±0.2*	1.5±0.1	1.5±0.1	1.3±0.1
bFF (DEAE-dextran)	1.2±0.2	1.5±0.1	1.6±0.1*	1.4 ±0.1
Androvax	1.3±0.2	1.9±0.2*	1.7±0.2*	1.6±0.2
bFF (DEAE-dextran) + Androvax	1.4±0.2*	1.8±0.2	1.7±0.1**	1.6±0.1*

Values are mean ± s.e.m. for 17-20 ewes/group.

Asterisks indicate values differing significantly from corresponding non-immunised values ( $\chi^2$  analysis).

or 1 or 5 ml pFF.

The following breeding season, in an effort to improve upon the increase in ovulation rate obtained by immunisation with bFF given in DEAE-dextran, ewes were immunised with bFF in the oil-based STM adjuvant. In addition, ewes were immunised with bFF and Androvax together to determine if the combined treatment would more effectively increase ovulation rate than each treatment individually. The results are shown in Tables 3 and 4. The mean ovulation rates were increased by 8 to 75% following bFF immunisation and there was a significant increase in the overall frequency of multiple ovulations. There was no significant difference between ewes immunised with bFF in STM or DEAE-dextran. Immunisation with Androvax increased monthly

**TABLE 4** Frequency table showing overall effect on ovulation rate of immunisation with 5 ml bFF and/or Androvax.

Treatment	Ovulation rate (OR)				Mean OR ± s.e.m.
	0	1	2	3	
Non-immunised	10	51	17	1	1.1±0.1
bFF (STM)	4	41	34	1	1.4±0.1**
bFF (DEAE-dextran)	3	44	31	2	1.4±0.1*
Androvax	3	34	31	10	1.6±0.1***
bFF (DEAE-dextran) + Androvax	3	29	36	7	1.6±0.1***

Asterisks indicate values differing significantly from corresponding non-immunised value ( $\chi^2$  analysis).

mean ovulation rates by 33 to 63% and significantly increased the overall frequency of multiple ovulations. However, immunisation with bFF and Androvax together was no more effective in increasing ovulation rates than immunisation with Androvax alone.

### DISCUSSION

Active immunisation of sheep with bFF increased mean ovulation rates by 8 to 100%, relative to non-immunised ewes, by increasing the frequency of double ovulations. The increases in mean ovulation rates were independent of the dose of FF administered (in the range 1 to 10 ml bFF), or whether an aqueous (DEAE-dextran) or oil (STM) based adjuvant was used. It is unlikely, however, that immunisation with bFF will be commercially useful as a means of increasing ovulation rates in sheep, and hence their fecundity. The increases in mean ovulation rates in response to bFF immunisation were very variable, and generally lower than those obtained with Androvax. There was also no advantage in immunising with Androvax and bFF together compared to immunising with Androvax alone.

Interestingly the greatest increase in mean ovulation rates, in response to immunisation with bFF or Androvax, was generally observed early in the breeding season (April) when mean ovulation rates of the non-immunised ewes were at their lowest. At this time 22 and 40% of the non-immunised ewes in each breeding season were still anovulatory whereas only 0 to 8% and 10 to 15% of the bFF or Androvax immunised ewes were anovulatory in each of the 2 breeding seasons respectively. Thus immunisation with bFF may advance the onset of the breeding season in some ewes, as has previously been demonstrated following immunisation with androstenedione (Gibb *et al.*, 1982).

The immunogen in FF responsible for the increase in ovulation rates is assumed to be inhibin, as active immunisation with semi-pure inhibin derived from follicular fluid will increase ovulation rates in sheep (Henderson *et al.*, 1984b, Cummins *et al.*, 1986). However, the possibility that other components may also be responsible

cannot be excluded. Indeed some factors in the FF may actually be aggravating the positive ovulatory response to the immunogen(s) involved. More consistent and higher increases in mean ovulation rates may be obtained using highly purified forms of inhibin. The use of the a subunit of inhibin as an immunogen, prepared by recombinant DNA methods, may offer some potential in this regard (Forage *et al.*, 1987).

The mechanism by which Androvax (androstenedione) immunisation increases ovulation rates in sheep is uncertain, but increased FSH secretion may be involved (McNatty *et al.*, 1988). Androgens can stimulate ovarian inhibin production *in vitro* (Henderson and Franchimont, 1983). Androvax immunisation may therefore cause a reduction in ovarian inhibin production. Thus, the consequences of both bFF and Androvax immunisation may be mediated through effectively reducing the negative feedback effects of inhibin on pituitary FSH secretion. Such a common mechanism would be consistent with the observation that immunisation with bFF and Androvax together was no more effective in increasing ovulation rates than immunisation with Androvax alone.

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**Authors:** K.M. Henderson, R.L. Ellen, A. Weaver, K. Ball & K.P. McNatty

**Title:** Relative effectiveness of active immunization with follicular fluid and/or steroids in increasing ovulation rates in Romney ewes.

**References:** *Animal Reproduction Science* (1990) 22, 121-130

## Relative Effectiveness of Active Immunization with Follicular Fluid and/or Steroids in Increasing Ovulation Rates in Romney Ewes

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### ABSTRACT

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Groups of Romney ewes were repeatedly immunized with either crude, unfractionated bovine (b) or porcine (p) follicular fluid (FF) and/or steroids (androstenedione or estrone), administered in aqueous DEAE-dextran, at monthly intervals during the breeding season. Laparoscopy was performed each month to determine ovulation rates. The proportion of ewes with multiple ( $\geq 2$ ) ovulations ranged from 0 to 39% in non-immunized ewes, 8 to 42% in pFF-immunized ewes, 35 to 56% in bFF-immunized ewes and 40 to 74% in steroid-immunized ewes. Immunization with bFF and androstenedione or estrone together was generally no more effective in increasing the proportion of ewes with multiple ovulations (range 45 to 70%) than immunization with steroids on their own. Similarly, immunization with androstenedione and estrone together was no more effective than either steroid alone. Of the multiple ovulations observed, 88% were doubles and 12% were triples (mostly occurring in the steroid-immunized ewes). Immunization with bFF in an oil- rather than an aqueous-based adjuvant consistently produced higher anti-bFF titres in serum. However, there was no significant difference in the proportion of ewes with multiple ovulations between the two groups. The results show that while active immunization with unfractionated bFF can increase the proportion of ewes with multiple ovulations, it offers no advantage over steroid immunization in this regard.

### INTRODUCTION

For practical usefulness, methods for increasing ovulation rates to increase prolificacy in sheep must be convenient to use and cost-effective. Active immunization against sex steroids, notably androstenedione, is an established method for increasing ovulation rates and fecundity in sheep (Smith, 1985). However, the variability of the ovulatory response can reduce the cost-effectiveness of this procedure. Over recent years, there has been increasing interest

in the potential use of inhibin-derived immunogens as an alternative for increasing ovulation rates. Ovulation rates in sheep can be increased by active immunization with partially purified inhibin preparations derived from fractionation of follicular fluid (Henderson et al., 1984; Cummins et al., 1986) or with inhibin  $\alpha$  subunit produced by recombinant DNA techniques (Forage et al., 1987). However, relative to the preparation of steroid immunogens, the preparation of these immunogens is time-consuming and costly. This may hinder their possible commercial usefulness.

Follicular fluid (FF) is rich in inhibin activity and readily obtained in large amounts from slaughterhouse waste products. An increased frequency of multiple ovulations occurs in sheep following repeated injections of unfractionated bovine FF daily during the luteal (Wallace and McNeilly, 1985) or follicular (Henderson et al., 1986) periods. For practical purposes, however, an immunization procedure to increase ovulation rates would be more convenient than one requiring multiple daily injections. The purpose of this study was to determine if active immunization with crude, unfractionated cow or pig FF, treated only with dextran-coated charcoal to remove endogenous steroids, might be an alternative practical way of raising ovulation rates in sheep, by increasing the frequency of multiple ovulations. In addition, the relative effectiveness in increasing ovulation rates of immunization with FF and steroid (androstenedione and estrone) immunogens, either alone or together, was studied.

#### MATERIALS AND METHODS

##### *Preparation of immunogens*

Ovaries were obtained from cows and pigs slaughtered at a local abattoir. Antral follicles on the surface of the ovaries were punctured with a 20-gauge needle, and the FF aspirated. Debris and red blood cells were removed from pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and the supernatant was stored frozen at -20°C. Several collections of FF were subsequently thawed, and the steroids removed by treatment with dextran T-70 (0.1%, Pharmacia Fine Chemicals AB, Uppsala, Sweden) coated charcoal (Norit A, 1% Fisher Scientific Co., Springfield, NJ) as described previously (Henderson et al., 1986). The FF was then lyophilized and stored at 4°C until needed.

Androvax<sup>TM</sup> (i.e., 6-hydroxyandrost-4-ene-3,17-dione-6-hemisuccinate coupled to bovine  $\alpha$ -lactalbumin), an androstenedione-based vaccine previously used for increasing ovulation rates in sheep (McNatty et al., 1988), was prepared at Wallaceville. The estrone immunogen was estrone 6 $\alpha$ -hemisuccinate conjugated to bovine serum albumin, and was generously provided by Dr. D. Crump, Chemistry Division, Department of Scientific and Industrial Research, Lower Hutt, New Zealand.

On the day before each immunization, the FF and steroid immunogens were formulated in a water-based adjuvant (5% aqueous DEAE-dextran, w/v (av-

erage MW of dextran component was 500 000); Pharmacia Fine Chemicals AB, Uppsala, Sweden) or in an oil-based adjuvant composed of Span 85 (sorbitan triolate; ICI Americas Inc., Washington, DC): Tween 85 (polyoxyethylene 20 sorbitan triolate; Sigma Chemical Co., St Louis, MO): Marcol 52 mineral oil (Exxon Co., Houston, TX) in the ratio 1:1:8  $v/v/v$  (STM).

FF in DEAE-dextran was prepared by dissolving lyophilized FF powder in 5% aqueous DEAE-dextran ( $w/v$ ) to yield the equivalent of 1, 5 or 10 ml FF in 1, 2 or 4 ml of DEAE-dextran solution respectively. FF in STM was prepared by dissolving lyophilized FF powder in distilled water and emulsifying with STM in the ratio 1:1.25 (aqueous:STM,  $v/v$ ) to yield the equivalent of 5 ml FF in 4.5 ml emulsion. The steroid immunogens were prepared only in 5% aqueous DEAE-dextran ( $w/v$ ) at a concentration of 5 mg/ml. All the formulations were stored at 4°C until administered to sheep the next day.

#### *Sheep and their treatment*

Parous New Zealand Romney ewes aged 2.5 to 3.5 years, and weighing an average of 51 kg (range 41 to 64 kg) were used in this study which consisted of three experiments undertaken over three consecutive breeding seasons. Different flocks of ewes, grazed on open pasture, were used in each of the three breeding seasons. The normal breeding season for Romney ewes at Wallaceville is from March to August.

#### *Experiment 1*

Seventy-eight ewes were randomly assigned to five groups of 12 and one group of 18. The groups of 12 ewes were immunized four times at monthly intervals (March to June) with either 1, 5 or 10 ml bovine (b) FF, or 1 or 5 ml porcine (p) FF administered in DEAE-dextran solution. The 18 ewes of the remaining group were immunized with 2 ml of DEAE-dextran solution alone (control ewes). Immunizations were given subcutaneously into sites in the neck region, or into the gracilar or axillary region. Ovulation rate (the number of ovulations) was determined at monthly intervals (in April, May, June and July) by laparoscopic examination of the ovaries of each ewe, and counting the number of visible corpora lutea.

#### *Experiment 2*

This study was undertaken in the breeding season following completion of Exp. 1. Its purpose was to determine if: (a) the small increase in the frequency of multiple ovulations resulting from immunization with bFF in DEAE-dextran could be improved upon by using a different adjuvant (STM); (b) if immunization with bFF and Androvax together might more effectively increase the frequency of multiple ovulations than each treatment individually.

One hundred ewes were randomly assigned to five groups of 20. Four groups were immunized four times at monthly intervals, as in Exp. 1, with either 5 ml



bFF given in DEAE-dextran or STM, Androvax (5 mg) or Androvax (5 mg) plus 5 ml bFF given in DEAE-dextran. The remaining 20 ewes served as a non-immunized control group. Ovulation rates were determined in all ewes at monthly intervals by laparoscopy as in Exp. 1. The control ewes and those immunized with bFF alone were blood sampled (5 ml) by venepuncture at fortnightly intervals for 16 weeks, commencing at the time of the first immunization. Serum was prepared from the blood samples and stored frozen ( $-20^{\circ}\text{C}$ ) until used for titre determinations.

### *Experiment 3*

This study was undertaken in the breeding season following the completion of Exp. 2. Its purpose was to determine if immunization with bFF and an estrone immunogen together, or with Androvax and an estrone immunogen together might more effectively increase the frequency of multiple ovulations than each treatment individually.

Groups of 18 to 20 ewes were immunized in March and again in April with either 5 ml bFF, estrone immunogen (5 mg), Androvax (5 mg), 5 ml bFF plus estrone immunogen (5 mg) or Androvax plus estrone immunogen (5 mg of each). All the immunogens were administered in aqueous DEAE-dextran. Eighteen untreated ewes served as a non-immunized control group. All ewes were laparoscoped 1 month after each immunization to determine ovulation rates.

### *Titre determination*

The titres of antisera generated against bovine FF were estimated using an enzyme-linked immunoassay. Unless stated otherwise, all reagents were obtained from the Sigma Chemical Co., St Louis, MO. A volume of  $0.1\ \mu\text{l}$  bFF in  $0.1\ \text{ml}$   $50\ \text{mM}$   $\text{NaHCO}_3$  buffer, pH 9.6 was added to wells of 96-well polystyrene microtitre plates (Nunc, Kampstrup, Denmark), and allowed to adsorb overnight at  $4^{\circ}\text{C}$ . Plates were emptied and washed four times with washing buffer ( $10\ \text{mM}$  phosphate buffer containing  $0.8\%$  NaCl and  $0.05\%$  Tween 20, pH 7.4). Thereafter,  $1\ \mu\text{l}$  of serum from bFF-immunized ewes, or from non-immunized control ewes, was added to the wells in  $100\ \mu\text{l}$  of assay buffer (washing buffer containing  $0.1\%$  bovine serum albumin, pH 7.4), and the plates were incubated for 1 h at  $37^{\circ}\text{C}$ . The plates were emptied and washed four times with washing buffer. Rabbit anti-sheep  $\gamma$ -globulin antibody conjugated to horseradish peroxidase was added to the wells in assay buffer, and the plates were incubated for 1 h at  $37^{\circ}\text{C}$ . The plates were again emptied and washed four times with washing buffer. Next,  $100\ \mu\text{l}$ /well of substrate-chromogen solution ( $50\ \text{ml}$  of  $0.1\ \text{M}$  citrate buffer, pH 5.0, containing  $20\ \text{mg}$  *o*-phenylenediamine and  $20\ \mu\text{l}$   $30\%$   $\text{H}_2\text{O}_2$ ) was added. Plates were incubated for 1 h in the dark at  $37^{\circ}\text{C}$ . The reaction was terminated by adding  $50\ \mu\text{l}$ /well of  $2\ \text{M}$   $\text{H}_2\text{SO}_4$ . Colour formation was measured by reading the absorbance of each well at  $490\ \text{nm}$ . The intensity

of the colour formed is proportional to the amount of anti-bFF immunoglobulin interacting with the bFF coating the plates, and so provides an indication of the titre of the anti-bFF serum.

When comparing, with respect to time, the relative titres of antisera generated against bFF, using STM and DEAE-dextran as adjuvant, the absorbance readings at 490 nm for each ewe were expressed as a percentage of the reading obtained using serum obtained at the time of the first immunization. This transformation reduced the contribution of inter-sheep variation to titre differences.

#### *Data analysis*

Chi-squared analysis was used to determine the statistical significance of differences between the treatment groups in the proportion of ewes each month with multiple ( $\geq 2$ ) ovulations. The level of significance was set at  $P < 0.05$ .

## RESULTS

### *Experiment 1*

There were no significant differences ( $P > 0.05$ ) between the doses of bFF (1, 5 or 10 ml) or between the doses of pFF (1 or 5 ml) in the proportions of ewes with multiple ovulations, in any month. The ovulation rate data for the three groups of bFF- and two groups of pFF-immunized sheep were therefore pooled, for each month. The percentage of ewes with multiple ovulations is shown in Table 1. In all months, a higher proportion of the ewes immunized with bFF had multiple ovulations compared to the control ewes, though this was only statistically significant in April and July, when the frequency of multiple ovulations in the control ewes was at its lowest. With pFF immunization, the proportion of ewes with multiple ovulations was in between that found in the control and bFF-immunized ewes, but not statistically different ( $P > 0.05$ )

TABLE 1

Effect of immunization with bovine (b) or porcine (p) FF on ovulation rates in Romney ewes

	N	Percentage of ewes with multiple ovulations			
		April	May	June	July
Controls	18	6 (1) <sup>a</sup>	33 (6) <sup>a</sup>	39 (7) <sup>a</sup>	0 (0) <sup>a</sup>
bFF-immunized	36	42 (15) <sup>b</sup>	44 (16) <sup>a</sup>	53 (19) <sup>a</sup>	36 (13) <sup>b</sup>
pFF-immunized	24	29 (7) <sup>ab</sup>	33 (8) <sup>a</sup>	42 (10) <sup>a</sup>	8 (2) <sup>a</sup>

Numbers in parentheses are the actual numbers of ewes with multiple ( $\geq 2$ ) ovulations at laparoscopy. Group values with different letter superscripts in the same month indicate statistically significant differences ( $P < 0.05$ ) between groups in the proportion of ewes with multiple ovulations.

from either group, except in July when the proportion was significantly lower than that for the bFF-immunized ewes.

None of the control or pFF-immunized ewes had ovulation rates  $> 2$  in any month, except for one pFF-immunized ewe which had a triple ovulation each month. In ewes immunized with bFF, triple ovulations were observed in one ewe in May, two ewes in June and one ewe in July. One ewe in July also had five ovulations. All other multiple ovulations were double ovulations. In April, four of the control ewes (22%), and one each of the bFF- and pFF-immunized ewes (3% and 4% respectively) were found to be anovulatory. Anovulation was not observed again until July when one control, one bFF-immunized and two pFF-immunized ewes were anovulatory.

### Experiment 2

In all months immunization with bFF in DEAE-dextran or STM, or immunization with Androvax, or Androvax plus bFF resulted in a higher proportion of ewes with multiple ( $\geq 2$ ) ovulations compared to the non-immunized control ewes, though this was not always statistically significant (Table 2). There was no significant difference ( $P > 0.05$ ) in any month, in the proportion of ewes with multiple ovulations between groups immunized with bFF in DEAE-dextran or STM, Androvax or Androvax plus bFF. An ovulation rate of 3 was the highest recorded for any ewe in any of the groups. Only one control ewe had a triple ovulation (in June). Two ewes immunized with bFF in DEAE-dextran (one in May and one in July) and one ewe (in June) immunized with bFF in STM had triple ovulations. Triple ovulations were recorded in April (0, 2), May (5, 3), June (2, 1) and July (3, 1) for ewes immunized with Androvax

TABLE 2

Effect of immunization with bFF and/or Androvax on ovulation rates in Romney ewes

Immunogen	Percentage of ewes with multiple ovulations			
	April	May	June	July
Non-immunized	15 (3) <sup>a</sup>	35 (7) <sup>a</sup>	20 (4) <sup>a</sup>	21 (4/19) <sup>a</sup>
bFF (STM)	45 (9) <sup>b</sup>	55 (11) <sup>ab</sup>	40 (8) <sup>ab</sup>	35 (7) <sup>ab</sup>
bFF (DEAE-dextran)	50 (10) <sup>b</sup>	40 (8) <sup>ab</sup>	55 (11) <sup>b</sup>	35 (7) <sup>ab</sup>
Androvax	40 (8) <sup>ab</sup>	68 (13/19) <sup>b</sup>	58 (11/19) <sup>b</sup>	45 (9) <sup>ab</sup>
bFF (DEAE-dextran) + Androvax	45 (9) <sup>b</sup>	70 (14) <sup>b</sup>	65 (11/17) <sup>b</sup>	56 (10/18) <sup>b</sup>

Numbers in parentheses show the actual number (for  $N=20$ ) or proportion (for  $N < 20$ ) of ewes with multiple ( $\geq 2$ ) ovulations. Although there were always 20 ewes in each treatment group, occasionally, because of technical difficulties, the ovaries of some ewes could not be seen when laparoscopy was performed. In these instances  $N$  was  $< 20$ . Group values with different letter superscripts in the same month indicate statistically significant differences ( $P < 0.05$ ) between groups in the proportion of ewes with multiple ovulations.

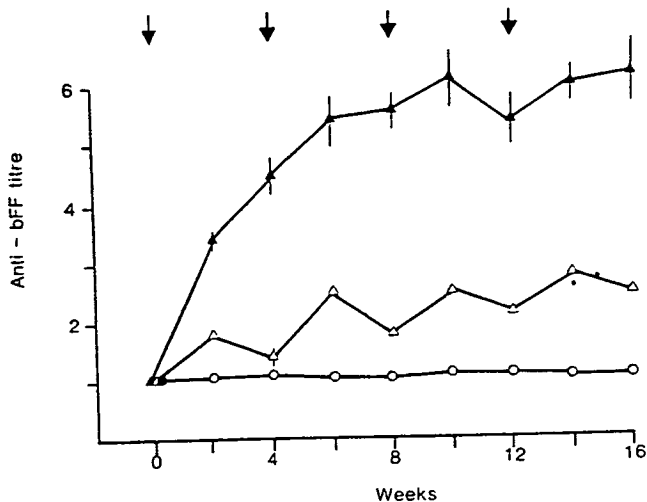


Fig. 1. Influence of adjuvant (STM or DEAE-dextran) on serum anti-bFF titres. Ewes were either non-immunized (O—O) or immunized monthly with 5 ml bFF using DEAE-dextran ( $\Delta$ — $\Delta$ ) or STM ( $\blacktriangle$ — $\blacktriangle$ ) as adjuvant. Values are means of 20 ewes per group with vertical lines showing the s.e.m. when larger than the size of the symbol. Arrows indicate the times of immunization.

and Androvax plus FF respectively. Eight control ewes (40%) were anovulatory in April, but only 3, 2, 2 and 3 of the ewes (10 to 15%) immunized with bFF in DEAE-dextran or STM, Androvax or Androvax plus bFF respectively.

Interestingly, although the proportion of ewes with multiple ovulations did not differ significantly between groups immunized with bFF given in DEAE-dextran or STM, the mean serum anti-bFF titres generated were consistently higher in ewes in which STM was the adjuvant (Fig. 1).

### Experiment 3

In the 2 months studied, immunization with bFF, Androvax and/or estrone resulted in a higher proportion of ewes with multiple ( $\geq 2$ ) ovulations compared to the non-immunized control ewes, though the increase was not always statistically significant (Table 3). Immunization with estrone together with either bFF or Androvax was no more effective in increasing the proportion of multiple-ovulating ewes than immunization with estrone alone. The highest ovulation rate recorded was 2 in the non-immunized ewes, and 3 in the immunized ewes. In the 2 months studied, triple ovulations were observed in one bFF, six estrone, four Androvax, two bFF plus estrone and five estrone plus Androvax immunized ewes. The number of non-ovulating ewes in each group ranged from 0 to 3 over the 2-month period.

TABLE 3

Effect of immunization with bFF, Androvax and/or estrone on ovulation rates in Romney ewes

Immunogen	N	Percentage of ewes with multiple ovulations	
		April	May
Non-immunized	18	22 (4) <sup>a</sup>	28 (5) <sup>a</sup>
bFF	18	50 (9) <sup>ab</sup>	56 (10) <sup>ab</sup>
Estrone	20	74 (14/19) <sup>b</sup>	70 (14) <sup>b</sup>
bFF + estrone	20	55 (11) <sup>b</sup>	70 (14) <sup>b</sup>
Androvax	20	68 (13/19) <sup>b</sup>	55 (11) <sup>ab</sup>
Androvax + estrone	19	63 (12) <sup>b</sup>	68 (13) <sup>b</sup>

Numbers in parentheses show the actual number (for  $N$  ewes) or proportion (for  $<N$  ewes) of ewes with multiple ( $\geq 2$ ) ovulations. In April, the ovaries of two ewes could not be viewed at laparoscopy. Group values with different letter superscripts in the same month indicate statistically significant differences ( $P < 0.05$ ) between groups in the proportion of ewes with multiple ovulations.

#### DISCUSSION

This study shows that active immunization of sheep with crude, unfractionated bFF produces a small increase in the proportion of ewes having multiple (mainly double) ovulations. In non-immunized ewes, the proportion having multiple ovulations in the months studied during the three breeding seasons ranged from 0 to 39%, compared to 35 to 56% for the bFF-immunized ewes. In sheep immunized with pFF, the proportion with multiple ovulations ranged from only 8 to 42%. Attempts to further increase the proportion of ewes having multiple ovulations by immunizing with bFF in an oil-based adjuvant (STM) rather than aqueous DEAE-dextran were unsuccessful. Although immunization with bFF in STM, instead of DEAE-dextran, consistently produced higher anti-bFF titres in serum, there was no significant difference between the two groups in the proportion of ewes with multiple ovulations.

Although not statistically significant, the proportion of ewes with multiple ovulations was generally higher following active immunization with Androvax or estrone (range 40 to 74%) than with bFF (range 35 to 56%). Attempts to further increase the effectiveness of the immunogens in producing multiple ovulations by combined immunization with bFF and steroids were unsuccessful. There was no significant difference in the proportion of ewes with multiple ovulations between groups of ewes immunized with bFF and steroids (Androvax or estrone), together or alone. Similarly, immunization with estrone and Androvax together was no more effective than immunization with either steroid alone.

Interestingly, early in the breeding seasons (April), 22 and 40% of the control ewes in Experiments 1 and 2 respectively were anovulatory compared to only 3% of the bFF-immunized ewes in Exp. 1 and 10 to 15% of the bFF and/or Androvax-immunized ewes in Exp. 2. Thus, immunization with bFF may advance the onset of the breeding season in some ewes, as has previously been demonstrated following immunization with androstenedione (Gibb et al., 1982).

The immunogen in FF responsible for the increase in multiple ovulations is assumed to be inhibin, as active immunization with semi-pure inhibin derived from fractionated follicular fluid is known to increase ovulation rates in sheep (Henderson et al., 1984; Cummins et al., 1986). However, the possibility that other components may also be responsible cannot be excluded. Indeed some factors in the FF may actually be aggravating the positive ovulatory response to the immunogen(s) involved (Li et al., 1987). More consistent and higher proportions of multiple ovulations may require the use of purer inhibin immunogens. The use of recombinant DNA-derived inhibin  $\alpha$  subunit (Forage et al., 1987) or synthetic peptide fragments of inhibin (Knight et al., 1989) as immunogens may offer some potential in this regard. However, whether using such immunogens will ever offer any practical advantages over steroid immunization as a means of producing multiple ovulations and increasing fecundity remains to be seen.

In summary, the results of this study show that while active immunization with unfractionated bFF can increase the proportion of ewes having multiple ovulations, it offers no advantage over steroid immunization.

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## Oocyte Production and Ovarian Steroid Concentrations of Immature Rats in Response to Some Commercial Gonadotrophin Preparations

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### Abstract

Four commercial gonadotrophin preparations, namely Folligon, F.S.H.-P., Folltropin and Ovagen, were examined for their effects on oocyte production and ovarian steroid concentrations in immature rats. The ratios of the FSH to LH concentrations of the preparations, determined by radioreceptor assays, were Folligon 5, F.S.H.-P. 18, Folltropin 49 and Ovagen 1090.

Forty-eight hours after administering each gonadotrophin preparation to immature rats, ovulation was induced by injection of chorionic gonadotrophin. Twenty-four hours later, oocytes were recovered from the oviducts and counted. Oocytes were produced after injection of chorionic gonadotrophin following a single injection of Folligon (10-50 i.u.). However, no oocytes were produced in response to the other gonadotrophin preparations unless they were administered by continuous infusion (30-1000  $\mu\text{g day}^{-1}$ ). When given by injection (Folligon) or infusion (others), the gonadotrophin preparations all promoted a dose-dependent increase in mean oocyte production, except at the highest doses when mean oocyte numbers either remained unchanged or declined significantly in the cases of Folligon and F.S.H.-P. The highest mean numbers of oocytes produced in response to Folltropin ( $48 \pm 9$  oocytes, mean  $\pm$  s.e.m.) and Ovagen ( $47 \pm 7$ ) were greater than those attained with Folligon ( $21 \pm 6$ ) or F.S.H.-P. ( $31 \pm 5$ ). Mean ovarian weights also increased in a dose-dependent fashion in response to each of the gonadotrophin preparations. Measurement of ovarian steroid concentrations 48 h after the onset of gonadotrophin treatment (i.e. immediately prior to ovulation induction with chorionic gonadotrophin) showed that the gonadotrophin preparations markedly influenced the ratios of ovarian oestradiol-17 $\beta$  and androgen (androstenedione plus testosterone) concentrations. At low doses the gonadotrophin preparations increased the ratio of oestradiol-17 $\beta$  to androgens, but at the highest doses, with the exception of Ovagen, the ratio was reduced relative to peak values. Co-infusion of ovine LH (NIADDK-oLH-25; 10-20  $\mu\text{g day}^{-1}$ ) with Ovagen (250  $\mu\text{g day}^{-1}$ ) or ovine FSH (10  $\mu\text{g day}^{-1}$ , NIADDK-oFSH-17), both low in LH content, increased the mean number of oocytes produced and also the ovarian oestradiol-17 $\beta$ :androgen concentration ratio. However, with 40  $\mu\text{g LH day}^{-1}$ , the oestradiol-17 $\beta$ :androgen ratio fell due to a continued increase in mean ovarian androgen concentrations and a decrease in mean ovarian oestradiol-17 $\beta$  concentration. The mean number of oocytes produced also fell significantly.

These studies show that the ovarian responses of immature rats is a convenient model system in which to examine the attributes of different gonadotrophin preparations. The differences in ovarian steroid concentrations and oocyte production in response to the four commercial gonadotrophin preparations examined in this study may be related, at least in part, to differences in their ratios of the concentrations of bioactive FSH to LH. For maximum superovulatory effectiveness, optimization of this FSH:LH ratio seems important, either too little or too much LH being deleterious to oocyte production.

### Introduction

Superovulation of farm animals by administration of exogenous gonadotrophins is an accepted and widely used technique. A variety of gonadotrophin preparations are now

available for this purpose. Such preparations are usually either serum gonadotrophins (e.g. pregnant mares serum gonadotrophin) or pituitary extracts of follicle stimulating hormone (FSH). A variety of doses and administration regimes have been recommended in attempts to optimize the superovulatory response in different species (Armstrong and Evans 1983; Armstrong *et al.* 1983; Monniaux *et al.* 1983; Ryan *et al.* 1984; McNatty *et al.* 1989). However, to make valid comparisons of the efficacy of the various gonadotrophin preparations available requires field trials of different dosage and treatment regimens in the species of interest. For farm animals, this can be impractical because of the large numbers of animals and amounts of gonadotrophin required.

Alternative bioassays are therefore sought that can provide objective information on the attributes of gonadotrophin preparations currently produced and that can assess the likely advantages of novel preparations as they become available. Radioreceptor assays to measure bioactive FSH and luteinizing hormone (LH) concentrations are frequently used for comparing gonadotrophin preparations (Monniaux *et al.* 1983; Murphy *et al.* 1984; Donaldson *et al.* 1986). However, such *in vitro* bioassays may not always be helpful in assessing the behaviour of a gonadotrophin preparation *in vivo*. For example, receptor assays provide no information on likely clearance rates from the circulation or on postreceptor events such as effects on ovarian steroid production, each of which could markedly influence superovulatory effectiveness. Early studies have shown that infusion of gonadotrophins is a useful way of monitoring their effects on ovarian function in laboratory animals (Garza *et al.* 1984). More recent studies have demonstrated that the ovulatory response of immature rats infused with gonadotrophins may be a useful model system to provide some information on the superovulating properties of gonadotrophin preparations (Armstrong and Opavsky 1988; Armstrong *et al.* 1989; Opavsky and Armstrong 1989).

The purpose of this study was to examine the effect of four commercial gonadotrophin preparations (Folligon, F.S.H.-P., Folltropin and Ovagen) on oocyte production and ovarian steroid concentrations of immature rats. Folligon is prepared from the serum of pregnant mares, whereas the others are porcine (F.S.H.-P. and Folltropin) or ovine (Ovagen) pituitary extracts. In addition to FSH, these hormones also contain varying amounts of LH activity (Monniaux *et al.* 1983; Murphy *et al.* 1984; McNatty *et al.* 1989). The effects of highly purified ovine FSH (NIADDK-oFSH-17) were also studied for comparison.

## Materials and Methods

### *Gonadotrophin Preparations*

Folligon (batch 488121) and Chorulon (batch 690891) were obtained from Intervet (Aust.) Pty Ltd, NSW, Australia. F.S.H.-P. (lot 548D86) was obtained from Burns-Biotec Laboratories, Nebraska, USA. Folltropin (lot V-028) was obtained from Vetrephearm Inc., London, Ontario, Canada. Ovagen (batch 8202) was obtained from Immuno-Chemical Products Ltd, Auckland, New Zealand. Ovine FSH (NIADDK-oFSH-17; FSH activity  $20 \times \text{NIH-FSH-S1 U mg}^{-1}$ , LH activity  $0.04 \times \text{NIH-LH-S1 U mg}^{-1}$ ) and ovine LH (NIADDK-oLH-25; LH activity  $2.3 \times \text{NIH-LH-S1 U mg}^{-1}$ , FSH contamination  $<0.5\%$  by weight) were provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, USA.

### *Treatment of Rats*

Sprague-Dawley rats aged 21 to 24 days, obtained from the Experimental Small Animal Breeding Unit at Wallaceville, were used in this study. The rats were housed under constant temperature and lighting conditions and were allowed pelleted food and water *ad libitum* throughout their treatment.

Folligon was administered in varying doses to rats 2 days before induction of ovulation (Day -2) as a single subcutaneous injection in 0.2 mL saline. In preliminary experiments, varying doses of F.S.H.-P., Folltropin and Ovagen were also administered in 0.2 mL saline either as a single subcutaneous injection or as once- or twice- (12 h apart) daily injections from Day -2 to Day 0. In later experiments, F.S.H.-P., Folltropin, Ovagen and NIADDK preparations of ovine FSH and LH were administered by continuous infusion in saline, using subcutaneously implanted Alzet mini-osmotic pumps (Model 2001, Alza Corporation, Palo Alto, California, USA). These pumps, which have a mean

pumping rate of approximately  $1 \mu\text{L h}^{-1}$ , were implanted into rats under light ether anaesthesia on Day -2. On Day 0, 48 h after either the first injection of gonadotrophin or implantation of the pumps, the rats received a subcutaneous injection of 25 i.u. chorionic gonadotrophin (Chorulon) in 0.2 mL saline to induce ovulation. Twenty-four hours later, the rats were sacrificed by overdosing with pentobarbitone sodium. The ovaries and oviducts were excised and trimmed free of fat. The ovaries were blotted dry and weighed individually. Under a dissecting microscope, oocytes were released from the swollen ampullae of the oviducts into Dulbecco's phosphate-buffered saline containing  $250 \mu\text{g mL}^{-1}$  hyaluronidase (Type IV-S, Sigma Chemical Co., St Louis, Missouri, USA). Once the clumped cumulus-oocyte masses had been dispersed by the enzyme solution, the number of oocytes released per pair of ovaries provided an estimate of ovulation rate.

Other groups of rats were sacrificed by overdosing with pentobarbitone sodium on Day 0 when Chorulon would normally have been given. The ovaries were excised, trimmed free of fat, weighed and homogenized individually in 2 mL ethanol to extract steroids. After incubation at room temperature overnight, the extracts were centrifuged at 500 g for 15 min. The ethanol supernatant was removed and stored at  $-20^\circ\text{C}$  until assayed for steroid. The pelleted residual ovarian tissue was then extracted again with a further 2 mL ethanol in the same manner. The first homogenization in ethanol was subsequently found to be sufficient to routinely extract more than 90% of ovarian oestradiol-17 $\beta$ , testosterone and androstenedione, as assessed by the additional amounts of steroid extracted following the second homogenization. Ovarian steroid data presented here are therefore based on the amounts extracted by the first homogenization only.

#### Radioimmunoassays

Aliquants of the ethanol extracts of ovarian tissue were evaporated to dryness under a stream of nitrogen gas and reconstituted in 0.1 M phosphate-buffered saline (pH 7) containing 0.1% gelatin and 0.1% sodium azide. Oestradiol-17 $\beta$ , androstenedione and testosterone concentrations were measured in these aqueous samples by specific radioimmunoassays described previously (McNatty *et al.* 1981, 1984a, 1984b). The steroids showing major cross-reactions with the antisera were: oestrone (7%), oestriol (1%) and oestradiol-17 $\alpha$  (1%) with the oestradiol-17 $\beta$  antiserum (WA-27); 4-androsten-3,11,17-trione (40%), 11 $\beta$ -hydroxyandrostenedione (31%) and testosterone (0.4%) with the androstenedione antiserum (WA-965); and 5 $\alpha$ -dihydrotestosterone (75%), 5 $\beta$ -dihydrotestosterone (75%), 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (17%) and androstenedione (0.1%) with the testosterone antiserum (WA-36). The limit of sensitivity of the assays (per tube) was 10 pg for oestradiol-17 $\beta$  and 20 pg for androstenedione and testosterone. The intra- and inter-assay coefficients of variation were <9% and <14% respectively. Steroid concentrations in the aqueous extracts were normalized with respect to milligrams of ovarian tissue.

#### Radioreceptor Assays

The bioactive FSH and LH content of the gonadotrophin preparations was estimated from radioreceptor assays, using methodologies described previously (Cheng 1975). Membrane fractions were prepared by the method of Abou-Issa and Reichert (1977) from bovine testis and bovine corpus luteum and were used as sources of FSH and LH receptor respectively. The radioactive tracers used were either iodinated NIAMDD-oFSH-I-1 or NIADDK-oLH-I-3 and were provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, USA. The reference standards were either NIADDK-oFSH-17 or NIADDK-oLH-25. Non-specific binding was determined by using either Gonadotrophin L.H. or Gonadotrophin F.S.H. (Paines & Byrne Ltd, Greenford, UK) as the saturating ligand. Bioactive FSH and LH values for the gonadotrophin preparations were calculated by interpolation from standard curves generated by plotting percentage of radioligand bound versus the logarithm of the concentration of reference standard.

#### Protein Determinations

The protein content of the gonadotrophin preparations was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the protein standard.

#### Data Analysis

Bioactive FSH and LH values determined from the radioreceptor assays were normalized with respect to the protein content of the gonadotrophin preparations. Mean values together with the standard error

of the mean (s.e.m.) were calculated. Ovarian weight data and oocyte number data were each summed for pairs of ovaries to give total values per rat. Ovarian steroid concentrations were determined for each individual ovary and the values averaged for each pair of ovaries. Mean and s.e.m. values for oocyte number, ovarian weight and ovarian steroid concentrations were calculated for each treatment group.

One-way analysis of variance (ANOVA) in conjunction with the Newman-Keuls (N-K) multiple-range test was used to test the statistical significance of differences between mean values. The level of significance was set at  $P < 0.05$ . Bartlett's test was performed on data prior to ANOVA, and heterogeneity of variance was indicated on several occasions. While variances could be equalized in these instances by logarithmic ( $\ln(x + 1)$ ) transformation of the data, performing the ANOVA and N-K tests on either transformed or untransformed data did not affect the statistical conclusions reached (i.e. differences being either significant or non-significant). Therefore, in all instances the untransformed data are presented as mean values together with the s.e.m.

## Results

### *Protein Content of Gonadotrophin Preparations*

The units of all the gonadotrophin preparations differed, so protein determinations were performed to provide a common parameter. The protein contents were: Folligon  $0.3 \mu\text{g i.u.}^{-1}$ , F.S.H.-P.  $0.6 \text{ mg mg}^{-1}$  Armour standard, Folltropin  $0.5 \text{ mg mg}^{-1}$  NIH standard, and Ovagen  $32 \text{ mg unit}^{-1}$ . The NIADDK ovine gonadotrophins contained more than 85% protein per unit weight.

**Table 1. Bioactive FSH and LH concentrations of commercial gonadotrophin preparations as assessed by radioreceptor assay**  
Values are mean  $\pm$  s.e.m. for  $n = 4$ . Mean values with different letter superscripts in the same column are significantly different ( $P < 0.05$ )

	Bioactive concentration		FSH:LH ratio
	FSH ( $\mu\text{g NIADDK-oFSH-17}$ $\text{mg}^{-1}$ protein)	LH ( $\mu\text{g NIADDK-oLH-25}$ $\text{mg}^{-1}$ protein)	
Folligon	$236 \pm 13^A$	$46 \pm 2^A$	5
F.S.H.-P.	$92 \pm 5^B$	$5.0 \pm 0.4^B$	18
Folltropin	$270 \pm 19^A$	$5.5 \pm 0.3^B$	49
Ovagen	$109 \pm 7^B$	$0.1 \pm 0.01^C$	1090

### *Radioreceptor Assays*

The displacement curves for the reference standards, generated by plotting percentage of radioligand bound versus the logarithm of the concentration of standard, were linear over the ranges 0.2 to 2 ng NIADDK-oLH-25 and 2-50 ng NIADDK-oFSH-17. Displacement lines parallel to that of the LH standard were obtained with the commercial gonadotrophin preparations over the ranges 30-300 ng protein for F.S.H.-P., 1000-10 000 ng protein for Ovagen, 50-500 ng protein for Folltropin, and 2-20 ng for Folligon. Displacement lines parallel to that of the FSH standard were obtained with the commercial gonadotrophin preparations over the ranges 30-300 ng protein for F.S.H.-P., 50-500 ng protein for Ovagen, and 10-100 ng protein for Folltropin and Folligon. Table 1 shows the bioactive FSH and LH concentrations of the four gonadotrophin preparations tested in the radioreceptor assay. Bioactive FSH concentrations varied approximately 3-fold between the preparations, whereas bioactive LH concentrations varied approximately 500-fold. Folligon had the lowest FSH:LH ratio, Ovagen the highest.

### *Effect of Gonadotrophin Treatment on Oocyte Numbers and Ovarian Weights*

Preliminary studies demonstrated that while immature rats could be induced to ovulate following a single injection of Folligon, ovulation could not be induced following admin-

istration of the other gonadotrophin preparations either as a single injection or as once- or twice-daily injections at total doses of up to 0.5 mg protein. Ovulation could, however, be induced following continuous infusion of F.S.H.-P., Folltropin or Ovagen via subcutaneously implanted mini-osmotic pumps. Because of the limited available amounts of the NIADDK preparations of ovine FSH and LH, these were administered only by infusion.

A single injection of Folligon or continuous infusion of the other gonadotrophin preparations increased the mean numbers of oocytes produced in a dose-dependent fashion except at the highest doses (Fig. 1). At the highest doses, Folltropin and Ovagen produced no further increase in mean oocyte number, while the Folligon and F.S.H.-P. mean oocyte numbers fell significantly relative to the second-highest dose tested. The highest mean  $\pm$  s.e.m. numbers of oocytes produced in response to the gonadotrophin preparations were  $48 \pm 9$  for Folltropin at  $125 \mu\text{g day}^{-1}$ ,  $47 \pm 7$  for Ovagen at  $1000 \mu\text{g day}^{-1}$ ,  $31 \pm 5$

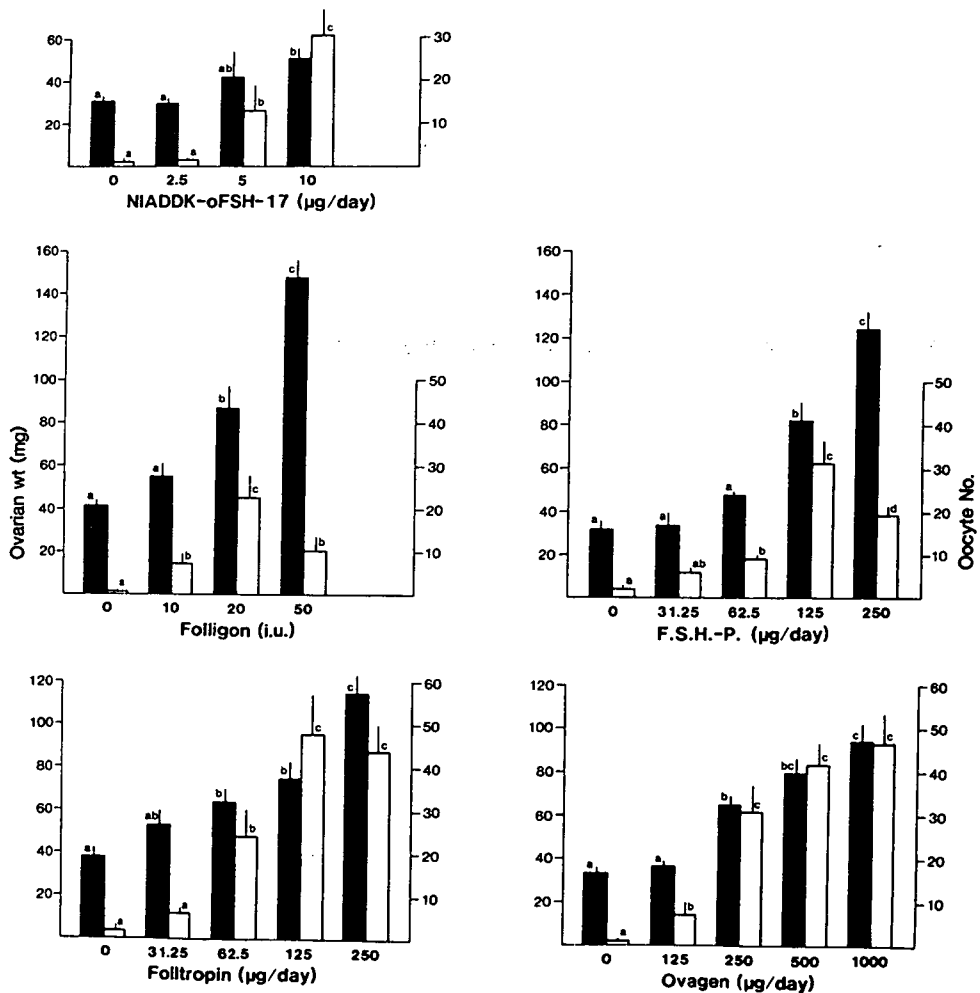


Fig. 1. Effect of gonadotrophin preparations on ovarian weights and oocyte numbers. Solid bars, mean ovarian weight; open bars, mean oocyte number. Doses are in  $\mu\text{g protein day}^{-1}$  (Folligon in i.u.). Values are means of five rats per dose, with vertical lines showing the s.e.m. Mean values with different letter superscripts indicate significant differences between mean ovarian weights or mean oocyte numbers ( $P < 0.05$ ).

for F.S.H.-P. at  $125 \mu\text{g day}^{-1}$ , and  $21 \pm 6$  for Folligon following a single injection of 20 i.u. The limited amount of highly pure NIADDK ovine FSH available for study meant that the highest dose that could be infused was  $10 \mu\text{g protein day}^{-1}$ . However, the high number of oocytes produced at this dose ( $31 \pm 6$ , mean  $\pm$  s.e.m.) was comparable to that produced by infusing  $\geq 125 \mu\text{g day}^{-1}$  of F.S.H.-P., Folltropin or Ovagen or by giving a single injection of 20 i.u. Folligon.

In addition to their effects on oocyte numbers, the gonadotrophin preparations caused a dose-dependent increase in mean ovarian weights (Fig. 1). Mean ovarian weights continued to rise significantly even at the highest doses of Folligon, F.S.H.-P. and Folltropin that had caused mean oocyte numbers to fall significantly, or remain unchanged, relative to the second-highest doses tested.

To test the possibility that the differences in response to the gonadotrophin preparations, particularly in oocyte numbers, might be related to the FSH : LH ratio of the preparations, the effect of co-infusing exogenous NIADKK ovine LH with either Ovagen or NIADKK ovine FSH was studied. The results are shown in Fig. 2. Relative to infusion with  $250 \mu\text{g}$

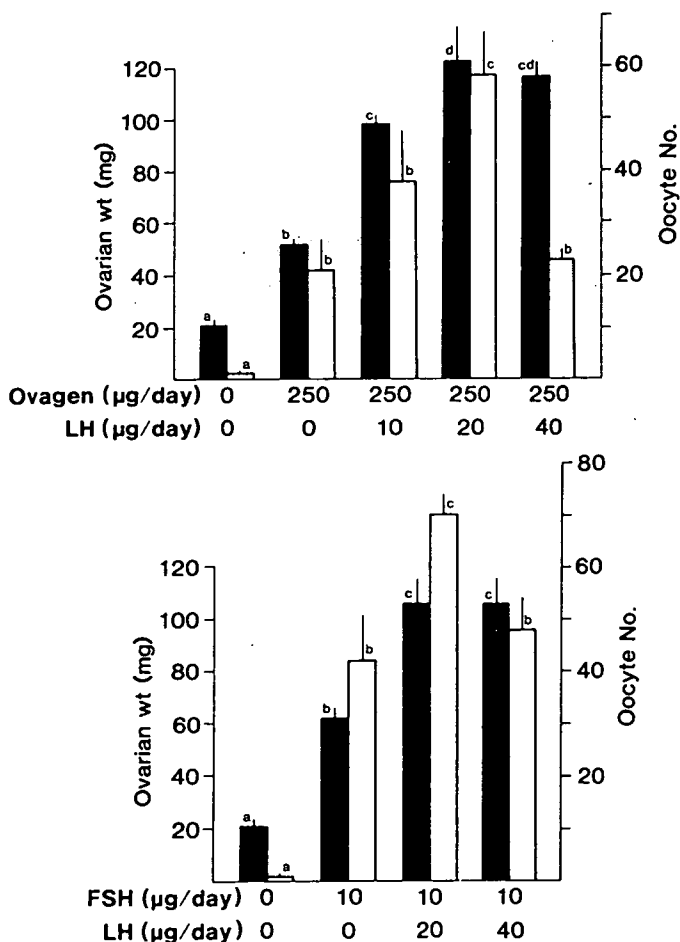


Fig. 2. Effect of co-infusion of NIADKK-oLH-25 with Ovagen or NIADKK-oFSH-17 on ovarian weights and oocyte numbers. Solid bars, mean ovarian weight; open bars, mean oocyte number. Doses are in  $\mu\text{g protein day}^{-1}$ . Values are means of five rats per dose, with vertical lines showing the s.e.m. Mean values with different letter superscripts indicate significant differences between mean ovarian weights or mean oocyte numbers ( $P < 0.05$ ).

Ovagen day<sup>-1</sup> alone, co-infusion with 10 µg LH day<sup>-1</sup> increased mean ovarian weight significantly (from 52 ± 2 to 98 ± 4 mg) and increased mean oocyte numbers (from 21 ± 6 to 38 ± 10), though this increase was not statistically significant. Increasing the dose of LH to 20 µg day<sup>-1</sup> caused a further significant increase in the mean ovarian weight (to 122 ± 13 mg) and a significant increase in mean oocyte numbers (to 58 ± 9). With the latter regime, the mean oocyte number and mean ovarian weight were comparable to those achieved with the highest dose of Follitropin (44 ± 6 oocytes, 115 ± 8 mg ovary; Fig. 1). Further increasing the dose of LH to 40 µg day<sup>-1</sup> significantly reduced the mean oocyte number to 23 ± 2 but had no significant effect on mean ovarian weight (116 ± 6 mg). These values were similar to those produced by the highest dose of F.S.H.-P. (19 ± 2 oocytes, 124 ± 8 mg ovary; Fig. 1).

Infusion of 20 µg LH day<sup>-1</sup> with 10 µg NIADDK ovine FSH day<sup>-1</sup> significantly increased both mean oocyte number and ovarian weight approximately 1.5-fold relative to infusion to FSH alone. Increasing the dose of LH to 40 µg day<sup>-1</sup> had no further effect

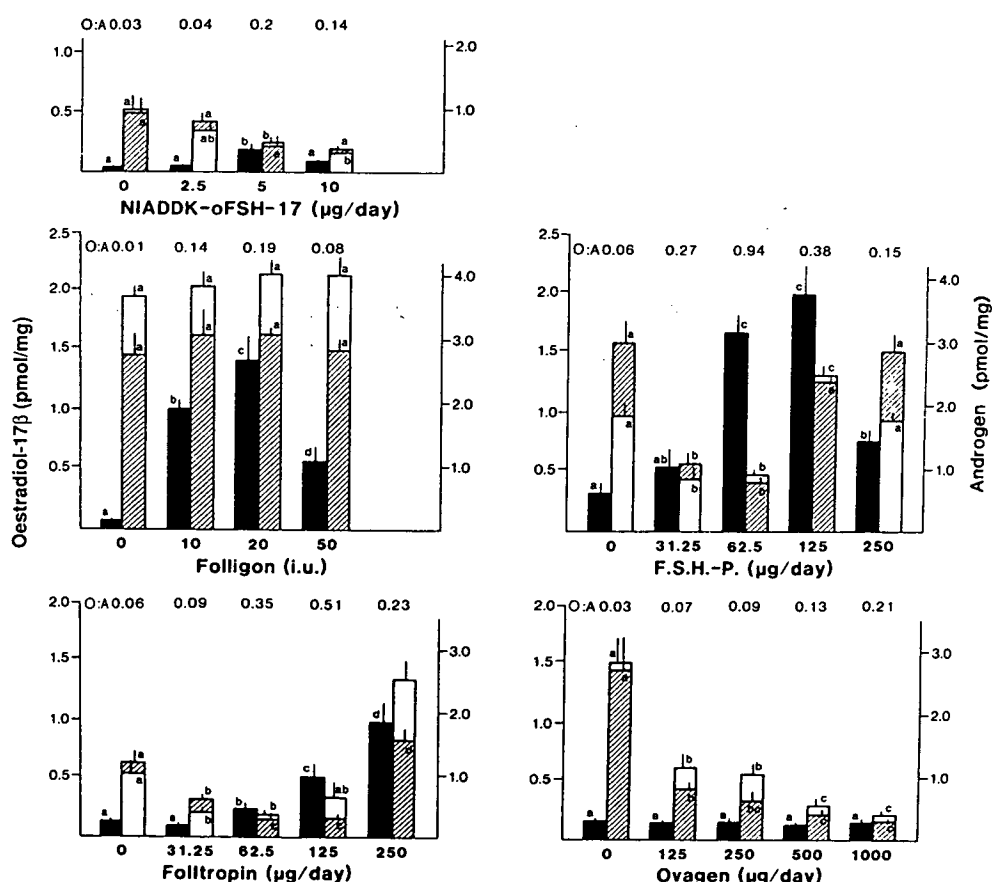


Fig. 3. Effect of gonadotrophin preparations on ovarian oestradiol-17β and androgen (androstenedione and testosterone) concentrations. Solid bars, mean oestradiol-17β; hatched bars, mean androstenedione; open bars, mean testosterone. Doses are in µg protein day<sup>-1</sup> (Folligon in i.u.). Values are means of five rats per dose, with vertical lines showing the s.e.m. For each steroid, mean values with different letter superscripts are significantly different ( $P < 0.05$ ). O:A values are the ratio of the mean ovarian oestradiol-17β concentration to the sum of the mean ovarian androstenedione and testosterone concentrations at each dose of each gonadotrophin preparation tested.

on mean ovarian weight but significantly reduced the mean oocyte number to that achieved with FSH alone. Infusion of LH on its own at doses from 10 to 40  $\mu\text{g day}^{-1}$  had no effect on mean oocyte numbers or ovarian weight relative to non-infused control rats.

*Effect of Gonadotrophin Treatment on Ovarian Steroid Contents*

Fig. 3 shows the steroid concentrations in ovarian tissue 48 h after the onset of gonadotrophin treatment. The ovarian steroid content of untreated (control) rats was inconsistent between experiments, with mean ovarian oestradiol-17 $\beta$  and androgen concentrations ranging from 0.1 to 0.3 pmol mg $^{-1}$  and from 0.8 to 3.6 pmol mg $^{-1}$  respectively. The

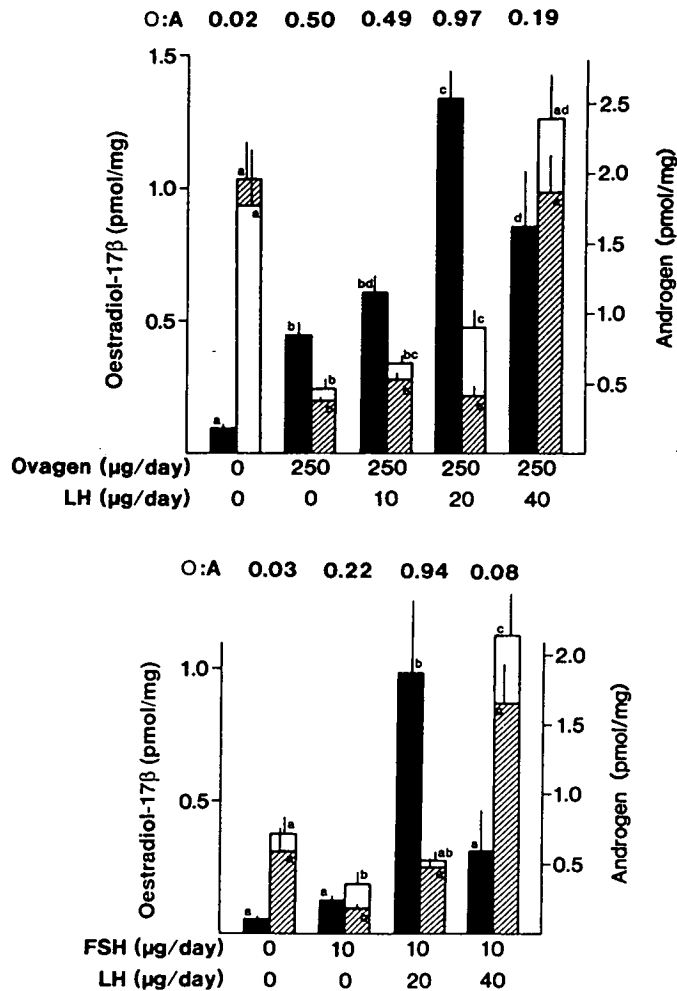


Fig. 4. Effect of co-infusion of NIADDK-oLH-25 with Ovagen or NIADDK-oFSH-17 on ovarian oestradiol-17 $\beta$  and androgen (androstenedione and testosterone) concentrations. Solid bars, mean oestradiol-17 $\beta$ ; hatched bars, mean androstenedione; open bars, mean testosterone. Doses are in  $\mu\text{g protein day}^{-1}$ . Values are means of five rats per dose, with vertical lines showing the s.e.m. For each steroid, mean values with different letter superscripts are significantly different ( $P < 0.05$ ). O:A values are the ratio of the mean ovarian oestradiol-17 $\beta$  concentrations to the sum of the mean ovarian androstenedione and testosterone concentrations at each dose tested.



reason for the between-group variability is uncertain and may reflect some inconsistency in the breeding colony from which the rats were derived. The ratio of the concentration of ovarian oestradiol-17 $\beta$  to androgen (testosterone plus androstenedione) (O : A) in the untreated (control) rats was in the range 0.01–0.06. Gonadotrophin treatment produced an increase in the O : A ratio by causing either an increase in mean oestradiol-17 $\beta$  concentration (Folligon), a decrease in mean androgen concentration (Ovagen), or increasing the oestradiol-17 $\beta$  and decreasing the androgen concentrations (F.S.H.-P., Folltropin and NIADDK ovine FSH). The highest doses of Folligon, F.S.H.-P. and NIADDK ovine FSH caused a reduction in the O : A ratio relative to the second-highest doses due to a significant fall in the mean ovarian oestradiol-17 $\beta$  concentrations. At these high doses of Folligon and F.S.H.-P., there was also a significant fall in the number of oocytes produced (Fig. 1). The highest mean ovarian oestradiol-17 $\beta$  concentrations produced in response to Folligon ( $1.4 \pm 0.2$  pmol mg<sup>-1</sup> ovary) and F.S.H.-P. ( $2.0 \pm 0.2$ ) was considerably higher than those produced in response to Folltropin ( $0.9 \pm 0.2$ ) or Ovagen ( $0.2 \pm 0.03$ ). In contrast, the highest mean numbers of oocytes produced in response to Ovagen and Folltropin were 1.5- to 2.3-fold higher than the highest mean oocyte numbers produced in response to Folligon and F.S.H.-P. (Fig. 1).

Infusion of 20  $\mu$ g LH day<sup>-1</sup> with 250  $\mu$ g Ovagen day<sup>-1</sup> caused a significant increase in mean ovarian oestradiol-17 $\beta$  and testosterone concentrations relative to Ovagen alone and a consequent increase in the O : A ratio from 0.50 to 0.97 (Fig. 4). However, increasing the dose of LH to 40  $\mu$ g day<sup>-1</sup> produced a significant reduction in the mean ovarian oestradiol-17 $\beta$  concentration but a significant increase in the mean ovarian androgen concentration relative to LH at 20  $\mu$ g day<sup>-1</sup>. The O : A ratio consequently fell from 0.97 to 0.19. Similarly, infusion of 20  $\mu$ g LH day<sup>-1</sup> with NIADDK ovine FSH (10  $\mu$ g day<sup>-1</sup>) significantly increased both mean ovarian oestradiol-17 $\beta$  and androstenedione concentrations relative to FSH alone. Again, however, 40  $\mu$ g LH day<sup>-1</sup> further significantly increased the mean ovarian androgen concentration while the mean ovarian oestradiol-17 $\beta$  concentration fell substantially.

## Discussion

Gonadotrophin preparations derived from the serum of pregnant mares (e.g. Folligon) have long been used to superovulate farm animals. However, attempts to reduce the variability of the response, increase ovulation rates, and improve embryo quality following fertilization of ovulated eggs have seen the introduction of pituitary gonadotrophin extracts (e.g. F.S.H.-P.) and, more recently, of pituitary extracts claiming a low content of LH activity (e.g. Folltropin and Ovagen). Examination by radioreceptor assays of the bioactive FSH and LH content of these four commercial gonadotrophin preparations showed a marked variation in the ratio of the concentrations of bioactive FSH to LH, with Folligon having the lowest FSH : LH ratio and Ovagen the highest (Table 1). Recent studies by Armstrong and Opavsky (1988) have shown that the numbers of oocytes produced by immature rats in response to exogenous gonadotrophins can be increased by raising the FSH : LH ratio. This was also evident in the present study to some extent. The highest mean numbers of oocytes produced in response to Folligon ( $21 \pm 6$ ) and F.S.H.-P. ( $31 \pm 5$ ), which had FSH : LH ratios of 5 and 18 respectively, were lower than those produced in response to Folltropin ( $48 \pm 9$ ) and Ovagen ( $47 \pm 7$ ), which had FSH : LH ratios of 49 and 1090 respectively. It could be argued that the low response to Folligon was influenced by its being administered as a single injection, whereas the others were infused. Although there was a 20-fold difference in the FSH : LH ratio of Folltropin and Ovagen (49 v. 1090), the highest mean numbers of oocytes produced in response to each were similar ( $48 \pm 9$  v.  $47 \pm 7$ ).

Adequate exposure to both LH and FSH is essential for follicles to mature and ovulate. Normally, because of the ever-changing relative concentrations of LH and FSH circulating

in blood, relatively few follicles are exposed to the correct gonadotrophic environment. Most are therefore destined to become atretic. By administration of exogenous gonadotrophins, the number of follicles receiving adequate gonadotrophin support to attain ovulatory maturity is raised and consequently the ovulation rate is increased. A major role of LH is to stimulate follicular production of androgens that, under the influence of FSH, can be metabolized to oestradiol-17 $\beta$ . Oestradiol-17 $\beta$  and FSH together further promote follicular development (Hillier 1985). Inadequate exposure to either LH or FSH is thus detrimental to follicular maturation. Follicular atresia can also be promoted by excessive exposure to androgens (Erickson *et al.* 1985). This situation may arise as a consequence of excessive stimulation by LH and/or inadequate exposure to FSH, thereby limiting the aromatization of androgen to oestradiol-17 $\beta$ .

Examination of the ovarian steroid environment immediately prior to induction of ovulation indicated that the gonadotrophin preparations had a marked, though varied, influence on the ovarian androgen and oestradiol-17 $\beta$  concentrations (Fig. 3). Relative to untreated (control) rats, Folligon had no effect on ovarian androgen concentrations but influenced ovarian oestradiol-17 $\beta$  concentrations. In contrast, Ovagen could influence ovarian androgen concentrations while having no effect on ovarian oestradiol-17 $\beta$  concentrations (Fig. 3). The other gonadotrophins influenced the ovarian concentrations of both androgens and oestradiol-17 $\beta$ . These differing effects may be related to the differences in the ratio of the bioactive concentrations of FSH and LH between the preparations. At the lower doses, the gonadotrophin preparations increased the O:A ratio. At the highest doses, with the exception of Ovagen, the O:A ratio was reduced relative to peak values due to either a decrease in ovarian oestradiol-17 $\beta$  content (Folligon, F.S.H.-P.) or an increase in androgen content (Folltropin). The reduction in the ovarian oestradiol-17 $\beta$  content at the highest doses of Folligon and F.S.H.-P. was also accompanied by a reduction in the number of oocytes produced. It is likely that at high doses the high LH bioactivity of these two preparations impaired normal follicular development, as indicated by the reduction in ovarian oestradiol-17 $\beta$  concentrations and subsequent fall in oocyte production. Previous studies have also shown that an excess of LH will reduce ovulation rates or oocyte or embryo numbers in sheep (Henderson *et al.* 1988), cows (Chupin *et al.* 1984; Murphy *et al.* 1984; Donaldson and Ward 1986), and rats (Armstrong *et al.* 1989; Opavsky and Armstrong 1989).

The importance of the ratio of the bioactive concentrations of FSH to LH was also shown by the co-infusion of LH with Ovagen or NIADDK ovine FSH, which are both low in LH content. At low doses of LH (10–20  $\mu\text{g day}^{-1}$ ), the mean numbers of oocytes produced increased (Fig. 2). Ovarian oestradiol-17 $\beta$  and androgen concentrations were also increased, though the increase in androgen was less marked than that of oestradiol-17 $\beta$ , producing an elevated O:A ratio. At the highest dose of LH (40  $\mu\text{g day}^{-1}$ ), the O:A ratio fell due to increased ovarian androgen but reduced oestradiol-17 $\beta$  concentrations. The mean number of oocytes produced also fell. Thus, while the presence of low amounts of LH can enhance the ovulatory response, raising the LH component too much had a deleterious effect. Similar findings have also been reported recently by Armstrong *et al.* (1989). It was of interest that by gradually increasing the amount of LH infused with Ovagen, the effects produced, in terms of increases in ovarian weight and oocyte numbers, were similar to those produced at the highest doses of first Folltropin and then F.S.H.-P. This is consistent with the FSH:LH ratios of these preparations.

Although all of the gonadotrophin preparations produced a dose-dependent increase in mean ovarian weights, the weight increase was not always accompanied by increased mean oocyte production (Fig. 1). At the highest doses of the gonadotrophin preparations, increases in mean ovarian weights were accompanied by either no change or a fall in the mean numbers of oocytes produced. Thus, bioassays based on increases in ovarian weight alone will not give a good indication of the superovulatory potency of gonadotrophin preparations. Armstrong *et al.* (1989) have also reached a similar conclusion.

While the highest mean numbers of oocytes were produced in response to Folltropin and Ovagen, they had to be administered by continuous infusion to be effective. This presumably reflects the short half-life of pituitary FSH in rats. In farm animals, too, for maximum superovulatory effectiveness, the manufacturers recommend that Folltropin and Ovagen be administered as several small doses over a period of days rather than as a single large dose. Multiple-dose regimes are, however, inconvenient to administer. Clearly, there is a need to develop long-acting or slow-release preparations of gonadotrophins.

In summary, the present study shows that the ovarian responses of immature rats is a useful additional bioassay to study the attributes of novel and established gonadotrophin preparations. However, while bioassays such as this and others (e.g. receptor assays) can provide useful information on the relative properties of gonadotrophin preparations, they cannot be used to accurately predict the ovulatory response in any particular species. Proper follow-up trials to assess superovulatory effectiveness in the species of choice remain essential. The present study demonstrated that there were marked differences in the ovarian responses to four commercial gonadotrophin preparations. These differences may be related, at least in part, to differences in the ratio of the bioactive concentrations of FSH and LH between the preparations. While the addition of LH to FSH preparations low in LH activity may enhance the ovulatory response, the addition of too much LH can be deleterious. The optimum FSH : LH ratio is, however, likely to vary from species to species of recipient animal, or even between breeds (Chupin *et al.* 1985), and it may also be dependent on the species from which the gonadotrophin is prepared.

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