

STEROID PRODUCTION BY HUMAN OVARIES IN CULTURE

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STATEMENT AS TO THE AUTHOR'S PARTICIPATION IN THE WORK SUBMITTED

The composition of this thesis is that of the author. The isolation of human Graafian follicles, aspiration of follicular fluid and collection of granulosa cells for tissue culture was carried out by the author. Techniques for 1) the in vitro culture of granulosa cells; 2) the radioimmunoassay of progesterone in peripheral plasma, culture media and follicular fluid; 3) the in vitro test of cytotoxic autoantibodies; 4) the neutralisation of endogenous gonadotrophic activity in culture medium; 5) the selective removal of endogenous gonadotrophins from serum; and 6) the determination of cell numbers were all developed solely by the author. Many studies were carried out in collaboration with other workers and their contributions have been duly acknowledged.

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Neal, P., Baker, T.G., McNatty, K.P. & Scaramuzzi, R.J. (1975) Influence of prostaglandins and human chorionic gonadotrophin on progesterone concentration and oocyte maturation in mouse ovarian follicles maintained in organ culture. *Journal of Endocrinology* (In press).

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Reproduction and Fertility (In press).

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Henderson, K.M. & McNatty, K.P. (1975). A biochemical hypothesis to explain the mechanism of luteal regression. *Prostaglandins* (In press).

Hagen, C., McNatty, K.P. & McNeilly, A.S. (1975). Immunoreactive subunits of luteinizing hormone in peripheral blood and follicular fluid throughout the menstrual cycle and their effect on the secretion rate of progesterone by human granulosa cells in tissue culture. *Journal of Endocrinology* (Submitted for publication).

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ABSTRACT OF THESIS

The morphology and function of the human Graafian follicle and corpus luteum, together with the factors which may control follicular development, ovulation and luteal regression, are reviewed.

Intact ovaries or ovarian wedge biopsies, endometria and peripheral blood samples were obtained from women (aged 21-52 years) who were at varying stages of the menstrual cycle and undergoing hysterectomy for various gynaecological conditions. Intact Graafian follicles were dissected from the ovarian specimens: the follicular fluid was aspirated for hormone assay and the granulosa cells harvested for culturing in vitro. The concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH) the α and β subunits of LH (LH α and LH β), prolactin, progesterone, oestradiol-17 β and androstenedione in peripheral plasma and follicular fluid were determined by various specific radioimmunoassays. In addition, the concentration of testosterone was measured in follicular fluid. Histological sections were prepared from Graafian follicles and corpora lutea at differing stages of the menstrual cycle. The steroidogenic potential, mitotic activity and viability of granulosa cells were examined after they were cultured in media containing varying amounts of pituitary hormone, the subunits of LH, oestradiol, prostaglandins N⁶, O² - dibutryl adenosine 3',5' cyclic monophosphate (DBC) or serum from patients with Addison's disease and premature ovarian failure.

From these procedures, it was possible to investigate the relationship between the concentrations of pituitary and steroid hormone in follicular fluid and relate them to concentrations in plasma, follicle size, stage of the menstrual cycle and the steroidogenic

potential of granulosa cells in tissue culture.

The results suggest that a precise sequence of hormonal changes occur within the microenvironment of the developing Graafian follicle: the order in which they occur may be of considerable importance for the growth of that follicle and the secretory activity of granulosa cells both before and after ovulation. These results suggest that the early appearance of FSH in follicular fluid is critical for the growth of that follicle: subsequent exposure to high levels of both FSH and oestradiol stimulates the mitotic activity of the granulosa cells and enhances their biosynthetic capacity. The final entry of significant amounts of LH into the follicle just before ovulation inhibits further mitotic activity and, together with prolactin, initiates the secretion of progesterone which can only be sustained in the presence of low physiological concentrations of FSH, LH and prolactin.

Serum from some patients with idiopathic Addison's disease and premature ovarian failure contained antibodies which together with complement were cytotoxic to granulosa cells in culture. It is suggested that in vivo, these cytotoxic antibodies may cross the blood-follicle barrier and may at least be partially responsible for the persistence of amenorrhoea in some patients with idiopathic Addison's disease and premature ovarian failure.

Prostaglandin $F2\alpha$, either alone or in combination with LH and FSH, inhibited the production of progesterone over a wide range of concentrations (1-8000 ng/ml) without affecting cell viability. By contrast PGE₂ over a similar range of concentrations markedly stimulated the production of progesterone by granulosa cells, and this was not prevented by the addition of PGF₂ α . The degree of

inhibition by $\text{PGF}_{2\alpha}$ or stimulation by PGE_2 was related to the biosynthetic capacity, that the cells had achieved prior to tissue culture.

Finally two hypotheses are proposed, the first of which may explain why only one follicle is selected for ovulation, and the second to explain a mechanism by which functional luteal regression may occur.

CHAPTER 1

GENERAL INTRODUCTION

The discovery of the ovary and its functions

Contemporary anatomists still refer to the human ovary as a rather uninteresting structure averaging about 35 mm in length, 20 mm in depth and 15 mm in thickness and weighing between 2 and 10 g. (Corner, 1947; Mossman & Duke, 1973a). It is not surprising therefore, to discover that the ancient anatomists were completely mystified as to its function since they did not appreciate that the female mammal produced eggs (Corner, 1947). In fact, it took almost 300 years from the time Andreas Versalius of Brussels first described the ovarian follicle (Versalius, 1555) until von Baer (1827) first described the mammalian egg as a very small speck inside the follicle, too small to be seen with the naked eye. Paradoxically however, it is Regnier de Graaf (1672) who gave his name to the ovarian follicle, although he and many others for years to come thought that the whole follicle was an egg. Nevertheless, de Graaf did provide the first accurate and detailed descriptions of the preovulatory follicle and the corpus luteum, which he correctly attributed to a consequence of coitus in the rabbit. Furthermore, he suggested that the number of corpora lutea provided an index of the number of embryos present. It was probably Malpighi (1697) who first suggested that the Graafian follicle never left the ovary, but served to protect the true ovum that lay within it. He also noted that the corpus luteum was glandular in nature, although he failed to appreciate that it was formed from the ruptured follicle. By the beginning of the 19th century the corpus luteum was thought to be scar tissue formed after ovulation (Prevost & Dumas, 1824) and another 70 years passed before Prenant, (1898) suggested that the corpus luteum was a gland of internal secretion releasing its products directly into the

circulation. About the same time Lataste (1886-1887) showed that changes in the vaginal epithelium of rodents correlated with the stage of follicular development.

Although Pott (1775) gave the first accurate clinical description of the consequences of ovariectomy in women and the first evidence to show that menstruation is under ovarian control, the inter-relationship between menstruation and ovarian function was not fully appreciated until the end of the 19th century (see review by Short, 1975). Thus by the beginning of the 20th century it was generally understood that both the Graafian follicle and the corpus luteum were capable of secreting substances which could influence menstruation and the vaginal epithelium.

During the early part of the 20th century, considerable debate arose as to the actual time in the menstrual cycle when ovulation takes place. Raciborski (1834) noted that girls who were married soon after a menstrual period became pregnant without menstruating again while those who married twelve or more days after menstruation invariably menstruated once more before becoming pregnant. However Pouchet (1842; 1847) thought that pregnancy could only occur if intercourse took place during or immediately after menstruation; thus his "safe period" actually coincided with the time of ovulation. Even though people slowly began to appreciate how menstruation was related in time to ovulation, it was not until the 1930's that it was realised that menstruation marked the end of a cycle, rather than a sign of the next impending ovulation (see review by Short, 1975).

In 1911, Leo Loeb, the father of American endocrinology, provided convincing experimental evidence to support an earlier suggestion by Beard (1897) that the corpus luteum controlled the time

of ovulation; Loeb showed that enucleation of corpora lutea from ovaries of guinea-pigs hastened the onset of the next oestrus. Not long after this discovery Stockard & Papanicolaou (1917) and Long & Evans (1922) some 30 years after Lataste's original observations showed that the changing cytology of vaginal smears from rodents provided a sensitive indicator of ovarian activity. These studies quickly led to the development of a suitable bio-assay system in which extracts of ovarian tissue were tested for their ability to alter the vaginal cytology of rats and mice (Allen & Doisey, 1923). In 1924, Doisey, Ralls, Allen & Johnston isolated and purified a substance from follicular fluid in sows which produced vaginal cornification when injected into rats and mice. They named this substance 'theelin' from the Greek 'thelys' meaning female, although it was later called oestrin (see review by Parkes, 1966). Before long oestradiol- 17β was isolated and purified from a pool of follicular fluid which had been obtained from four tons of sow's ovaries (MacCorquodale, Thayer & Doisey, 1937). A few years earlier Doisey in St. Louis, Butenandt in Gottingen, Laqueur in Amsterdam and Marrian in London had isolated and characterised oestrone and oestriol (see reviews by Marrian, 1966; Parkes, 1966; Short, 1975). In 1929, Corner & Allen prepared an ethanolic extract of pig corpora lutea which produced a progesterone proliferation of the endometrium in the rabbit, and in the following year, they found that the extract successfully maintained pregnancy in rabbits up to term, even after the animals had been ovariectomized only eighteen hours after mating (Allen & Corner, 1930). The speed at which endocrinology was proceeding continued unabated when in 1934 progesterone was isolated in crystalline form from luteal tissue and its structure elucidated by

four independent groups (Allen & Wintersteiner, 1934; Slotta, Rusching & Fels, 1934; Butenandt, Westphal & Cobler, 1934; Hartmann & Wettstein, 1934). In retrospect however, perhaps the most significant endocrinological discovery of these exciting years was by Makepeace, Weinstein & Friedman (1937) because they found that the administration of progesterone to rabbits inhibited ovulation. This discovery was to be of immense importance to social medicine. However sixteen years were to pass before Pincus & Chang (1953) put theory to practice and provided a method for controlling fertility, and thus "The Pill" was born.

Conceptually the endocrine control of menstruation was a problem which concerned many researchers during the early part of the 20th century. Although it was known that the regression of the corpus luteum was followed by the onset of menstruation the difficulty was to explain how menstruation could occur in the primate in the absence of a corpus luteum (Corner, 1923). Allan (1927) found that menstruation could be induced in castrated rhesus monkeys following a period of oestrogen treatment and later it was shown that progesterone could prevent oestrogen withdrawal bleeding (Smith & Engle, 1932; Engle, Smith & Shelesnyak, 1935; Hisaw, 1935). Finally the problem was solved when it was conclusively demonstrated that menstruation occurred normally after the regression of the corpus luteum but, if no corpus luteum was present, menstruation could still occur in response to a declining secretion of oestrogen (Zuckerman, 1937; Hisaw & Greep, 1938).

The control of the life span of the corpus luteum is still a problem which remains with us today and especially so in women (see review by Short, 1972). Loeb (1923; 1927) showed that hysterectomy

in the guinea-pig could prolong the life of the corpus luteum and he went on to postulate that uterine secretions may be responsible for terminating normal corpus luteum function. However almost 70 years were to pass before prostaglandin F_{2α} was identified as the uterine luteolytic hormone in sheep (see review by Goding, 1974).

Another equally important era of research began at the beginning of the 20th century; this was the relationship between the anterior pituitary and the ovary. Research in this area proceeded at considerable speed following the experiments of Carmichael & Marshall (1908) who showed that unilateral ovariectomy led to compensatory hypertrophy of the contralateral gland. In 1927, Smith at Columbia University, New York, showed that after rats were hypophysectomised, there was a marked increase in their ovarian weights after they had received implants of pituitary extract. During the following year Evans & Simpson (1928) found that alkaline extracts of pituitaries induced luteinization. These studies led to the postulate that two separate gonadotrophic factors controlled ovarian activity (see review by Chester-Jones & Ball, 1962; Greep, 1967). About the same time, two fractions of the anterior lobe of the pituitary were prepared, one with follicle-stimulating and the other with luteinizing properties (Fevold, Hisaw & Leonard, 1931; van Dyke & Wallen-Lawrence, 1933; Evans, Korpi Pencharz & Simpson, 1936). Using the very latest techniques in protein chemistry Greep, van Dyke & Chow (1942) in a classic series of experiments isolated some very highly purified preparations of FSH and LH. They then found that the administration of a highly purified FSH preparation to hypophysectomized rats enhanced follicular development but that the uterus remained atrophic as in the untreated animals. Thus it appeared

that in the absence of LH, oestrogen secretion did not occur despite the development of large follicles. These findings provided convincing evidence to suggest that pituitary hormones acted in concert (see review by Greep, 1967).

In 1941, Astwood suggested that a third gonadotrophin, luteotrophin, activated corpus luteum function in rats; this hormone was later to be known as prolactin. However, in man there was no evidence before 1961 to suggest that pituitary prolactin existed. The initial experimental evidence for the separate existence of prolactin came from in vitro cultures of human pituitaries, which in the absence of the hypothalamus produced large amounts of a substance which was distinct from growth hormone but similar to the pigeon crop-stimulating substance, i.e. prolactin (see review by Pasteels, 1973). Antibodies raised in rabbits to this substance neutralised the pigeon crop-stimulating activity of human blood collected from either lactating women or women with amenorrhoea and galactorrhoea. Finally the separate existence of human prolactin was confirmed when Lewis, Singh and Seavey (1972) successfully extracted it from human pituitary glands.

By the 1950's despite the lack of knowledge about the role of prolactin in man it was clear that the follicular phase of the ovarian cycle is dominated by the growth of Graafian follicles which secretes oestrogen and that follicular growth and secretion is controlled by the gonadotrophins. It was also clear that the luteal phase is dominated by the corpus luteum which secretes progesterone under the influence of LH and also prolactin in some species (Rothchild, 1965).

The way was then open to explore how the follicle prepared itself for the transition from an oestrogen to a progesterone

secreting gland following follicular rupture. Investigators began to examine the cell types of the follicle to examine whether steroid secretion originated from a specific cell type. Histologists had for many years postulated that the theca interna cells might produce oestrogens but the first direct evidence to support this concept came from the elegant studies of Falck (1959). He transplanted granulosa and theca interna cells into the anterior chamber of the rat's eye, together with a piece of vagina to serve as an indicator of oestrogen secretion. He observed that the granulosa cells by themselves always grew into luteal cells and failed to cornify the vaginal transplant, whilst the theca cells produced cornification provided some granulosa cells were also present. He suggested that the production of oestrogen was dependent on an interplay between the theca interna and the granulosa cells. A few years later Short (1962a; 1964a) after studying the ovarian cell types of the mare advanced the concept that the theca interna cells possessed all the enzyme systems necessary for oestrogen secretion by the follicle, and that the luteinized granulosa cells produced mainly progesterone because of their relatively inactive 17-hydroxylase and desmolase enzyme systems. Further evidence from the mare to support the earlier concept proposed by Short came from studies of Younglai & Short (1970); they found that the rate of progesterone released from follicular fluid was very slow. This observation suggested that progesterone produced by the granulosa cells was unlikely to be a suitable substrate for the theca interna cells. In addition this study drew attention to the possible importance of follicular fluid as an endocrine microenvironment in which granulosa cells could be 'programmed' for their subsequent secretory activity in the corpus luteum

However at the present time the factors influencing the maturation and biosynthetic potential of the granulosa cells within the developing Graafian follicle, and the granulosa-derived lutein cells of the corpus luteum remain obscure.

The morphology of the ovarian follicle

The changes in morphology of the follicle during its growth has been extensively reviewed (Corner, 1947; Brambell, 1956; Harrison, 1962; Maulean, 1969; Perry, 1971; Baker, 1972a; Hertig & Barton, 1973; Mossman & Duke, 1973a, b; Peters, 1973; Schwartz, 1973; Zamboni, 1974). The system of nomenclature used here to define the various stages of follicular growth is that described by Baker (1972a, b).

The PRIMORDIAL FOLLICLE (Plate 1) is the most immature stage. This follicle is separated from the surrounding stroma by a basement membrane and contains a primary oocyte in prophase of the first meiotic division (Baker, 1972b) together with a single layer of spindle-shaped cells which are the undifferentiated granulosa cells. The oocyte originates from the primordial germ cells which migrated into the embryonic gonad from the yolk sac stalk near to the developing allantois (Witschi, 1948). However, the origin of the granulosa cells is much less certain. It has been suggested that they are derived from the embryonic cells of the stroma (see review by Mossman & Duke, 1973b) and also the rete ovarii (Byskov & Lintern-Moore, 1973; Byskov & Rasmussen, 1973). Ohno & Smith (1964) have suggested that the presence of follicular cells determines the fate of the oocyte; without these cells, oocytes are unable to enter meiosis. It has also been suggested that the granulosa cells inhibit the final stages of meiosis in the oocyte (Foot & Thibault, 1969). A functional interrelationship seems to persist between the

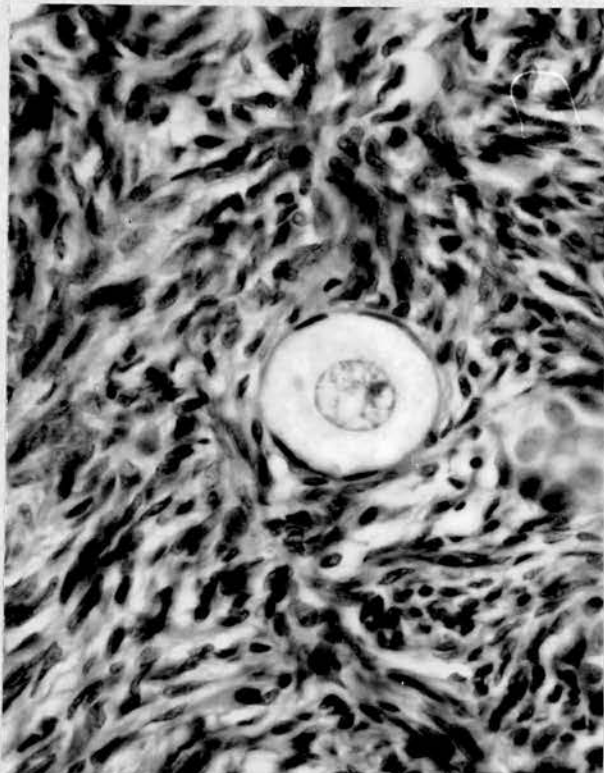


Plate 1. Human primordial follicle. Note the lack of orientation of the cells beyond the 'flattened' epithelial cells which are positioned around the circumference of the oocyte. Mag. X 580.

(Lacassagne, Duplan, Marcovich & Raynaud, 1962; Short, 1971)

Most follicles in the adult ovary are quiescent primordial follicles (Peters, Byskov & Faber, 1973a) within the cortex. When the flat, spindle-shaped cells inside the basement membrane become cuboidal (Plate 2), the follicle is then defined as a PRIMARY FOLLICLE. Successive mitotic divisions of these cuboidal cells gives rise to a multilayered lining of granulosa cells (Plate 3) and the oocyte is separated from the granulosa cells by the zona pellucida (see Plates 3 and 4). Protoplasmic processes from the adjacent granulosa cells traverse the zona pellucida and interdigitate with microvilli arising from the oocyte (see reviews, Baker, 1972a; Zamboni, 1974). Presumably the transfer of nutrients to the oocyte occurs by diffusion (Zamboni, 1974) or by pinocytosis of granulosa cell projections (Hope, 1965; Baker, 1972c).

Coincident with proliferation of the granulosa cells, adjacent stromal cells outside the basement membrane become orientated in concentric perifollicular layers (Plate 2 and 3). It is suggested that these changes are induced by the proliferating granulosa cells (Dubreuil, 1957). At the time of antrum formation the concentric layers of 'compressed' stromal cells differentiate into theca interna and theca externa. The theca interna cells assume a cuboidal shape and develop a vacuolated cytoplasm and vesicular nuclei (Harrison, 1962). Unlike the granulosa cells which are avascular the theca interna cells in the growing follicle are surrounded by an extensive capillary and lymphatic network which terminates at the basement membrane (see review, Reynolds, 1973). With continued proliferation of the membrane granulosa a confluent fluid filled space within the follicle develops which is called the ANTRUM. The follicle is now

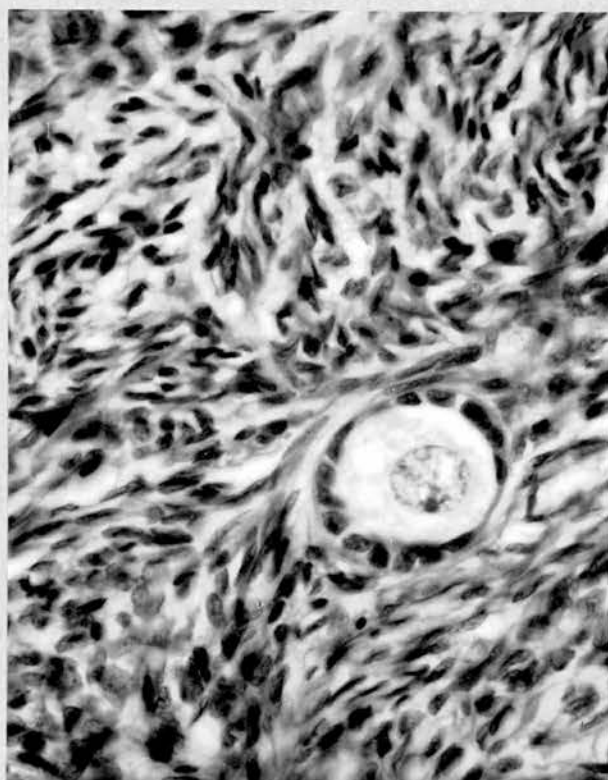


Plate 2. Human primary follicle. Note the single layer of cuboidal epithelial cells (granulosa) around the circumference of the oocyte. The cells adjacent to the granulosa are beginning to align themselves concentrically to the oocyte. Mag. X 580.

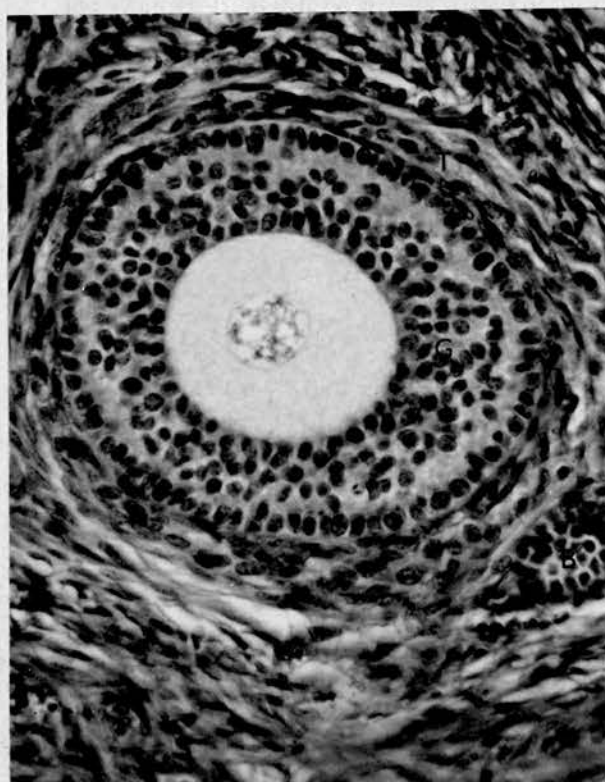


Plate 3. A primary follicle with 4-5 layers of granulosa cells around the oocyte. Note the well defined concentric layer of theca (T) cells surrounding the granulosa (G) cell layers and the prominent blood vessels (B).
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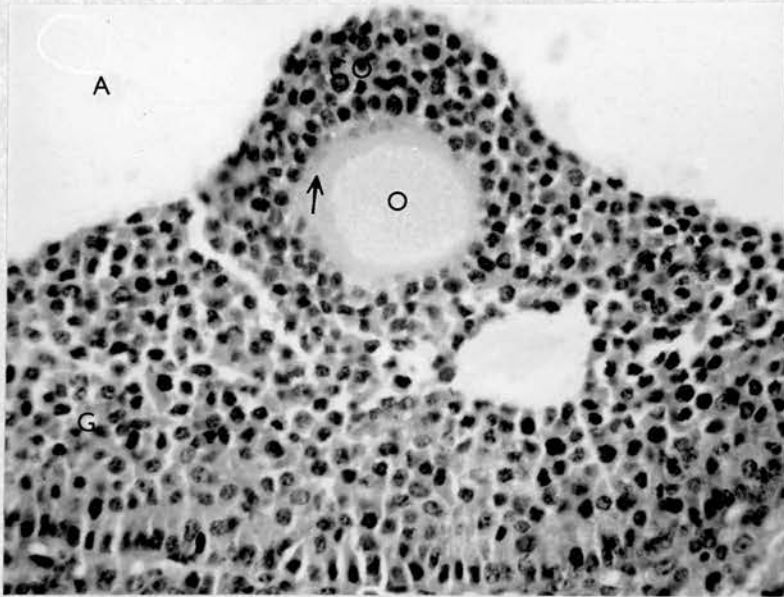


Plate 4. Section through a human Graafian follicle showing the oocyte (O), antrum (A), granulosa (G) and the cumulus oophorus cells (CO). Note the zona pellucida ('arrowed') around the oocyte. Mag. X 380.

defined as the GRAAFIAN FOLLICLE (Plate 5) and is visible to the naked eye. The antral fluid is presumed to consist of transudates of plasma and secretory products of the granulosa cells (Perloff, Schultz, Farris & Balin, 1955; Caravaglios & Cilotti, 1957; Shivers, Metz & Lutwak-Mann, 1964; Desjardins, Kirton & Hafs, 1966; Manarang & Menge, 1971; Edwards, 1974). By the time the antrum has appeared, the oocyte has enlarged to its final 'resting' size which, in the human female, is $\sim 80 \mu\text{m}$ in diameter (Green & Zuckerman, 1951; Baker, 1972a). Proliferation of the granulosa in the mouse is said to be greatly increased after the formation of the antrum (Bullough, 1942). Peak mitotic activity appears to occur during the mid to late follicular phase of the human menstrual cycle when there are rising concentrations of oestradiol in peripheral plasma (Dyrenfurth, Ferin, Jewelewicz, Warren, Rizkallah & Mikhail, 1970; Delforge, Thomas, Roux, Carneiro de Siqueira & Ferin, 1972). Granulosa cells show little sign of secretory activity during the period of intense mitotic activity; they are small and cuboidal and contain a granular cytoplasm and a densely stained nucleus (Harrison, 1962). Mitotic activity of the granulosa cells falls abruptly during the late follicular phase when there are rising concentrations of luteinising hormone (LH) in peripheral plasma; this has been attributed to high concentrations of LH and/or progesterone (Delforge et al., 1972).

The theca cells in the developing human Graafian follicle show a marked degree of hypertrophy (Solomons & Gatenby, 1924; Harrison, 1962) and an increase in histochemical activity (McKay & Robinson, 1947) indicating secretory activity. Little information however, is available on the temporal relationships between the morphology of theca cells in human Graafian follicles and their

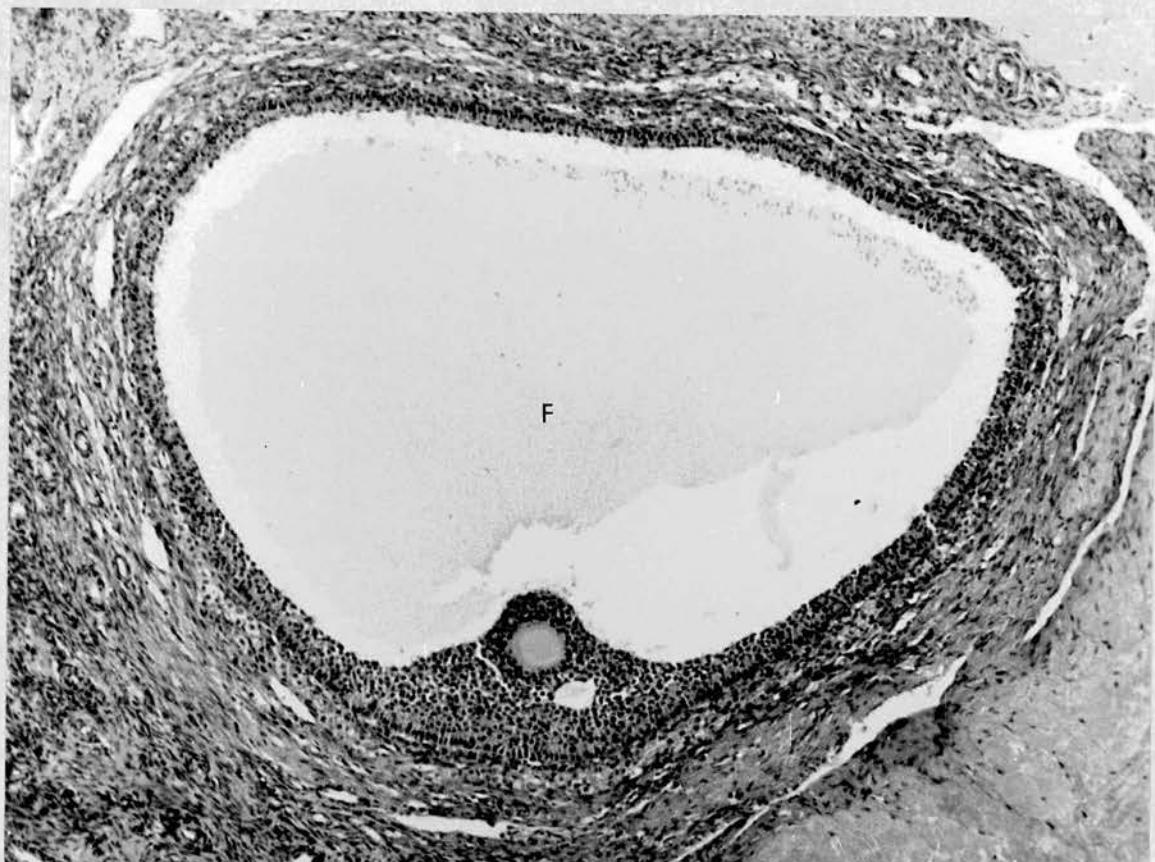


Plate 5. Human Graafian follicle. Note the size of the follicular cavity (F) in relation to the cells of the follicle. Mag. X 72.

secretory activity (Baird, Baker, McNatty & Neal, 1975).

The cells attached to the oocyte (Plate 4) remain morphologically indistinguishable from the granulosa cells until just prior to ovulation when they become columnar in shape and are called the corona radiata or cumulus oophorus cells. After ovulation these cells remain attached to the oocyte for 2 to 3 days (Harrison, 1962; Baker, 1972a); degenerative changes in these cells are a strong indication that the oocyte is atretic (Baker, 1972b).

In women it is not known how long it takes for a primordial follicle to develop into a mature preovulatory follicle whereas it takes 10 - 11 days in the guinea pig (Bland & Donovan, 1968) and rabbit (Desaive, 1947, 1948), but 26 days in the mouse (Pedersen, 1969). However, follicular development and ovulation can be induced in anovulatory women (whose ovaries previously contained no visible antral follicles) with exogenous gonadotrophins administered over a period not exceeding the follicular phase of the spontaneous ovulatory cycle (see review, Ross & Vande Wiele, 1974). Therefore, it is reasonable to assume that complete follicle maturation, beginning with a primary or a small antral follicle can be completed within 10 - 15 days, which is similar to the time taken from the primary follicle stage in the mouse (Pedersen, 1969). There is a 2 to 4 hundred-fold increase in follicle diameter (i.e. from 0.05 to 10-20 mm) and the follicle prior to rupture contains up to 4 mls of antral fluid. Similarly, the oocyte increases 4 to 5 fold in diameter from 30-40 μ m in the primordial follicle to 150 μ m in a mature preovulatory follicle (Baker, 1963; Ross & Vande Wiele, 1974).

The morphology of the preovulatory follicle

The human preovulatory follicle averages 15 mm in diameter and occupies up to 20% of the entire volume of the ovary. Little is known however about the changes which occur prior to rupture although extensive studies have been made in the following species: rodents (Blandau & Rumery, 1963; Blandau, 1955; Byskov, 1969); rabbit (Bjersing & Cajander, 1974a, b, c; Espey, 1967, 1971a, 1971b; Espey & Lipner, 1963, 1965; Espey & Rondell, 1968; Lipner, 1973; ewe (Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai, 1972; Moor, Hay & Seamark, 1975) and the mare (Short, 1964a).

In women, following the mid-cycle peak of LH in plasma, the abrupt fall in mitotic activity of the granulosa cells is accompanied by morphological changes consistent with luteinization. (Hertig, 1967; Delforge et al., 1972). Furthermore the electron microscope reveals an increase in these cells of the amount of smooth endoplasmic reticulum and mitochondria (Delforge et al., 1972; Crisp, Dessouky & Denys, 1970) and histochemically, the presence of 3β -ol steroid dehydrogenase can be demonstrated (Friedrich, Breitenecker, Sulzer & Holzner, 1974). Changes in the viscosity of follicular fluid occur as the mucopolysaccharides become depolymerised (Jensen & Zachariae, 1958). The fluid is highly viscous in preovulatory follicles in women and rhesus monkeys (Koering, 1969; Steptoe & Edwards, 1970; Butler, 1971). A general haemorrhagic appearance has been described in some human preovulatory follicles, and in those of macaques (Steptoe & Edwards, 1970; Jewett & Dukelow, 1972). There is a marked increase in capillary flow to the developing human follicle (Gillet, Kovitke, Muller & Juliens, 1968) and in the ewe a 2-fold increase in blood flow occurs some 12 to 18h before ovulation (Moor et al., 1975). Burr & Davies (1951) have suggested that

the follicle destined to ovulate not only receives the largest volume of blood in absolute terms (ml/min) but also has capillaries which are more permeable than those in other follicles. The high levels of oestrogen and LH during the preovulatory period probably have an important role in increasing the rate of blood flow to follicles (Moor et al., 1975).

The morphological changes occurring in human theca cells before ovulation are not clear although the levels of oestradiol fall in plasma and probably in follicular fluid (Edwards, 1974; Baird et al., 1975). Presumably degenerative changes occur as is the case in the sheep (Moor et al., 1975), rabbit (Bjersing & Cajander, 1974a) and rat (Byskov, 1969).

A number of hypotheses have been proposed to explain the mechanism of follicular rupture (see review, Lipner, 1973). The most recent is that based on studies in the rabbit (Lipner, 1973) and is possibly the most attractive since it integrates the many diverse changes which take place in and around the follicle following the mid-cycle 'surge' of LH. This hypothesis relates the increased rate of steroidogenesis (which will be discussed more fully elsewhere), blood flow and prostaglandins (Bjersing & Cajander, 1975) to the production of proteolytic enzymes, theca cell degeneration and increased distensibility of the follicle wall. Lipner (1973) then suggests that ovulation occurs when at constant intrafollicular pressure, the modulus of elasticity of the follicle wall approaches a minimal value, and, as the antral volume increases, wall stress achieves rupture stress.

The dynamics of follicular growth

Follicular growth from the time of completion of meiosis is a continuous process uninterrupted by pregnancy or other periods of

anovulation. Follicles grow sequentially and continue to grow until they either become atretic or ovulate (Peters, Byskov, Himelstein-Braw & Faber, 1975).

Most available evidence, based on studies in rodents, suggests that the initiation of follicular growth is independent of gonadotrophins (Pfeiffer & Hooker, 1942; Fainstat, 1968; Mauleon, 1969; Eshkol, Lunenfeld & Peters, 1970; Eshkol & Lunenfeld, 1971; Odor & Blandau, 1971; Peters & Braathen, 1973; Peters et al., 1973a). The rate at which follicles leave the non-growing pool of primordial follicles also appears to be independent of gonadotrophins (Peters, Byskov, Lintern-Moore, Faber & Andersen, 1973b). It is postulated that the order in which follicles begin to grow is related to the time of first contact with the cells of the rete ovarii (Byskov & Lintern-Moore, 1973; Byskov, 1975). This may or may not be consistent with the earlier hypothesis of Henderson & Edwards (1968) which suggests that oocytes develop in the order in which they are formed in foetal life.

Intra-ovarian mechanisms are thought to regulate the number of antral follicles present in the ovary (Vande Wiele, Bogumil, Dyrenfurth, Ferin, Jewelwicz, Warren, Rizkallah & Mikhail, 1970; Short, 1971; Peters et al., 1973a; Taylor & Short, 1973) since a reduction in the pool of non-growing follicles reduces the number beginning growth (Krarup, Pedersen & Faber, 1969; Peters Sorenson, Byskov, Pedersen & Krarup, 1970). It has also been suggested that antral fluid from degenerating follicles may reduce the number of follicles leaving the pool (Peters et al., 1973a).

Follicular development in the absence of gonadotrophins is retarded and disorganised (Eshkol et al., 1970; Eshkol & Lunenfeld,

1971; Baker & Neal, 1973) suggesting that pituitary gonadotrophins are obligatory for the maturation of follicles at all stages of growth (see reviews by Baker, 1972b; Biggers & Scheutz, 1972; Schwartz, 1973; Yoshinaga, 1973; Ross & Vande Wiele, 1974). In addition the gonadotrophins regulate the proportion of follicles that will mature and the number that will ovulate (Baker, 1972a; Peters, et al., 1975). This has been demonstrated most dramatically by the incidence of multiple births following exogenous gonadotrophin treatment in anovulatory women (Gemzell, 1965; Brown, Evans, Adey, Taft & Townsend, 1969; Brown, 1971; see Baird, 1974a, for review). The distribution and size of Graafian follicles fluctuates throughout the menstrual cycle, with the largest follicles occurring at mid-cycle and again during the mid to late luteal phase (Block, 1951a). This variation appears to be related temporally to the cyclic changes in the concentrations of the pituitary hormones (Ross & Vande Wiele, 1974).

At birth the human female has 2×10^6 oocytes (follicles) and this population declines throughout life until at menopause it is almost totally depleted (Hertig, 1944; Block, 1951b; Baker, 1973). During the period from menarche to menopause (~15 to 50 y) only a maximum of ~430 follicles will ovulate. Clearly the fate of most follicles is atresia. Conversely the process of selecting a follicle for ovulation must be very precise and depend on the interplay of several mechanisms which lie partly outside the ovary and partly within it.

Steroid secretion by the Graafian follicle

The human ovary probably secretes most of the steroids in the biosynthetic pathway from pregnenolone to oestradiol (Baird, 1971).

It has conclusively been demonstrated that the concentrations of progesterone, 17 α -hydroxyprogesterone, androstenedione, oestrone, and oestradiol-17 β are higher in the vein draining the ovary containing a large Graafian follicle than in the contralateral vein during the follicular phase (Mikhail, 1970; Baird, 1971; Lobotsky, Baird, McCracken, Weisz, Pupkin, Zanartu & Puga, 1971; Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). Neither dehydroepiandrosterone nor testosterone seem to be major secretory products of the Graafian follicle (de Jong et al., 1974), although their concentrations are higher in ovarian than peripheral venous plasma (Horton, Romanoff & Walker, 1966; Rivarola, Saez, Jones, Jones & Migeon, 1967).

The changing concentration of oestradiol-17 β in peripheral plasma during the follicular phase of the menstrual cycle provides an index of follicular growth (Baird, 1971), since more than 90% of this steroid is derived solely from actively growing follicles (Baird, 1972; Baird & Fraser, 1974a). These actively secreting follicles also contain high concentrations of oestradiol (Smith, 1960; Short & London, 1961; Giorgi, 1965; Edwards, Steptoe, Abraham, Walters, Purdy & Fotherby, 1972; Baird & Fraser, 1974b; Sanyal, Berger, Thompson, Taymor & Horne, 1974). Furthermore, during the immediate preovulatory phase there is an increase in the concentration of progesterone in follicular fluid (Edwards et al., 1972; Sanyal et al., 1974) which is also reflected in ovarian (Lloyd et al.; de Jong, 1974) and peripheral venous plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970). The high correlation between ovarian venous concentrations of oestradiol and oestrone or androstenedione suggests that these steroids arise largely from the same structures (Baird et al.,

1974; de Jong et al., 1974) and perhaps even the same cell type (Younglai & Short, 1970).

The theca cells of the human Graafian follicle can apparently synthesise de novo the complete range of ovarian steroids when incubated in vitro, whereas the granulosa cells do not appear to have the biosynthetic capacity to make oestradiol (Ryan & Petro, 1966; Ryan, Petro & Kaiser, 1968; Channing, 1969a). In addition when theca cells are cultured in vitro, they secreted quantitatively more oestradiol than progesterone while granulosa cells secreted more progesterone than oestradiol (Channing, 1969a). It is difficult however to conclude from this study that the granulosa cells actually secrete oestradiol since high concentrations of this steroid are present in follicular fluid, a trace of which may have been transferred with the cells into culture medium. Studies in the mare have shown that granulosa cells have extremely low desmolase and aromatase activity in vivo (Younglai & Short, 1970) thus it is unlikely that these cells have the capacity to secrete significant amounts of androstenedione and oestradiol-17 β . These studies are consistent with the "two-cell-type" theory proposed earlier by Short (1962a).

The synthesis of steroids by human granulosa and theca cells in vitro is increased when both cell types are incubated together, as compared to each cell type alone (Ryan et al., 1966) however this could not be confirmed by Channing (1969a). It is of interest to note that in Channing's study the cells were obtained from an ovary during the mid-proliferative phase whilst those from Ryan's study were obtained from a preovulatory follicle. These studies perhaps

highlight the importance of the "two-cell-types" in steroid secretion from the preovulatory follicle and corpus luteum rather than from the small immature Graafian follicle since in women and rhesus monkeys, the avascular granulosa cells of Graafian follicles show neither histological, histochemical nor ultrastructural indications of steroidogenic activity until just prior to rupture (Harrison, 1962; Hope, 1965; Hertig & Adams, 1967; Christensen & Gillim, 1969; Crisp & Channing, 1972; Delforge et al., 1972; Friedrich, Breitenecker, Sulzer & Holzner, 1974). The evidence obtained from a number of species including women strongly suggests that oestradiol- 17β is secreted by the theca cells and progesterone by the luteinising granulosa cells (Falck, 1959; Deane, Lobel & Romney, 1962; Short, 1964a; Younglai & Short, 1970; Seemark, Moor & McIntosh, 1974).

Why don't the granulosa cells in small Graafian follicles which are not preovulatory secrete progesterone in vivo? There is substantial evidence to suggest that granulosa cells harvested from these follicles are capable of secreting some progesterone in vitro (Channing, 1969a, 1970a, 1972). It has been assumed that granulosa cells from these follicles are inhibited from expressing their biosynthetic potential in vivo or that they are deprived of an essential nutrient or pituitary hormone. One hypothesis was that the oocyte exerts the 'inhibitory influence' which can be overcome by luteinising hormone (LH) (El-Fouly, Cook, Nekola & Nalbandov, 1970). It is difficult to interpret the results of experiments involving surgical removal of an oocyte from a Graafian follicle in vivo ('oovectomy'), since it is impossible to avoid damaging the basement membrane, with the consequent haemorrhage into the follicular cavity

and the development of follicular cysts (Hunter & Baker, 1975). This hypothesis of oocyte inhibition has not been confirmed in in vitro experiments since 'ovectomy' has no effect on the production of progesterone by murine Graafian follicles (Lindner, Tsafiriri, Lieberman, Zor, Koch, Bauminger & Barnea, 1974). In addition, granulosa cells harvested from small immature Graafian follicles of women, rhesus monkeys and mares when cultured in the absence of the oocyte will still secrete only low amounts of progesterone even in the presence of LH (see review Channing, 1970a), and the fine structure of these cells is not consistent with functional or structural luteinization (Crisp & Channing, 1972). It is likely therefore that granulosa cells from small follicles do not secrete large amounts of progesterone because they do not have the biosynthetic capacity (Channing, 1970a) and are devoid of receptors for LH (Channing, 1972, 1975; Channing & Kammerman, 1973a; Zeleznik, Midgely & Reichert, 1974). It is also possible that they may be deprived of LH which is perhaps unable to cross the blood-follicle barrier in appreciable amounts until the mid-cycle 'surge'. Human adrenal cortical cells in culture medium devoid of adrenocorticotrophic hormone (ACTH) proliferate actively and secrete only low amounts of steroid; however, in the presence of ACTH, increased secretion occurs and cell proliferation is arrested (O'Hare & Munro-Neville, 1973a, b). The measurement of LH in follicular fluid may help to confirm whether similar changes occur within the Graafian follicles

The pituitary-ovarian axis in relation to follicular development

During the last decade considerable advances have been made in our understanding of the interaction between the ovary and the

pituitary (see reviews by Gemzell, 1965; Rothchild, 1965; Short, 1967, 1972b; Ross, Cargille, Lipsett, Rayford, Marshall, Strott & Rodbard, 1970; Vande Wiele et al., 1970; McCracken, Baird & Goding, 1971; Knobil, Dierschke, Yamaji, Karsh, Hotchkiss & Weick, 1972; Baird, 1974a;; Barraclough, 1974; Yoshinaga, 1974).

Since the development and secretory activity of a follicle is totally dependent on the pituitary gonadotrophins, the selection of a developing follicle for ovulation must relate to the changes in their concentrations throughout the ovarian cycle. In women it is believed that a primary follicle is stimulated to further growth by rising concentrations of plasma FSH (and to a lesser extent LH) which occur during the late luteal phase of the previous cycle, when the secretion of oestradiol and progesterone from the regressing corpus luteum ceases (Ross et al., 1970; Ross & Vande Wiele, 1974). By the 6th or 7th day following the onset of menstruation a significant increase in plasma oestradiol is detectable and the developing follicle is clearly established as the major source of ~~this steroid~~ (Baird & Fraser, 1974a). The rise in the secretion of oestradiol inhibits FSH by negative feedback, so that the concentration of this gonadotrophin in peripheral plasma falls progressively (Midgely & Jaffe, 1968). Despite falling levels of FSH in plasma, follicular maturation is probably unaffected since the rising concentrations of oestradiol may enhance the sensitivity of the follicle to FSH (Bradbury, 1961; Goldenberg, Vaitukaitis & Ross, 1972a). Moreover when the level of FSH in peripheral plasma is falling, that of LH is actually rising slightly. The reason for this is obscure but it may be due to the differential sensitivity of the hypothalamus and/or the anterior lobe of the pituitary to the feedback effects of oestrogen. The secretion of FSH is more

sensitive than that of LH to the negative feedback effect of oestrogen in both men (Kulin & Reiter, 1972) and women (Cargille, Vaitukaitis, Bermudez & Ross, 1973). The basal level of LH is probably important for maintaining the secretion of oestrogen and androstenedione by the theca cells of the follicle. In sheep, LH is known to stimulate the production of oestradiol and androstenedione by the follicle in vivo (Baird, Goding, Ichikawa & McCracken, 1968; McCracken, Uno, Goding, Ichikawa & Baird, 1969), and in vitro (Moor, 1973). It would seem that a certain minimal concentration of LH is probably required for steroidogenesis to occur in ovarian cells.

During the mid to late follicular phase there is a marked increase in the size of the largest Graafian follicle in both women (Block, 1951a) and rhesus monkeys (Koering, 1969) at a time when there is a sharp increase in the concentration of oestradiol in plasma (Ross & Vande Wiele, 1974). These two events are probably causally related since the exogenous administration of oestradiol early in the menstrual cycle elicits a discharge of LH, which results in either anovulation or a shortened luteal phase probably because the follicle was not sufficiently mature (Tsai & Yen, 1971; Vaitukaitis, Bermudez, Cargille, Lipsett & Ross, 1971; Cargille et al., 1973). Thus as the follicle matures and the secretion of oestradiol reaches between 200 and 300 μ g per day the cyclic centre in the hypothalamus is stimulated by positive feedback (Harris & Naftolin, 1970) and the mid-cycle release of LH and FSH occurs (Knobil, 1973). Although both progesterone (Swerdloff & Odell, 1969; McCann, Kalra, Donoso, Bishop, Schneider, Fawcett & Krulich, 1972; Leyendecker, Wardlaw & Nocke, 1972) and oestradiol

(Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969; Taleisnik, Caligaris & Astrada, 1969; Knobil et al., 1972; Leyendecker et al., 1972) can elicit an acute release of LH, progesterone is unlikely to be involved in the preovulatory 'surge' since its plasma concentrations do not precede those of LH in women (Neill, Johansson, Datta & Knobil, 1967; Vande Wiele et al., 1970; Thomas, Hertogh, Pizzaro, van Exter & Ferin, 1973), rhesus monkeys (Monroe, Atkinson & Knobil, 1970), rats (Barracough, Collu, Massa & Martini, 1971) or sheep (A. G. Wheeler, R. B. Land, D.T. Baird & R.J. Scaramuzzi, unpublished observations). By contrast, the circulating levels of oestradiol clearly and consistently rise several days before the initiation of the LH 'surge' in women (Vande Wiele, et al., 1970) and in the rhesus monkey (Hotchkiss, Atkinson & Knobil, 1971) and the neutralisation of circulating oestrogen with antisera to oestradiol effectively blocks the LH 'surge' in both rats and rhesus monkeys (Ferin, Tempone, Zimmering & Vande Wiele, 1969; Cowchock, Ferin, Dyrenfurth, Carmel, Zimmerman, Brinson & Vande Wiele, 1971).

During the LH 'surge' in women the plasma levels of progesterone rise (Johansson & Wide, 1969; Yussman & Taymor, 1970; Thomas et al., 1973) and this may have a role in facilitating the continued discharge of LH (Leyendecker et al., 1972). High levels of progesterone similar to those found during the luteal phase will however inhibit the positive feedback of oestradiol (sheep, Goding et al., 1969; monkey, Diershke, Yamaji, Karsch, Weick, Weiss & Knobil, 1973; women, Leyendecker et al., 1972). Knowledge is lacking with regard to the possible effects of other ovarian steroids on the release of gonadotrophins although preliminary evidence from the ewe suggests that

androstenedione may influence the release of LH (Martensz, Scaramuzzi, Van Look & Baird, 1975). The increased secretion of androgens (Abraham, 1974) at mid cycle and during the luteal phase may also be important for the maintenance of sexual libido in primates (Everett & Herbert, 1971).

Although the rapidly maturing follicle dictates the timing of the gonadotrophin discharge, it is the pituitary which is the final regulator since high levels of LH terminate the secretion of oestradiol (Moor, 1974) and initiate the production of progesterone from the granulosa cells which are undergoing hypertrophy at this time (Delforge *et al.*, 1972).

The action of FSH and LH on the Graafian follicle until recently remained obscure. Studies of the uptake of biologically active iodine-labelled (¹²⁵I) pituitary hormones, and measurement of the rate of oxygen consumption following exogenous gonadotrophin stimulation of the rat or pig have produced conflicting results. Luteinizing hormone does not bind to porcine granulosa cells until the immediate preovulatory stage (see Channing, 1975 for review) although it is retained by the plasma membrane of the theca cells in the rat (Rajaniemi, Hirschfield & Midgely, 1974; Hans, Rajaniemi, Cho, Hirschfield & Midgely, 1974). By contrast, FSH binds to granulosa cells in the rat but not to theca cells (Midgely, Zeleznik, Rajaniemi, Richards & Reichert, 1973; Zeleznik & Midgely, 1973). Paradoxically however, LH stimulates oxygen consumption by rat granulosa cells, whilst FSH stimulates consumption by the theca cells (Ahren, Hamberger & Rubinstein, 1969).

The role of prolactin in the control of follicular growth and steroidogenesis remains uncertain (Knobil, 1973) although it has

been known for some time that women with high plasma levels of prolactin and apparently normal levels of LH and FSH commonly have amenorrhoea (McNeilly, 1974). The reduction of these high prolactin levels leads to the restoration of normal menses; thus the possibility remains that prolactin has a direct effect on the follicle, although most of the evidence is against this (Vande Wiele, et al., 1970).

Much is known about the dynamics of follicular growth and the levels of hormones in plasma and urine throughout the menstrual cycle, and yet we know very little about how the gonadotrophins act on the follicle to prepare it for subsequent luteinization, or indeed why only one follicle is selected for ovulation. Some answers to these questions may be provided by examining the role of the gonadotrophins within the follicular fluid of the developing and regressing Graafian follicles. Furthermore, by relating the steroidogenic potential of human granulosa cells in vitro to a) the hormones which they were previously exposed to in follicular fluid and b) those in peripheral plasma, it may be possible to relate the maturation of the follicle to its endocrine environment.

The morphology of the corpus luteum

The corpus luteum is formed as a consequence of follicular rupture. The newly formed corpus luteum is characterised by marked vascularization of the granulosa cells and a considerable amount of haemorrhage into the follicular cavity. Changes suggestive of luteinization of the membrana granulosa can be demonstrated before rupture of the follicle in women and these are almost certainly induced by LH (White, Hertig, Rock & Adams, 1951; Hertig, 1967;

Delforge et al., 1972). During follicular rupture, whilst the granulosa cells are undergoing hypertrophy, the theca cells are degenerating quickly (Mossman & Duke, 1973a). However, within three to five days the immediately adjacent stromal cells undergo hypertrophy and form the paraluteal cells (the theca lutein cells of human corpus luteum; Corner, 1956; Crisp, Dessouky & Denys, 1970). The fine structure of the granulosa lutein cell differs from that of the paraluteal cell in that they have a more homogenous and electron-lucent nuclear matrix with more abundant mitochondria, isolated Golgi complexes and granular and agranular endoplasmic reticulum (Crisp et al., 1970). The paraluteal cells remain as a definitive cluster of cells around the periphery of the gland and also in 'folds' or 'streaks' of tissue which have penetrated deeply into the dispersed layers of granulosa lutein cells, during follicular rupture. According to Corner (1956) capillaries invade the granulosa and finally reach the cavity between Days 2 and 4 after ovulation. A few mitoses are seen in the granulosa and paraluteal cells before Day 4, but none afterwards. Haemorrhage into the follicular cavity occurs regularly at ovulation and again from Day 9 to menstruation. Capillary dilation reaches a peak on Day 7 and thin walled venules appear along the border of the antral cavity. By Day 8 the gland has reached its secretory peak and a definitive connective tissue layer lines the central cavity.

In 1911, Meyer described the human corpus luteum of the cycle as showing 'full bloom' or maturity between the 8th and 10th Day following ovulation which probably covers the time when the blastocyst implants (Harrison, 1962). During a non-pregnant cycle,

cell shrinkage and vacuolation occurs from Days 9 to 12 and degenerating nuclei are seen from Day 14. The subsequent rate and degree of cellular degeneration is said to be variable (Corner, 1956). Degeneration of the corpus luteum results in a distinctive corpus albicans which is composed of a dense 'cottony' mass of very fine collagenous fibres with very few cells. In women the functioning corpus luteum is red in colour and when regressing the colour goes to pale yellow and then finally to white (Mossman & Duke, 1973a).

Whilst there is no doubt as to the origin of the granulosa lutein cell, there remains some controversy as to whether paraluteal cells are derived from stromal cells incorporated into the tissue folds as the follicle collapses following rupture, or whether they are 'regenerated' theca interna cells which did not regress completely during or just prior to follicular rupture (see review, Mossman & Duke, 1973b). According to Mossman & Duke, new luteal cells commonly form from the undifferentiated thecal and stromal cells immediately adjacent to the corpus luteum. These differentiating cells are said to be the paraluteal cells, which can usually be seen to be located centrally with the granulosa lutein cells and peripherally with undifferentiated stromal cells.

The presence of the paraluteal cell in the human corpus luteum may be of considerable importance since in the ewe (Harrison, 1948) and cow (Hansel, Concannon & Lukaszewska, 1973) these cells are much less prominent. This may account for the differences in the biosynthetic potential of the corpus luteum between women and sheep (Baird et al., 1975).

Steroid secretion by the corpus luteum

The human corpus luteum is almost unique amongst mammals in synthesizing and secreting androgens and oestrogens as well as prostagens (Savard, March & Rice, 1965). At least 8 steroids, pregnenolone, progesterone, 17α -hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, oestrone and oestradiol- 17β have all been isolated and characterized as conversion products following incubations of slices of human corpora lutea in vitro with radioactively labelled acetate (Huang & Pearlman, 1963; Savard et al., 1965). Although all eight steroids have also been found in higher concentrations in ovarian venous than in peripheral venous blood (Baird, 1971), the most important secretory products appear to be progesterone, androstenedione and oestradiol (Mikhail, 1970; Lloyd et al., 1971). The secretion of oestradiol by the corpus luteum is responsible for the peak concentrations of this hormone in plasma during the luteal phase of the menstrual cycle (Brown, 1971; Abraham, 1974; Baird & Fraser, 1974a). As the corpus luteum develops to full maturity about 8 days after ovulation there is a marked rise in the secretion of both oestradiol (3 fold) and progesterone (~ 20 fold) with a smaller rise in androstenedione (1.5 fold) (Abraham, 1974).

The cellular origin of these steroids (paraluteal versus granulosa lutein cells) is difficult to identify because of the difficulty in separating these cell types from the intact gland. Although theca cells from the Graafian follicle synthesize and secrete virtually every steroid in the biochemical pathway from pregnenolone to oestradiol in vitro, granulosa cells probably do not secrete oestradiol (Ryan & Petro, 1966; Ryan et al., 1968;

Channing, 1970a); there is compelling evidence to suggest that when both cell types are recombined there is a marked quantitative increase in steroidogenesis (Ryan et al., 1968). These findings taken together with the 'two-cell-type' hypothesis proposed by Short (1962a) suggest that the theca (or stroma) and granulosa derived cells of the corpus luteum may each secrete the same steroids as the isolated cells of the Graafian follicle in vitro but there may be marked quantitative differences in output.

It is of interest to compare the secretory products of the human corpus luteum to those of the rhesus monkey and the ewe since the mechanism of luteal regression in primates probably differs from that in other mammals (Neill, Johansson & Knobil, 1969; Baird, 1973). The secretion of oestradiol by the corpus luteum of the rhesus monkey must be small since there is a relatively small peak in plasma during the luteal phase (Hotchkiss et al., 1971). Nevertheless it is significant that in the rhesus monkey the concentration of oestradiol in ovarian venous plasma draining the ovary containing the corpus luteum is higher than on the contralateral side (Weiss, Dierschke, Karsh, Hotchkiss, Butler & Knobil, 1973), indicating some secretion by the corpus luteum. By contrast, the corpus luteum of the sheep does not have the capacity to synthesise oestrogen when incubated in vitro (Kaltenbach, Cook, Niswender & Nalbandov, 1967) and in vitro it secretes only progesterone and 20 α -dihydroprogesterone (Short, 1964a; Baird, McCracken & Goding, 1973). It has been clearly established in the sheep that the secretion of oestradiol

and androstenedione throughout the luteal phase originates from the Graafian follicles (Baird et al., 1973). In addition the secretion of oestradiol as measured by cannulating the utero-ovarian vein in situ is not significantly different in the ovary containing the corpus luteum to that from the contralateral ovary (D. T. Baird & R. J. Scaramuzzi; unpublished results).

Thus the pattern of steroid secretion by the human corpus luteum is clearly different to that of the sheep. In the latter, oestradiol originates entirely from the Graafian follicle with the corpus luteum secreting progestins while during the luteal phase in women the follicle makes insignificant contributions to the total ovarian secretion of oestradiol, over 90% of which originates from the corpus luteum (Baird & Fraser, 1974a).

Although there are now numerous descriptive accounts of the changing hormone levels in blood and urine throughout the menstrual cycle, we still do not really understand which factors are responsible for the maintenance of the corpus luteum in women. This is an area of great potential interest for the development of new forms of contraception. Luteinizing hormone and prolactin together constitute at least part of the lutetrophin complex necessary for the maintenance and secretory activity of the corpus luteum in the rat (Evans, Simpson, Lyons & Turpeinen, 1941; Astwood, 1941), mouse (Kovacic, 1964), rabbit (Spies, Hilliard & Sawyer, 1968), hamster (Greenwald & Rothchild, 1968), ferret (Donovan, 1963), pig (Du Mensil du Buisson, 1973) and sheep (Denamur, Martinet & Short, 1973). Recent evidence suggests however, that prolactin may have little to do with luteal function in women (Dyrenfurth et al., 1970; Huang, Guyda & Friesen, 1971; Midgley & Jaffe,

1972; McNeilly, Evans & Chard, 1973; Robyn, Delvoe, Nokin, Vekemans, Badawi, Perez-Lopez & L'Hermite, 1973; Tyson & Friesen, 1973). Perhaps the most convincing argument against the involvement of prolactin in luteal function is that in hypophysectomized women the corpus luteum can be maintained with LH alone (Vande Wiele et al., 1970). Nevertheless in this study the levels of prolactin in plasma were not determined and since it is virtually impossible to remove all the pars tuberalis in women (Purves, 1966; Daniel & Prichard, 1972) it is likely that significant levels of prolactin remained in plasma. Thus it is still possible that prolactin could play a 'permissive' role as in other species, where alterations in circulating prolactin levels within limits, have little effect on luteal activity.

It is also generally assumed that during follicular rupture and corpus luteum formation, FSH is not required for either the initiation or maintenance of steroidogenesis (see Nalbandov, 1973 for review). It has been strongly suggested that FSH is involved in the formation of receptors for LH as the granulosa cells mature within the developing follicle (Channing, 1975; Zeleznik, Midgley & Reichert, 1974) although conclusive evidence is still required. Nevertheless during a fertile cycle when it is presumed that the corpus luteum is required to function for longer than 12 days it is essential that receptors for LH or human chorionic gonadotrophin (HCG) are maintained. Thus like prolactin FSH may have a 'permissive' role in luteal maintenance. It is suggested that until conclusive proof is demonstrated for the integrity of corpus luteum function in the total absence of a pituitary gonadotrophin it is probably

safer to infer that all three gonadotrophins are required, as has been demonstrated in the hamster (Greenwald & Rothchild, 1968). The demise of the human corpus luteum during the non-fertile menstrual cycle.

It is somewhat surprising to find that we still do not know how the human corpus luteum regresses during a non-fertile menstrual cycle (see review by Short, 1972a); it is not known whether there is a luteolytic substance, or indeed how it might act. To have control over the life-span of the corpus luteum is of considerable interest since it opens up new possibilities of fertility control.

It has been suggested that the luteolytic substance in women may well be prostaglandin $F2\alpha$ ($PGF2\alpha$) (Baird, 1973; Powell, Hammarstrom & Samuelsson, 1974a) and that it is produced locally within the corpus luteum rather than in the uterus as is the case in the ewe (Wiltbank & Casida, 1956; Goding, 1974). High concentrations of $PGF2\alpha$ have in fact been found in the ovaries of rhesus monkeys (Wilks, Forbes & Norland, 1972). Although women appear to have a different pattern of venous drainage (Goding, Cumming, Chamley, Brown, Cain, Cerini, Findlay, O'Shea & Pemberton, 1972; Ginther, Diershke, Walsh & del Campo, 1974) when compared to the sheep (Dobrowolski & Hafez, 1970) 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 (Whitelaw, 1958; Brown, Keller & Mathew, 1959; Beavis, Brown & Smith, 1969; MacRae & Mohammedally, 1969; Beling, Marcus &

Markam, 1970; Doyle, Barclay, Duncan & Kirton, 1971; Fraser, Baird, Hobson, Michie & Hunter, 1973). It is difficult to imagine how the release of prostaglandins which are present in high concentrations in the endometrial lining of the uterus (Pickles, 1957; Downie, Poyser & Wunderlich, 1974) can continue to be released into the uterine vein during the late luteal phase when 'sloughing' of the endometrium is taking place. There is circumstantial evidence to suggest that oestrogen has an important role in controlling the life span of the corpus luteum in women (Rivera & Shearman, 1969), sheep (Nalbandov, 1973) and the rat (Christiansen, Keyes & Armstrong, 1970) since X-irradiation of the ovary at a dose which destroys Graafian follicles and perhaps also the theca-lutein cells of the human corpus luteum, removes the source of oestrogen and prolongs the life of the corpus luteum. By contrast, implantation of oestrogenic substances into the ovary containing a corpus luteum in women (Hoffman, 1960) or to either ovary in the ewe (Cook, Karsch, Foster & Nalbandov, 1973) during the mid-luteal phase significantly shortens the length of the luteal phase.

It is of interest to examine the experimental data from the sheep more closely to see how oestradiol can influence the life span of the corpus luteum, because in the ewe, oestradiol originates from developing follicles but in women during the luteal phase it originates from the corpus luteum. Oestrogen can release significant amounts of PGF₂α from the uterus in sheep (Caldwell, Tillson, Brock & Speroff, 1972; Scaramuzzi, Boyle, Wheeler, Land & Baird, 1974) although the uterus has to be primed with progesterone before the maximum effect is seen (Caldwell et al., 1972; Scaramuzzi et al., 1974). In the ewe there is a 2 to 5 fold increase in the

concentration of PGF₂ α in utero-venous plasma on Days 12-13 when Progesterone starts to decline (Baird & Scaramuzzi, 1975); this prostaglandin secretion is presumably a consequence of progesterone priming of the uterus. By Days 15 to 16 there is a dramatic rise in utero-ovarian concentrations of both oestradiol (5-fold) and PGF₂ α (10-fold) and these events appear to be causally related (Scaramuzzi, et al., 1974; Baird & Scaramuzzi, 1975). Although the falling levels of progesterone are accompanied by some structural changes in luteal cells from Day 13 onwards (Deane, Hay, Moor, Rowson & Short, 1966; Bjersing, Hay, Moor, Short & Deane, 1970), functional regression can still be halted by hysterectomy (i.e. removal of PGF₂ α) as late as Day 15 (Moor, Hay, Short & Rowson, 1970). Thus it appears that functional regression occurs prior to structural degeneration of the gland. It is probably the massive amount of PGF₂ α , released as a consequence of rising levels of oestradiol from the developing Graafian follicles, which induces the final irreversible morphological and functional demise of the ovine corpus luteum. Thus in the ewe, the progesterone from the corpus luteum and oestradiol from the developing follicles influence both the synthesis and release of uterine prostaglandins which in turn induces luteal regression. There is convincing experimental evidence to suggest that in sheep the uterine prostaglandins act on the corpus luteum by travelling directly to the ovary via the utero-ovarian vein and ovarian artery (see review by Baird & Scaramuzzi, 1975). Transplantation of the ovary in the sheep to the neck, leaving the uterus in the abdomen results in luteal maintenance, whilst transplantation of the ovary and uterus together to the neck results in normal luteal regression

(McCracken, Caldwell, Tillson, Thorneycroft & Scaramuzzi, 1969). This suggests that prostaglandins are unlikely to be sufficiently concentrated and/or able to survive long enough in the peripheral circulation to exert its effect. The most convincing evidence to support these results is that peripheral infusion of $\text{PGF}_2\alpha$ even at extremely high doses are unable to cause luteal regression in sheep (McCracken, 1971). The negative results with systemic infusions of $\text{PGF}_2\alpha$ could therefore be attributed partly to a dilution effect and partly to the rapid metabolism of $\text{PGF}_2\alpha$ in blood and especially by the lungs (Piper, Vane & Wyllie, 1970; Granstrom, 1972). It is perhaps not surprising therefore to find that attempts to induce luteal regression in women by short-term intravenous infusions have been unsuccessful (Hillier, Dutton, Corker, Singer & Embrey, 1972; Jones & Wentz, 1972; Jewelewicz, Cantor, Dyrenfurth, Warren & Vande Wiele, 1972; Lehmen, Peters, Breckwoldt & Bettendorf, 1972; Le Maire & Shapiro, 1972; Wentz & Jones, 1973). It is also possible that the human corpus luteum is refractory to the luteolytic effects of $\text{PGF}_2\alpha$ during much of its life as is the case in the pig (C. Polge, personal communication). Although PGE_2 is used as a therapeutic abortifacient in women (see Csapo, 1974) it is unlikely to be the luteolytic hormone since it has been shown to mimic the actions of trophic hormones in both primate and non-primate ovarian tissue. Prostaglandin E_2 stimulates the production of progesterone by the human corpus luteum (March & Le Maire, 1974), bovine corpus luteum (Speroff & Ramwell, 1970), rat follicle (Lindner et al., 1974), mouse follicle (Neal, Baker, McNatty & Scaramuzzi, 1975) and also monkey and porcine granulosa cells (Channing & Crisp, 1972; Kolena &

Channing, 1972).

The human and ovine corpora lutea have plasma membrane receptors for $\text{PGF}_2\alpha$ (Powell et al., 1974a, b), however the human corpus luteum unlike the sheep secretes both oestradiol and progesterone. Thus the most attractive working hypothesis to explain luteal regression in women is that progesterone and oestradiol produced within the corpus luteum induces $\text{PGF}_2\alpha$ production by the corpus luteum, which in turn causes luteolysis.

The exact mechanism by which $\text{PGF}_2\alpha$ exerts its lytic effect in non-primates is obscure, although several have been postulated, including vascular effects. One suggestion was that $\text{PGF}_2\alpha$ caused a constriction of the utero-ovarian vein which reduced the ovarian flow and thus luteolysis was a consequence of anoxia (Pharris, 1970). However many experiments have now shown that luteolysis does not arise as a result of reduced total ovarian blood flow (Baird, 1974b; Bruce & Hillier, 1974; Jansen, Albrecht & Ahren, 1975) and Jansen et al., (1975) have shown that the observed reduction in ovarian blood flow following $\text{PGF}_2\alpha$ administration is probably an artifact resulting from a 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 blood flow to the corpus luteum and a subsequent increase in blood flow to the follicles and interstitial tissue (Jansen et al., 1975). 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 luteal tissue in vitro (Speroff & Ramwell, 1970;

Behrman, Yoshinaga & Greep, 1971a) thus seeming to rule out the possibility of a direct luteolytic action on the luteal cell. Although some kind of vascular effect, such as vascular redistribution within the ovary still seems plausible, there are now convincing reports of $\text{PGF}_2\alpha$ inhibiting the secretion of progesterone by luteal tissue in vitro (Caldwell, Auletta, Gordon & Speroff, 1972; O'Grady, Kohorn, Glass, Caldwell, Brock & Speroff, 1972).

Granulosa cells harvested from large Graafian follicles just prior to ovulation undergo hypertrophy and secrete large amounts of progesterone in culture (see Channing, 1970a). Furthermore these cells display a fine structure similar to the granulosa lutein cells of the corpus luteum (Crisp & Channing, 1972). By contrast cells harvested from small Graafian follicles do not secrete much progesterone (see Channing, 1970a) and retain a fine structure similar to follicular cells (Crisp & Channing, 1972). Thus human granulosa cells of differing steroidogenic potentials when cultured in vitro would provide a simple in vitro biochemical model with which to examine the effects of $\text{PGF}_2\alpha$ and PGE_2 on the human corpus luteum.

The mechanism of hormone action in relation to ovarian steroid secretion

The mechanism of hormone action appears to be initiated by the selective binding of the hormone to a specific receptor in the target cell, either in the membrane or some other part of the cell. The binding of pituitary hormones to plasma membrane receptors has been discussed previously. In terms of ovarian steroid secretion, the interaction of LH with the granulosa-derived luteal cell is perhaps the best understood, although our knowledge is far from complete

(Lee & Ryan, 1971; Channing, 1975). Following the preovulatory surge, LH binds to its plasma membrane receptors on the granulosa cells (Channing & Kammerman, 1973a; Channing, 1975; Channing & Tsafiriri, 1975) which become saturated with this gonadotrophin, and morphological and functional luteinization ensue. The steroidogenic response to LH is mediated indirectly by the activation of the adenylate cyclase system (Savard et al., 1965; Dorrington & Kilpatrick, 1967) in the cell membrane to produce the "second messenger", adenosine 3', 5' cyclic monophosphate (C-AMP), an activation system common to many hormones such as ACTH, glucagon, vasopressin, catecholamines and some prostaglandins, e.g. PGE₂; (Marsh, 1971; Robison, Butcher & Sutherland, 1971). 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 lutein cell will secrete progesterone whereas lipolysis will be induced in adipose tissue. The morphological changes occurring on luteinization are probably also mediated by C-AMP, since Channing has shown that the addition of C-AMP (or the dibutyl derivative, DBC) to cultures of porcine and monkey granulosa cells stimulates both morphological luteinization and progesterone secretion (Channing & Seymour, 1970; Channing, 1970a).

It has been shown that C-AMP interacts with a protein receptor consisting of two components, (a) a regulatory unit (R) to which C-AMP binds and (b) a catalytic unit (c) which has protein kinase (P.K.) activity (Garren, Gill, Masui & Walton, 1971), see Fig. 1a. Should the stimulation of adenylate cyclase cease, the intracellular pool of C-AMP will be rapidly depleted, since its conversion to C-AMP

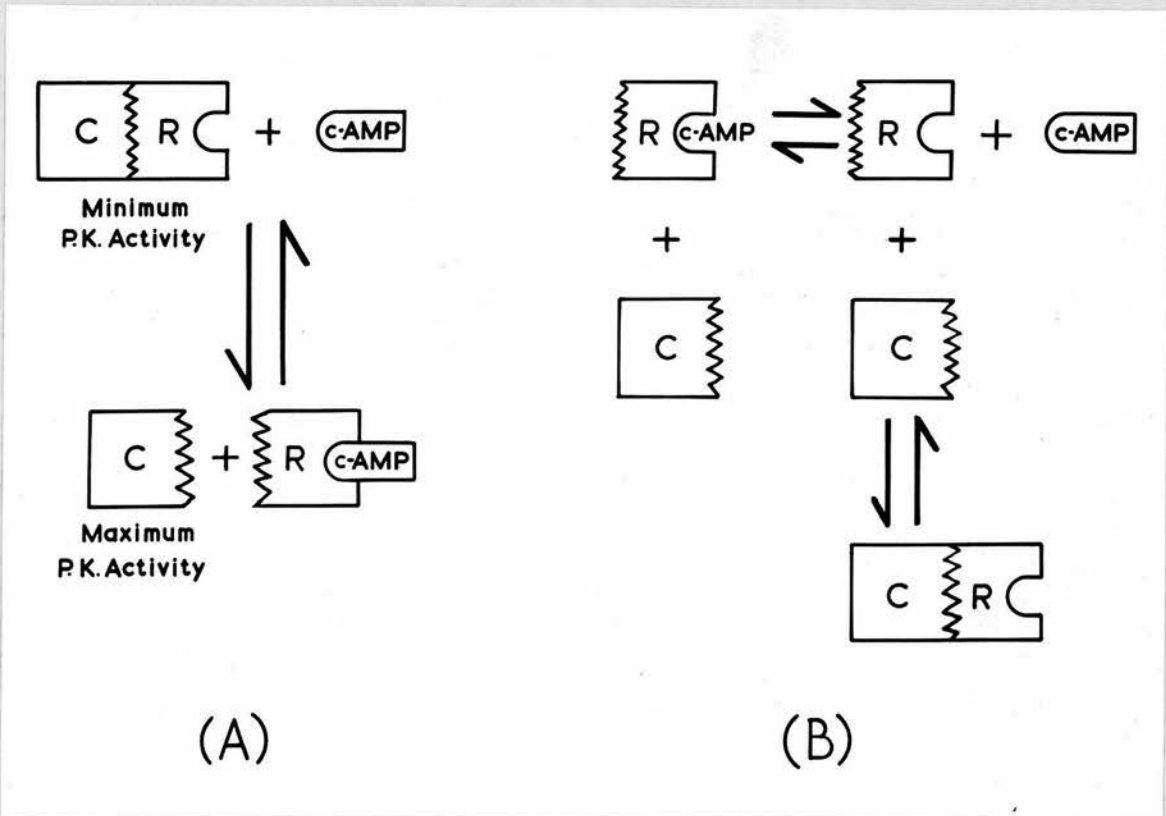


Fig. 1. A simplified model showing how C-AMP effects protein kinase activity.

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 is limited only by the availability of substrate. Thus when C-AMP is depleted, protein kinase activity is also depleted (see Fig. 1b).

The C-AMP dependent protein kinases are non-specific phosphorylators (Krebs, 1972). They can phosphorylate particular protein components of the nucleoprotein complex, and in so doing can probably 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 (Langan, 1968). Cyclic-AMP dependent protein kinases can also phosphorylate many different enzymes and in so doing, modify their activity (Krebs, 1972). In this respect, the most important enzyme in the luteal cell is cholesteryl esterase. From studies on the bovine adrenal cortex a C-AMP dependent protein kinase is capable of phosphorylating an inactive form of the cholesteryl esterase and converting it into an active form (Trzeciak & Boyd, 1974). It is reasonable therefore 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 (Claesson, 1954) into free cholesterol which on entering the mitochondria is readily metabolised to progesterone. 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 (Krebs, 1972), will reconvert the cholesteryl esterase to its original, non-phosphorylated inactive form, and the consequence is a rapid reduction in progesterone

synthesis by the luteal call. However mammalian m-RNA is very stable (Gelehrter, 1971), and the disappearance of C-AMP will not immediately effect the production of the new proteins induced by phosphorylation of the nucleoprotein complex. The biochemical mechanism of luteinization is summarized in Fig. 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 probably involved in the supply of cholesterol to the metabolic enzyme systems in the mitochondria (Hermier, Combarous & Jutisz, 1971).

The role of FSH in functional or structural luteinization is largely unknown. This is probably because some difficulty has been experienced in tagging a radioactive label to FSH without destroying its biological activity. It appears that FSH is more readily bound to granulosa cells within small Graafian follicles than to those which are more mature (Rajaniemi & Vanha-Pertulla, 1972). It has also been suggested that FSH may have a fundamental role in protecting granulosa cells from undergoing atresia (Peters et al., 1975) although the mechanism involved is obscure. The possibility of FSH involvement in the formation of receptors for LH has been discussed previously.

There is evidence to suggest that both LH and prolactin control the ovarian enzymes, cholesterol ester synthetase and esterase (Behrman & Armstrong, 1969; Behrman, Orczyk, MacDonald & Greep, 1970). These enzymes are maintained by prolactin and the esterase is activated by LH (see Greep, 1971). The implication is that prolactin may have an essential role in ovarian steroidogenesis; however, specific receptors for this hormone have not been demonstrated

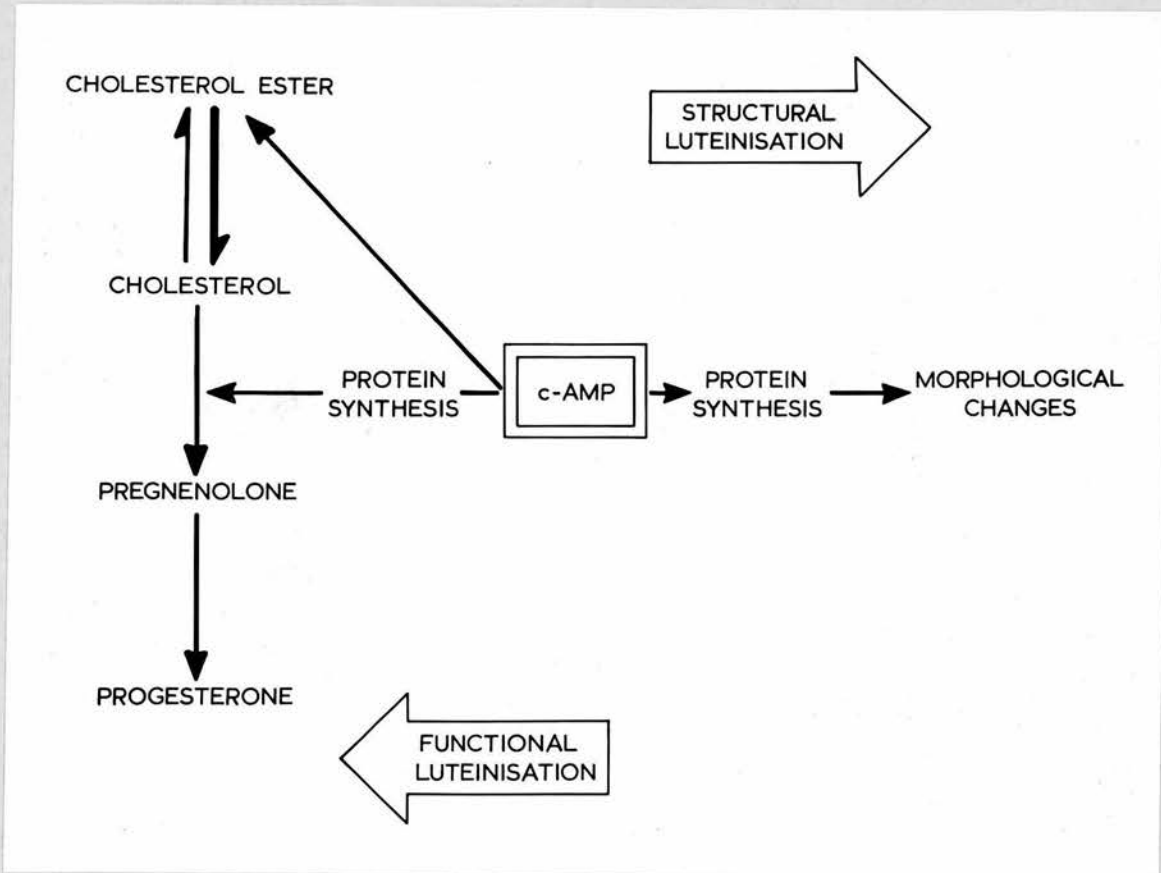


Fig. 2. The role of C-AMP in structural and functional luteinization.

Oestrogen enhances the secretion of progesterone by luteinizing granulosa cells in tissue culture (Goldenberg, Bridson & Kohler, 1972b); the stimulatory action is blocked by inhibitors of protein synthesis. Studies in the rat adrenal suggest that oestrogen may be involved in the synthesis of enzymes involved in steroidogenesis (Purvis, Canick, Mason, Estabrook, McCarthy, 1973).

It has been postulated that prostaglandin E₂ (PGE₂) may mediate the action of LH on cyclic AMP (Kuehl, Humes, Tarnoff, Cirillo & Ham, 1970), since prostaglandin antagonists block the actions of both prostaglandin and LH. However the actions of LH plus PGE₂ on luteal tissue, Graafian follicles and isolated granulosa cells have subsequently been found to be additive; thus it appears unlikely that prostaglandins play the role of an intermediate between the membrane effect of LH and C-AMP formation, (Marsh, 1971; Kolena & Channing, 1972; Jonsson, Shelton & Baggett, 1972; Herlitz & Rosberg, 1973; Lindner et al., 1974; Moor et al., 1975). Nevertheless PGE₂ has a specific plasma membrane receptor (Rao, 1973) and it activates the adenylate cyclase system (Marsh, 1971) yet its role in the physiological control of ovarian steroidogenesis remains obscure.

Granulosa cells in tissue culture

Granulosa cells can readily be obtained from Graafian follicles as a dispersed cell suspension without the need for chemical or enzymatic treatment. Granulosa cells have been used extensively in vitro to investigate various aspects of ovarian steroidogenesis (Bjersing, 1962; Bjersing & Carstensen, 1964, 1967; Ryan & Short, 1965, 1966; Channing, 1966, 1969a, b, c, 1970a, b, c, d; Ryan & Petro, 1966; Ryan et al., 1968; Goldenberg, Bridson & Kohler, 1972b;

Merk, Botticelli & Albright, 1972; Shirley & Stevenson, 1973; Challis, Erickson & Ryan, 1974; Seamark, Moor & McIntosh, 1974). However very limited use has been made of this technique for studies on ovarian steroid secretion in women (Channing, Butt & Crooke, 1968; Channing, 1969a).

It is often assumed incorrectly that granulosa cells 'spontaneously' luteinize in vitro and consequently do not reflect the activities of the cell in vivo. Studies on granulosa cells from women, rhesus monkeys and mares have shown clearly that morphological and functional luteinization can only occur 'spontaneously' when the cells are harvested from large preovulatory follicles or after the cells are stimulated with gonadotrophins, C-AMP or PGE2 in culture (Channing, 1970a; Channing & Crisp, 1972). Even to say that luteinization is 'spontaneous' is probably misleading since there is now evidence from women, rhesus monkeys and sheep to suggest that the initiation of luteinization occurs in vivo following the mid-cycle LH surge but prior to follicular rupture (Neill, Johansson & Knobil, 1967; Baird et al., 1975; A. G. Wheeler, R. B. Land, D.T. Baird, R. J. Scaramuzzi, unpublished data). Thus granulosa cells from large preovulatory follicles are probably undergoing hypertrophy and secretory changes prior to collection. By contrast cells harvested from smaller follicles do not undergo morphological and functional luteinization in vitro, unless stimulated in culture with either gonadotrophins, C-AMP or PGE2. Granulosa cells were said to luteinize in culture when they increased in size, accumulated eosinophilic granules and lipid droplets in the cytoplasm and secreted large amounts of progesterone (Channing, 1970a). However it is difficult to assess morphological

luteinization in cultured cells by light microscopy, since the degree of cellular enlargement may be related to the density of the cell suspension growing on the coverslips (Erickson et al., 1974; Challis, personal communication). Nevertheless the validity of granulosa cells in tissue culture as a model for ovarian function in vivo was further enhanced by the electron microscopy studies of Crisp & Channing (1972); these authors found that the fine structural appearance of granulosa cells after tissue culture was similar to those found in vivo.

Incubations of isolated granulosa cell suspensions with radioactive precursors of steroid hormones (e.g. Ryan et al., 1968) provides information as to which enzymes are present but tells us little about which are the important ones in vivo. In a study where granulosa cells and Graafian follicles were cultured in vitro, and their secretions were compared to those which occurred in vivo it is abundantly clear that the qualitative pattern of steroidogenesis by the granulosa cells in vitro is similar to that occurring in vivo (Seamark et al., 1974). It has also become clear that the steroidogenic capacity of granulosa cells in culture is markedly influenced by the endocrine environment that they were subjected to prior to harvesting and to the hormonal environment in which they were cultured (Channing, 1970a; Seamark et al., 1974). In addition isolated granulosa cells appear to retain their biosynthetic capacity and also their morphological integrity for at least 16 days in tissue culture (Channing, 1969a).

The fact that the steroidogenic potential of these cells is influenced by their previous hormonal environment provides an ideal way of assessing the degree of maturation a follicle has achieved

at the time of cell harvest. It would be of interest therefore to measure the hormone levels in follicular fluid and to culture granulosa cells from individual follicles. By comparing the hormone levels in plasma to those in follicular fluid it may be possible to determine which hormones influence the biosynthetic potential of the granulosa cells in culture.

Ideally an in vitro technique provides a method for culturing cells in an environment devoid of gonadotrophin. In practice this is difficult to achieve since a minimal volume of serum (5 to 20%, v/v) is normally required to maintain the viability of cells in culture (C. P. Channing, personal communication, but see also Channing, 1975). From a physiological point of view this is not inconvenient since granulosa cells are previously exposed to follicular fluid which contains most serum proteins (see Edwards, 1974, for review). However to examine whether granulosa cells require a certain gonadotrophin for steroidogenesis or to remain viable the problem remains as to how to remove the endogenous gonadotrophin(s) from the culture medium. Three methods of preparing such a culture medium are possible. The first method is to culture cells in a medium containing serum collected from patients who have been hypophysectomized or who have had some type of treatment to suppress pituitary function. However the search for a serum totally devoid of gonadotrophins has thus far been unrewarding (W. M. Hunter, A. S. McNeilly, personal communication). A second possibility involves neutralising the endogenous gonadotrophic activity in culture medium by adding an excess of a highly specific anti-serum. This technique has been widely used in vivo and in vitro (Ahren, Herlitz, Nilsson, Perklev, Rosberg, & Selstam, 1973; Laurence & Hassouna, 1973; Koch, Zor, Pomerantz, Chobsiang & Lindner, 1974;

Lindner et al., 1974). The concentration of gonadotrophin in culture medium can be measured both before and after treatment since the antibody-antigen complex can be absorbed out at the end of culture using a solid phase second antibody. The third possibility is to incubate the culture medium plus serum with excess antibody, then remove the antibody-antigen complex prior to tissue culture. Thus, at least two possible techniques could provide a method for investigating the dependence of granulosa cells on trophic hormone.

The scope of the in vitro culture system could also be extended to investigate some aspects of luteal function in women. Human granulosa cells harvested from large preovulatory follicles undergo hypertrophy and secrete large amounts of progesterone in vitro (Channing, 1969a); these cells are probably similar to the granulosa derived cells of the corpus luteum since preovulatory cells from the rhesus monkey have a fine structure after culture which is similar to that found in the luteal cell (Crisp & Channing, 1972). Thus the role of LH, FSH and prolactin together with those of the prostaglandins on luteal function in women could be investigated using this simple in vitro system.

What then are the disadvantages of in vitro culture? The cells are removed from an avascular environment and also from any further contact with the ~~theca~~ theca cells, the oocyte and transudates of plasma which appear in follicular fluid. Thus any physiological interpretations based on results obtained from in vitro culture must be related to in vivo histological, histochemical and endocrine data. At the present time there is a lack of information regarding the production of steroids after perfusion of human ovarian tissue or dispersed cell types in vitro. This is rather unfortunate since an important limitation of any static in vitro culture system is that



it may well be a rate limiting system because any increase in steroidogenesis (as a consequence of C-AMP production), is accompanied by a rise in phosphodiesterase activity which in turn may limit the rate of steroidogenesis (Channing, 1970a). However from in vitro studies using whole sheep follicles no clear differences in steroid production were observed when the culture medium was changed daily instead of every 3 days (Moor, 1973). In addition the daily production of steroid into culture medium can be comparable to that achieved in vivo (Moor, 1973). Thus provided the nutrient supply is adequate it appears that steroid production in vitro is not seriously handicapped by the static nature of the system.

A suitable method for confirming the validity of the in vitro tissue culture system for use as a model for investigating various aspects of ovarian steroidogenesis, is to compare the 24h secretion from ovarian tissue in vitro to that which occurs in vivo. This method requires knowledge of the 24h production rate of steroid both in vivo and in vitro and in addition an estimate of the number of cells both in the gland and in culture if a dispersed cell culture technique is used. Despite the widespread use of tissue and cell culture techniques, only Moor (1973) and Seamark et al., (1974) using an organ culture technique have compared the production of steroid in vitro with that occurring in vivo. In most studies employing isolated cell culture techniques the number of cells present either before, during or at the end of culture were not determined. This is particularly regrettable since there is no way in which a retrospective study on most of the published data can be made in order to determine whether the secretion rates achieved in vitro are comparable to those achieved in vivo. However, in one study where cell numbers were determined (Channing, 1969a) showed

that human granulosa cells when cultured as a monolayer, after being harvested from a preovulatory follicle, secreted about 5 pg of progesterone per cell each day (5 pg/cell/day). The preovulatory follicle or newly formed corpus luteum contains between 2×10^8 and 1×10^9 granulosa cells (K. P. McNatty & D. K. Green, unpublished observations). Thus if all the luteinizing granulosa cells of the preovulatory follicle or of the newly formed corpus luteum were cultured in vitro their total secretion of progesterone would have been between 1 and 5 mg per day. In vivo, the production rate (PR) of progesterone can be calculated from the equation $PR = MCR \times c$ where the metabolic clearance rate (MCR) reflects the rate of metabolism of the hormone, c is the concentration of the hormone in peripheral blood and PR is the rate at which the hormone is introduced de novo into the systemic circulation (Tait & Burstein, 1964). The MCR in women is 2,200 litres/day (Tagatz & Gurpide, 1973) and in plasma the concentration of progesterone is about 2 ng/ml or 2 μ g/litre during the very late follicular phase of the menstrual cycle (Johansson & Wide, 1969); thus the PR is 4.4 mg/day. Since more than 80% of the progesterone during the late follicular phase is secreted by the ovaries (D. T. Baird, personal communication), the PR is a close estimate of the total ovarian secretion of progesterone. Therefore a secretion of progesterone of 5 pg/cell/day in vitro closely correlates to the estimated secretion rate/cell in vivo. Since these estimates are based on the total number of granulosa-lutein cells in vivo they provide further evidence to suggest that most if not all of the progesterone from the preovulatory follicle or newly formed corpus luteum originates from the granulosa cells. The PR of progesterone during the mid-luteal phase is about 25 mg/day which is,

if no cell mitoses has taken place, 5 times higher than the amount of progesterone luteinized granulosa cells can secrete in vitro. Thus the marked increase in progesterone secretion during the mid-luteal phase, which is probably in excess of any increase in cell mitoses (Corner, 1956), may well be due to the presence of the two cell types in the corpus luteum, i.e. the granulosa and theca-lutein cells (Ryan et al., 1968). Alternatively, it may be that granulosa cells are not fully matured as steroid secreting cells until the mid-luteal phase of the menstrual cycle.

Interpretation of data obtained using luteinized granulosa cells as an in vitro model of human corpus luteum function probably requires the greatest caution because this gland consists of two cell types. In addition, the role of the vascular system in both the regulation of steroidogenesis by, and regression of, the corpus luteum cannot be discounted. However the absence of a vascular system can also be an advantage when examining the biochemical effects of prostaglandins on the luteal cell.

There are considerable ethical advantages in studying certain aspects of ovarian steroid secretion in women with an in vitro system. The results can provide information which is otherwise impossible or unethical to obtain; this information can complement that obtained from clinical or experimental studies on human volunteers. Further knowledge concerning the control of human ovarian steroid secretion is urgently required in order to develop new and more acceptable, methods of contraception.

The scope and aims of studies undertaken

The experiments described in this thesis were all carried out using human material. Intact ovaries or ovarian wedge biopsies, endometria and peripheral blood samples were obtained from women

(aged 21 - 52 years) who were at varying stages of the menstrual cycle and undergoing hysterectomy for various gynaecological conditions. Intact Graafian follicles were dissected from the ovarian specimens: the follicular fluid was aspirated for hormone assay and the granulosa cells harvested for culturing in vitro. The concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), the α and β subunits of LH (LH α and LH β), prolactin, progesterone, oestradiol-17 β and androstenedione in peripheral plasma and follicular fluid were determined by various specific radioimmunoassays. In addition, the concentration of testosterone was measured in follicular fluid. Histological sections were prepared from Graafian follicles and corpora lutea at differing stages of the menstrual cycle. The steroidogenic potential, mitotic activity and viability of granulosa cells were examined after they were cultured in media containing varying amounts of pituitary hormone, the subunits of LH, oestradiol, prostaglandins, N⁶, O²-dibutryl adenosine 3',5'-cyclic monophosphate (DBG) or serum from patients with Addison's disease and premature ovarian failure.

It was hoped that the results of these studies could be integrated to provide new information on:- a) the importance of the endocrine microenvironment of the follicle in preparing its granulosa cells for their subsequent secretory activity in the corpus luteum; b) how a follicle is selected for ovulation; and c) on the factors influencing the maintenance and the regression of the corpus luteum in women.

CHAPTER 2

MATERIALS AND METHODS

Patients

One hundred and sixty five women (aged 21 - 48 years) who were at varying stages of the menstrual cycle were undergoing hysterectomy for various gynaecological conditions. The indications for surgery were stage 0 carcinoma of the cervix, (22 patients), menorrhagia due to fibroids (52), dysfunctional uterine bleeding (10), endometriosis (15), or chronic pelvic pains or dysmenorrhoea (66). Those with stage 0 carcinoma of the cervix had regular menstrual cycles (21 - 32 days) and were considered to be endocrinologically normal (Baird & Fraser, 1974a). The previous menstrual cycles of the group with menorrhagia varied in length from 21 - 34 days and about 40% of these subjects had ovulated in the cycle under study, as indicated by the presence of a secretory endometrium and of at least one corpus luteum at the time of the operation. The remaining subjects were in the proliferative phase as assessed by their endometrial histology and date of the last menstrual period. Full details relating to the clinical histories of all the patients are shown in Appendix 1 and a summary of this data is shown in Table 1.

Dating the Menstrual Cycle

An endometrial biopsy and a peripheral blood sample (15 - 20 ml collected before oophorectomy) were obtained during the operation. The endometrium was examined histologically and dated according to the criteria of Noyes, Hertig & Rock (1950). The stage of the menstrual cycle was assessed in all subjects from:- (a) the date of the last menstrual period; (b) the concentrations in plasma of LH, FSH, oestradiol and progesterone; (c) endometrial histology; and (d) the presence or absence of a corpus luteum. The menstrual cycle was divided into six phases: early follicular, still menstruating, (EF); mid-follicular (MF); late follicular (LF); early luteal (EL); mid-

Table 1. Clinical details of all the patients studied.

<u>Experiment (Chapter)</u>	<u>Number of Patients</u>	<u>Number of Follicles</u>	<u>Indications for Surgery</u>	<u>% Patients in follicular phase</u>	<u>% Patients in luteal phase</u>
3	97	176	Stg. 0 (17); CPP (25); F. (35); CGH (1); DUB. (10); E. (9).	50	50
4	4	4	CPP (2); E. (2).	80	20
5	55	138	Stg. 0 (4); CGH (1); F. (7); CPP (39); E. (4).	50	50
6	10	19	CPP (5); E (2); F. (3).	68	32
7	47	80	Stg. 0 (3); DUB (4); F (8); CPP (29); E (3).	50	50
8	26	30	Stg. 0 (3); F (9); CPP (14).	100	none
9	13	21	F (3); CPP (10).	36	64
10	12	35	E (2); CPP (10).	58	42

Alliquots of granulosa cells harvested from follicles were frequently subdivided for use in the different experiments described (Chapters 3 - 10). Consequently the follicles from one patient may have been used in more than one experiment. The numbers in parentheses refer to the number of patients. The abbreviations used are:- Stg. 0 = stage 0 carcinoma of the cervix; F = fibroids; DUB = dysfunctional uterine bleeding; E = endometriosis; CPP = chronic pelvic pain or dysmenorrhoea and CGH = cystic glandular hyperplasia.

luteal (ML) and late luteal (LL).

Ovarian morphology

Ovaries were examined in situ to assess gross morphology and to record the presence or absence of a corpus luteum. In some cases histological sections of the excised ovaries were examined by light microscopy after removal of the Graafian follicles to ascertain that no major ovarian pathology existed.

Collection of follicular fluid

The follicular fluid which was analysed for pituitary and steroid hormones was obtained from the ovaries of the women described above. Two different methods were used for the collection of the fluid.

In 23 subjects, antral fluid was aspirated from 36 Graafian follicles through a 23G needle into a syringe during surgery. For technical reasons it was not possible to measure the size of these follicles accurately, consequently they were classified as large (≥ 8 mm) or small (< 8 mm).

In 142 subjects, ovarian specimens (whole ovaries or wedge biopsies) were collected into chilled media (Medium 199 containing Hank's salts and HEPES* buffer (20 mM), gentamicin (50 $\mu\text{g}/\text{ml}$), amphotericin-B (2.5 $\mu\text{g}/\text{ml}$), (Flow Laboratories, Irvine, Scotland)) and all follicles which were ≥ 4 mm diameter (238) were dissected out within 2h of surgery. The diameter of each isolated follicle was measured and the antral fluid aspirated through a 27G needle into a 500 μl Hamilton syringe. Fluid collected by either method was frozen at -20° until assayed. No attempt was made to recover the oocyte from the follicular fluid. Follicles without a lining of granulosa cells, irrespective

*HEPES: N-2-Hydroxyethylpiperazine N¹-2-ethanesulphonic acid.

of size were defined as 'cystic'. Subsequent data reported for the cystic follicles are considered separately from those obtained for normal follicles.

Technique for culturing granulosa cells

The method of collecting and culturing human granulosa cells was a modification of that described by Channing (1969a). The granulosa cells were harvested from each of the follicles and each culture chamber contained cells from individual follicles. The only exception to this procedure was adopted in the experiment described in Chapter 9, where, in many cases cells from different follicles were pooled to provide the large number of cultures required. The collapsed follicle (after removal of the follicular fluid) was slit open and the granulosa cells scraped into culture medium using a platinum loop. The remainder of the follicle was fixed in Bouin's aqueous fixative for subsequent histological examination to ensure that the basement membrane was still intact (and thus that the culture was free of theca cells). An aliquot of the suspension of granulosa cells was counted using a haemocytometer, and cell viability was determined with either nigrosin (Paul, 1972) or lissamine green (Channing, 1969b). The remaining cell suspension was subdivided into aliquots each containing a minimum of 5×10^4 "live cells", and layered onto 18 mm^2 , No. 3 coverslips which had previously been washed in acetone, dried with lens tissue and then heated to 200°C . These coverslips were placed in a tissue culture box containing twenty-one 19.0 mm^2 compartments (Flow Laboratories, Irvine, Scotland). Whenever possible cultures were carried out in triplicate although the low viabilities (< 10%) of some cell preparations meant that some studies had to be carried out on individual cultures. Each culture chamber received 1 ml of

medium containing antibiotics and 20% serum (v/v). Granulosa cells in the experiments described in Chapters 4, 5, 7 and 10, were cultured in medium containing 20% calf serum (batch 40498, Flow Laboratories, Irvine, Scotland), while in the experiments described in Chapters 6 and 8 they were cultured in medium containing 20% human serum (batch 40515, Flow Laboratories, Irvine, Scotland), with or without antiserum treatment. In the experiment described in Chapter 9 the cells were cultured in medium containing variable amounts of human serum (batch 40515) and/or serum from patients with Addison's disease with fresh serum or heat inactivated fresh serum being added as a source of complement or complement control. Calf serum (Flow Laboratories, batch 40498) was used as the main source of serum for tissue culture because only a limited supply of pooled human sera (batch 40515) was available. Thus all experiments which involved the addition of hormones, etc., to cells in culture were carried out using the same batch of calf serum so that the results from different experiments could be compared. Similarly all studies involving antibodies to human pituitary hormones were carried out using the same batch of human serum.

All cultures were incubated at 37°C using air as the gas phase. Each culture was maintained for 1 to 30 days and the culture medium was replaced daily and stored frozen at -20°C until assayed for progesterone. At the end of culture the coverslips were removed and were extensively rinsed in medium without serum, and fixed with Smearfix (Raymond A. Lamb, London). The coverslips were stained with haematoxylin and eosin and the cell numbers determined by counting the number of cells within a 0.12 mm^2 area at 23 uniformly spaced sampling points within the 18 mm^2 of the coverslip boundaries.

A PDP-9 computer, mechanical microscope and Quantimet television system was used (Green, 1974).

Histology

Endometria, follicle walls and in some cases sections of intact ovaries were fixed overnight in Bouin's aqueous fluid and were subsequently dehydrated with ethanol and embedded in paraffin wax. Serial sections cut at $6\mu\text{m}$ were stained with Harris's haematoxylin and eosin and mounted with DePex (Raymond A. Lamb, London). The endometria were examined in order to assess the stage of the menstrual cycle as described above.

All the follicle walls from which granulosa cells had been removed were examined to see whether the basement membrane was still intact; two or three sites on each follicle wall were sectioned. In most cases a recognisable lining of granulosa cells was observed. In approximately 9% of all the follicles examined (i.e. $\frac{20}{236}$) there were indications of extensive damage to the basement membrane. Of this group of follicles, 14 were 'cystic' (i.e. $< 50,000$ cells recovered) and the results obtained following culture of the granulosa cells from the remaining 6 damaged follicles were discarded.

Serial sections ($6\mu\text{m}$) were cut from 2 ovarian wedges and 2 intact pieces of follicle wall to obtain histological specimens of the walls of large antral follicles and the corpora lutea for the experiment described in Chapter 4.

Pituitary and steroid preparations

The LH and FSH which were added to the cultures were: human LH (Hartree IRC-2, 24.6.69) containing 7550 u. LH/mg and < 25 u. FSH/mg; human FSH (Butt CPDS/6) containing 2,200 u. FSH/mg and 90 u. LH/mg and human FSH (MRC 73/519) containing 2,200 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, assumed 32.8 u./ampoule (MRC National Institute for Biological Standards and Control). The concentrations of LH and FSH were expressed as μ u./ml. The gonadotrophins were dissolved in culture medium and stored at -20°C until used in culture.

The human prolactin preparation which was added to the cultures was Friesen prolactin of which $1\text{ ng} \equiv 20\mu\text{U}$ MRC standard 71/222. The Friesen prolactin preparation was 90% pure and the human pituitary hormone contaminants were found by radioimmunoassay to be negligible: growth hormone (HGH), HLH, HFSH, all $< 0.4\%$ and the concentration of human placental lactogen was undetectable. The concentration of prolactin is expressed as ng/ml. Prolactin was dissolved in culture medium and stored at -20°C until used. The biological activity of human prolactin in aqueous solutions even at -20°C , falls rapidly with time (H. G. Friesen, personal communication) consequently prolactin solutions were discarded after storage for three months.

Millipore filtration ($0.22\mu\text{m}$, Millipore Corporation, Bedford, U.S.A.) of LH, FSH or prolactin in medium 199 effectively removed $>95\%$ of the protein. Non-protein filtrates of all the gonadotrophins were examined for their ability to alter steroidogenesis by granulosa cells in culture and all were found to be inactive.

The gonadotrophin subunits which were added to the cultures were the α and β subunits of human pituitary LH, MRC 72/40 and MRC 71/342, respectively (MRC National Institute for Biological Standards and Control). The α subunit contained approximately 15% intact LH contamination whilst the β subunit contained $< 8\%$ intact LH (A. S. Hartree, personal communication). The concentrations of the subunits were expressed as ng/ml. The subunits were diluted in culture medium and

stored at -20°C until used in culture.

Oestradiol- 17β at a concentration of 10 g/ml and N^6, O^2 - dibutryl adenosine 3', 5' - cyclic monophosphate (DBC), $465\mu\text{g/ml}$, were made up in the culture medium and 0.1 ml aliquots were used for each culture. The final concentration of oestradiol ($1\mu\text{g/ml}$) which was present in the culture medium is similar to that found in actively developing human Graafian follicles while that for DBC ($46.5\mu\text{g/ml}$) was the maximum dose which did not cause cellular necrosis.

Prostaglandin $\text{F}2\alpha$ and $\text{E}2$ preparations

Prostaglandin $\text{F}2\alpha$ and $\text{E}2$ (Upjohn Company, Kalamazoo, U.S.A.) were each dissolved in 70% ethanol and stored in ampoules at 4°C until added directly to the cell cultures in a volume of 5 or $10\mu\text{l}$. All other cultures in the prostaglandin control experiments not receiving the hormone received a similar volume of 70% alcohol alone.

Determination of the biological activity of the gonadotrophins

Human granulosa cells were cultured in medium containing calf serum and the progesterone secretion compared to that achieved by cells cultured in media containing human sera with known concentrations of LH and FSH. In this way the endogenous gonadotrophic activity of calf serum was related to further additions of human gonadotrophins.

The biological activity of the exogenous LH, FSH, prolactin, and α and/or β subunits of LH preparations which were added to the cultures was examined by measuring the daily production of progesterone by granulosa cells in response to varying amounts of the pituitary hormones.

Preparation and specificity of the antisera to gonadotrophins

The LH antisera was raised in a rabbit (RG) and a guinea pig (7252) against human pituitary LH (Hartree, DE Fraction) which had a potency which was about 60% of the Hartree IRC-2, human LH preparation (Hartree,

1966). The major contaminant of the LH antigen (DE Fraction) was denatured LH (W. M. Hunter, personal communication). The anti-LH serum (R6 or 7252) bound 50% of 125 I LH (IRC-2, 100 pg) at a final dilution of 1:102,400 or 1:160,000 respectively. The LH antisera also bound 50% of 125 I human FSH (Butt CPDS/6, 100 pg) at a final dilution of 1:200 or 1:10,000 respectively but did not bind significant amounts of 125 I human prolactin (supplied by Dr. H. G. Friesen, University of Manitoba, Winnipeg, Canada). Thus the LH antiserum (7252) contained antibodies capable of binding significant amounts of FSH as well as LH. The LH antisera (7252 and R6) were used to remove the endogenous LH (and FSH) from human serum prior to its use in tissue culture.

The prolactin antiserum A_3 was raised in a rabbit against a crude pituitary prolactin extract (supplied by Dr. R. V. Davies & Dr. A. S. Hartree, University of Cambridge) containing about 30% prolactin, 60% human growth hormone (HGH) and 10% LH and FSH. Antiserum A_3 bound 50% 125 I human prolactin (Friesen, 100 pg) at a final dilution of 1:128,000, 50% 125 I human LH at a final dilution of 1:6000 and 50% 125 I human FSH at 1:400. The prolactin antiserum A_4 was obtained from the same rabbit following a booster immunisation with a different pituitary prolactin extract (supplied by Dr. R. V. Davies & Dr. A. S. Hartree) which was approximately 50% prolactin and 25% HGH. Antiserum A_4 bound 50% of 125 I human prolactin at a final dilution of 1:16,000, 50% of 125 I human LH at 1:2500 with insignificant binding of 125 I human FSH. Thus the prolactin antisera, A_3 and A_4 , also contained antibodies capable of binding significant amounts of LH. These antisera were used to remove or neutralise the endogenous prolactin (and/or LH) in human serum prior to or during tissue culture.

The HGH antiserum (W 105) (supplied by Dr. B. Hurn, Wellcome

Laboratories, Kent, England) was raised in a rabbit against Raben HGH and bound 50% ^{125}I prolactin at a final dilution of 1:400 with insignificant binding of both ^{125}I LH and ^{125}I FSH. Thus the HGH antiserum did not appear to bind appreciable amounts of LH, FSH or prolactin and was therefore used as a control.

The anti-bovine serum albumin (anti-BSA) was raised in a rabbit against crystallised BSA and did not bind significant amounts of iodine -125 labelled LH, FSH or prolactin, and was used as a non-specific antiserum control.

Treatment of human serum with antiserum to gonadotrophins

In order to assess whether prolactin was an essential hormone for the maintenance of steroidogenesis by human granulosa cells (see Chapter 6) the cells were cultured in a medium containing 20% human serum, 20% human serum and normal rabbit serum or rabbit antiserum to prolactin, HGH or BSA. Normal rabbit serum, rabbit anti-BSA, rabbit anti-human HGH (W105) or rabbit anti-human prolactin (A_4) which were previously dialysed extensively (4-5x) against medium 199 containing Hank's salts, Hepes' buffer (20mM), gentamicin (50 $\mu\text{g}/\text{ml}$) and amphotericin -B (2.5 $\mu\text{g}/\text{ml}$) in order to remove any cytotoxic preservatives, were added to the culture medium at a final dilution of 1:500 v/v. These culture media including the control with rabbit serum (1:500 v/v) were incubated at 37°C for 24h before being used in tissue culture. After these media had been used in culture, aliquots of each daily change of culture medium were treated with an equal volume of solid phase immunosorbent (sheep anti (rabbit γ -globulin) (Organon, Oss, Holland))* suspended in medium 199. The mixture was

*Antibodies to purified rabbit γ globulin raised in a sheep.

continuously slurried by gentle mechanical agitation for 8 hours at 40°C. After this time, the precipitated antibody-antigen complex was centrifuged at 2000 rpm for 15 min.: the precipitate was discarded and the supernatant was assayed for LH, FSH and prolactin. Using this procedure it was possible to examine the progesterone content of the culture medium and relate the biological and immunological neutralisation of endogenous prolactin to changes in the daily rate of progesterone secretion.

In the experiments described in Chapter 8, granulosa cells were cultured in medium containing 20% human serum which was devoid of either FSH, LH, prolactin or prolactin and LH. The procedure for removing selectively the endogenous gonadotrophins in the human serum was similar to that described for the experiment in Chapter 6. Serum from rabbits immunised against the human pituitary hormones LH (R6), prolactin (A₃ and A₄), GH (W105) or BSA together with normal rabbit serum (all previously stored at -20°C) were dialysed as described above. Serum from a guinea-pig immunised against human LH (7252), and normal guinea-pig serum, both previously stored at -20°C were dialysed in the same manner. Each dialysed immune or control serum was incubated at 4°C for 4 days with an aliquot of the same batch of pooled human serum (40515, with known concentrations of LH, FSH and prolactin) at a final dilution of between 1:200 and 1:400 (v/v). On the fourth day of incubation a solid phase immunosorbent (sheep anti- (rabbit γ globulin) or sheep anti- (guinea-pig γ globulin)) suspended in medium after being extensively washed (4-5x) with medium to remove any cytotoxic preservatives, was added (50/50, v/v). The supernatant was obtained as previously described and filtered through 0.22 μ m MillexTM filter (Millipore Corporation, Bedford, U.S.A.) and was subsequently diluted

with medium so that the serum fraction was 20% by volume. Aliquots were assayed for LH, FSH and prolactin while the remainder was stored frozen at -20°C until used for tissue culture.

In addition a further series of culture media were used as controls. For these, serum from patients with antibodies* to gastric parietal cells (3 patients), thyroid microsomal fraction and thyroglobulin (3), adrenal (3) and corpus luteum (6) (supplied by Dr. W. J. Irvine, Edinburgh) were each added to culture medium containing 20% untreated human serum so that the final dilution of antiserum was 1:400 (v/v). These culture media were added to HeLa or granulosa cell cultures without any further treatments.

Use of HeLa cells to test for non-specific antiserum toxicity

All sera treated with antibodies, together with control sera previously diluted to 20% (v/v) with medium, were then added to monolayer cultures of HeLa cells (adapted for culture in human serum, Flow Laboratories, Irvine, Scotland). The cells were grown on coverslips in Leighton tubes (16 X 120 mm) in 2 mls of culture medium at 37°C using air as the gas phase. Each serum was tested for its cytotoxic properties in triplicate and the culture medium changed daily. After six days the coverslips were removed, washed extensively with medium without serum, fixed with Smearfix (Raymond A. Lamb, London) and stained with haematoxylin and eosin.

Autoimmune serum and the test for cytotoxicity

(i) Serum

Serum was obtained from 21 women and 2 men with idiopathic

*These organ, cell or subcellular specific antibodies were obtained from patients with Addison's disease (Irvine & Barnes, 1974).

Addison's disease and stored at -20°C . All 23 sera were found by an indirect immunofluorescence technique to contain antibodies to human luteal tissue (Irvine, Chan & Scarth, 1969); nine gave "clumpy", eight gave "patchy" and four gave "confluent staining" patterns.* Two sera did not react with luteal tissue, one reacted with theca interna alone and one with Leydig cells.

The clinical histories of these patients, together with immunofluorescent staining pattern and antibody titre (as assessed on air dried, cryostat sections of human corpus luteum) are shown in Table 2.

Control sera were obtained from women who were either showing normal menstrual cycles (20) or who were post-menopausal with a previous normal menstrual history (20). In addition sera were obtained from patients with 'anti-nuclear factor antibodies' (4), with antibodies to gastric parietal cells and to intrinsic factor (3), with antibodies to thyroid microsomal antigen and to thyroglobulin (2), with antibodies to adrenal cortical antigens not shared with other steroid producing tissues (2), and with mitochondrial antibodies (1). Since these sera contained antibodies which did not react with luteal cells, they were suitable for use as controls.

*These refer to the staining patterns in the cytoplasm of cells after slices of tissue were incubated with serum from patients with Addison's disease and horse anti-human IgG - fluorescein isothiocyanate (Wellcome Laboratories, Kent, England).

Table 2. Clinical features of patients with Addison's disease

<u>Sex</u>	<u>Age at Study</u>	<u>Reproductive History</u>	<u>Associated Diseases</u>	<u>IMMUNOFLOURESCENCE</u>	
				<u>Staining Pattern on Corpus Luteum</u>	<u>Titre</u>
Female	22	Ovarian failure	Diabetes Mellitus		32
Female	22	Ovarian failure	-	Confluent	32
Female	34	Ovarian failure	-		8
Female	42	Ovarian failure	-		Neat
<hr/>					
Female	18	Primary amenorrhoea	Hypoparathyroid		8
Male	16	Atrophic testis	-		16
Female	23	Ovarian failure	-	Patchy	8
Female	26	Ovarian failure	-		64
Female	35	Ovarian failure	-		4
<hr/>					
Female	9	Prepubertal	Arthralgia		4
Female	36	Menorrhagia	Thyrototoxicosis	Patchy	16
Female	40	Ovarian failure	Pernicious anaemia		64
<hr/>					
Female	14	Menstruating	Hypoparathyroid		128
Male	14	Undergoing normal puberty	Hypoparathyroid	Clumpy	4
Female	15	Primary amenorrhoea	Keratitis & Chronic Monilia, Hypoparathyroid		16

Cont

Table 2 (cont.)

<u>Sex</u>	<u>Age at Study</u>	<u>Reproductive History</u>	<u>Associated Diseases</u>	<u>Immunofluorescence</u>		<u>Titre</u>
				<u>Staining Pattern on Corpus Luteum</u>	<u>Corpus Luteum</u>	
Female	26	Ovarian failure	Hashimoto thyroiditis			32
Female	29	Oligomenorrhoea	-			128
Female	30	Ovarian failure	-			128
Female	36	Ovarian failure	-	Clumpy		2
Female	40	Ovarian failure	Thyrototoxicosis, Asthma			4
Female	46	Ovarian failure	-			64
Female	51	Menorrhagia	Pernicious anaemia			0
Female	55	Normal menopause	-	Neg.		0

The clinical features of 23 patients with idiopathic Addison's disease together with the type of immunofluorescent staining pattern the individual sera gave on unfixed, air dried, cryostat sections of human corpus luteum and the reciprocal titre of the antibody in the sera.

(ii) Test for cytotoxicity

The technique for culturing granulosa cells was identical to that described previously. In some cases granulosa cells were pooled from follicles of similar size from the same ovary to obtain sufficient numbers of 'live' cells in order to culture eight separate aliquots simultaneously.

Sera from patients with Addison's disease or control sera were added to the culture media at the start of each culture period and at each medium change. The 'Addisonian' serum and controls were added in the following volumes and made up to 1 ml with medium; 0.5 ml (6 experiments); 0.2 ml (8); with smaller volumes (0.1 ml, 0.05 ml, 0.025 ml and 0.01 ml, 8 experiments each), the serum volume was made up to 0.2 ml with heat inactivated fresh human serum. In addition to varying the amount and nature of the antiserum it was also important to check for the effects of complement.* To each 1 ml of culture medium plus serum was added either 0.1 ml of fresh human male serum as a source of complement or 0.1 ml of the same serum after heat inactivation (56°C for 30 mins.) as a complement control. All the experiments using the test antiserum together with the controls were carried out in quadruplicate.

After 3 days of culture the coverslips were fixed and stained with haematoxylin and eosin. The number of damaged and intact granulosa cells on each coverslip were counted using a PDP - 9 computer mechanical microscope, and a Quantimet 720-D cell counter as described previously. When less than 15% of the total number of granulosa cells

*Complement: Serum protein components (m.w. between 180,000 and 400,000) whose activation by antibody may lead to a cytotoxic reaction (Roitt, 1972).

were damaged this was regarded as non-significant and scored -. However, when between 15 and 80% of the cells were damaged the preparations were scored at +, and greater than 80% as ++. Clumping of the cells which was a consequence of the short period of culture led to some difficulty in counting the proportion of damaged cells and hence the broad classification of degrees of cytotoxicity.

Coverslips containing granulosa cells which were cultured in serum from patients with Addison's disease in the absence of complement were incubated at 37°C with horse anti-human IgG - fluorescein isothiocyanate (IgG - FITC) (Wellcome Laboratories, Kent, England) and were examined daily to see if affinity for the antibody changed with time. The cells were examined under ultraviolet light and the intensity of staining was assessed subjectively as ++, +, ⁺- or negative.

Radioimmunoassay of pituitary and steroid hormones

Luteinising hormone and follicle-stimulating hormone

The radioimmunoassays for LH and FSH in plasma and follicular fluid were based upon those previously described (Hunter, Edmond, Watson & McLean, 1974). Some modifications were made to accommodate the small volumes of antral fluid recovered from the follicles. The original concentrations of standards, tracer and antiserum were retained but the incubation volume was reduced to 150 μ l of which 50 μ l was neat (or diluted) follicular fluid, or standard. The time taken to count the radio-activity was increased proportionately so that in this respect assay precision was not diminished. The following standards were used: LH, MRC 68/40, assumed 77 units/ampoule; FSH, MRC 63/39, assumed 32.8 units/ampoule (MRC National Institute for Biological

Standards and Control). The concentrations of LH and FSH are expressed as $\mu\text{u./ml}$, where $1 \mu\text{u. LH} = 11.6 \text{ ng LER 907}$ and $1 \mu\text{u. FSH} = 44.6 \text{ ng LER 907}$. These assays would normally measure LH and FSH over the ranges $0.8 - 12.8$ and $0.4 - 6.4 \mu\text{u./ml}$ respectively in undiluted follicular fluid. With the fluid obtained from large follicles ($\geq 8 \text{ mm diameter}$) assays were in general carried out in duplicate, whilst those for follicles $< 8 \text{ mm diameter}$ were single determinations. A number of LH assays were also carried out on larger volumes of pooled fluid from small follicles.

Prolactin

Prolactin was measured in samples of peripheral plasma and follicular fluid using a specific double antibody radioimmunoassay (McNeilly, 1973; McNeilly & Hagen, 1974). The purified human prolactin for standards and labelling with ^{125}I was generously supplied by Dr. H. G. Friesen, University of Manitoba, Winnipeg, Canada. The concentration of prolactin is expressed as ng Friesen prolactin of which $1 \text{ ng} = 20 \mu\text{U MRC 71/222}$. The minimum detectable level of prolactin was 1.5 ng/ml for both follicular fluid and plasma. Assays were carried out in duplicate in specimens of both plasma and fluid.

α and β subunits of LH

The α and β subunits of LH ($\text{LH}\alpha$ and $\text{LH}\beta$, respectively) in peripheral plasma and follicular fluid were measured using specific double antibody radioimmunoassays (Hagen & McNeilly, 1975a; Hagen & McNeilly, 1975b). The purified human pituitary preparations $\text{LH}\alpha$ (MRC 72/20), $\text{LH}\beta$ (MRC 71/342) were used for standards and labelling with ^{125}I . The concentrations of $\text{LH}\alpha$ and $\text{LH}\beta$ were expressed as ng/ml . The minimum detectable level of $\text{LH}\alpha$ and $\text{LH}\beta$ in plasma or undiluted follicular fluid was 0.12 ng/ml and 0.04 ng/ml respectively.

Oestradiol-17 β

Antiserum for the assay of oestradiol-17 β was raised in a rabbit to a conjugate of oestradiol 6 - carboxymethyloxime and bovine serum albumin. Cross-reactions of other steroids in the routine assay procedures were: oestrone, 3% oestriol, 0.4%; 6-Oxo-oestradiol, 100%; testosterone, 0.003%; progesterone, 0.0002%.

For estimations of oestradiol in plasma an ether extract was evaporated to dryness, equal volumes of 0.05 M NaOH and carbon tetrachloride were added and shaken together with the residue. Samples of the aqueous phase were then neutralised in a slightly acid diluent for incubation in the radioimmunoassay system described by A. Bolton & F. Rutherford (personal communication). Oestradiol in follicular fluid was assayed without prior extraction (Abraham, Odell, Edwards & Purdy, 1970). The assays were carried out in duplicate and the minimum detectable level of oestradiol was 8 ng/ml in follicular fluid and 20 pg/ml in plasma.

(v) Progesterone assay

Progesterone in peripheral plasma, follicular fluid or culture medium was measured using modifications of published radioimmunoassay techniques (Abraham, Swerdloff, Tulchinsky & Odell, 1971; Thorneycroft & Stone, 1972; Dighe & Hunter, 1974).

(1) Solvents and reagents

Analar grade petroleum ether (40 - 60^o) benzene, chloroform, ethanol, heptane and water (B.D.H.) were used without further purification. The assay buffer, phosphate buffered saline, 0.1M, 0.1% gelatin, pH 7.0 was prepared by dissolving sodium dihydrogen phosphate (5.38g), disodium hydrogen phosphate (8.67g), sodium chloride (9.00g) (all BDH, Analar grade) and gelatin (1.0g) (BDH)

in one litre of water. Sodium azide (0.1%, v/v) was added to the buffer as a preservative. Norit A charcoal (25 mg) and dextran T-70 (250 mg) (Sigma Chemical Co.) per 100 ml of assay buffer (D/C) was prepared as a fresh suspension just prior to use.

(ii) Steroids

Non radioactive steroids (Sigma Chemical Co.) were dissolved in ethanol and stored at 4°C at a concentration of 1 mg/ml.

Radioactive progesterone (1,2- ^3H -progesterone, $\sim 160\mu\text{Ci}/\text{mg}$, (The Radiochemical Centre, Amersham) was diluted to a concentration of 20-25 $\mu\text{Ci}/\text{ml}$ in benzene and ethanol (9/1, v/v) for storage at 4°C for up to 12 months.

(iii) Materials

Disposable glass test tubes and pipettes were used throughout the assay. The counting fluid used, was prepared by dissolving 10g of 2,5 - diphenyloxazole (PPO) and 0.75g of p-bis (2-(2-phenyloxazolyl)) - benzene (POPOP) in 2.5 litres of toluene (analytical grade, Koch-Light) which was then added to 1.25 litres of Triton X-100 (analytical grade, Koch-Light) and mixed thoroughly until a homogeneous solution was obtained. Glass counting vials were of the low background type (Packard Instrument Company).

(iv) Progesterone antiserum

The antiserum to progesterone (R1-4), generously supplied by Dr. K. K. Dighe (MRC Radioimmunoassay Team, Edinburgh) was raised in a rabbit against the progesterone - 11 α hemisuccinate-bovine serum albumin conjugate. The dilution of antiserum (1/2000, v/v) was that which bound 30-50% of 1,2 - ^3H - progesterone (30,000 d.p.m., ~ 50 pg).

(v) Extraction of progesterone from plasma

Aliquots of plasma (0.2 ml) were pipetted into 125 X 15 mm glass test tubes. The extraction was carried out by vigorous mechanical

('vortex') mixing of the plasma with 20 volumes of petroleum ether for at least 60 sec. After clear separation of the 2 phases, the lower phase (plasma) was quick-frozen by dipping in alcohol containing chips of dry ice. The petroleum ether was then decanted into a 75 X 9 mm test-tube partially submerged in a water bath at 60°C and evaporated to dryness under nitrogen.

(vi) Radioimmunoassay

The dried residue from the plasma extraction was redissolved in 0.1 ml buffer. For the measurement of progesterone in follicular fluid or culture medium, 0.1 ml aliquots (either undiluted or diluted from 1/10 to 1/1000, v/v, with buffer), were added to 75 X 9 mm test tubes and assayed without extraction. To replicate aliquots (0.1 ml) of the samples to be assayed, 0.1 ml of diluted antiserum (1/2000, v/v) in buffer was added to each tube and mixed, followed by 0.1 ml of buffer containing 30,000 d.p.m. of 1,2-³H - progesterone and further mixing. A standard curve was constructed for each assay by serially diluting with buffer a solution of progesterone (100 ng/ml) previously prepared in buffer so that a series of tubes contained known amounts of progesterone, each in triplicate and ranging from 10 to 5000 pg. The incubation volume for the standard curve assay tubes after the addition of antiserum (0.1 ml) and radioactive progesterone (0.1 ml) was kept the same as the unknowns, by adding further amounts of buffer if necessary, so that the final volume in each tube was 0.3 ml. A further six tubes were added to the assay with each containing 0.2 ml assay buffer and 0.1 ml of 1,2-³H - progesterone; three were used to evaluate the blank of the assay (water blank) while the other three were used as controls so that the average amount of radioactive progesterone added to each tube could be checked at the end of the assay.

After incubation at 4°C overnight, 1.0 ml of D/C suspension at 4°C was added to the tubes, which were then 'vortex' mixed and centrifuged (2,500 rpm) for 15 min. at 3-4°C. The same procedure was adopted for the three radioactive control tubes except that 1.0 ml of buffer was added instead of the D/C suspension. The supernatant was decanted into counting vials, 10 ml of scintillation fluid was then added and the contents mixed. The vials were allowed to equilibrate in the scintillation counter for 2 hours at 4°C prior to counting.

(vii) Counting

The liquid scintillation counter (Packard, Model 3375) had a counting efficiency of 30% for ^3H in the system described with a background of 18-20 cpm. Samples were counted for sufficient time so that the counting error was less than 2% (i.e. ~10 mins).

(viii) Calculations

The concentration of progesterone in aliquots of the unknown samples was calculated by interpolation on the standard curve. The three water samples treated like the unknowns, served as a check on the blank of the system. The blank values were consistently below the sensitivity of the assay in all but two of the 192 separate assays conducted. In the two assays where the blank values were unacceptable the cause was attributed to incorrect amounts of D/C used in the separation procedure and these results were discarded.

(ix) Effect of time of incubation

No significant alteration in binding was observed after 4h incubation at 4°C (Fig. 3). However overnight incubations of 16-18h were chosen for convenience.

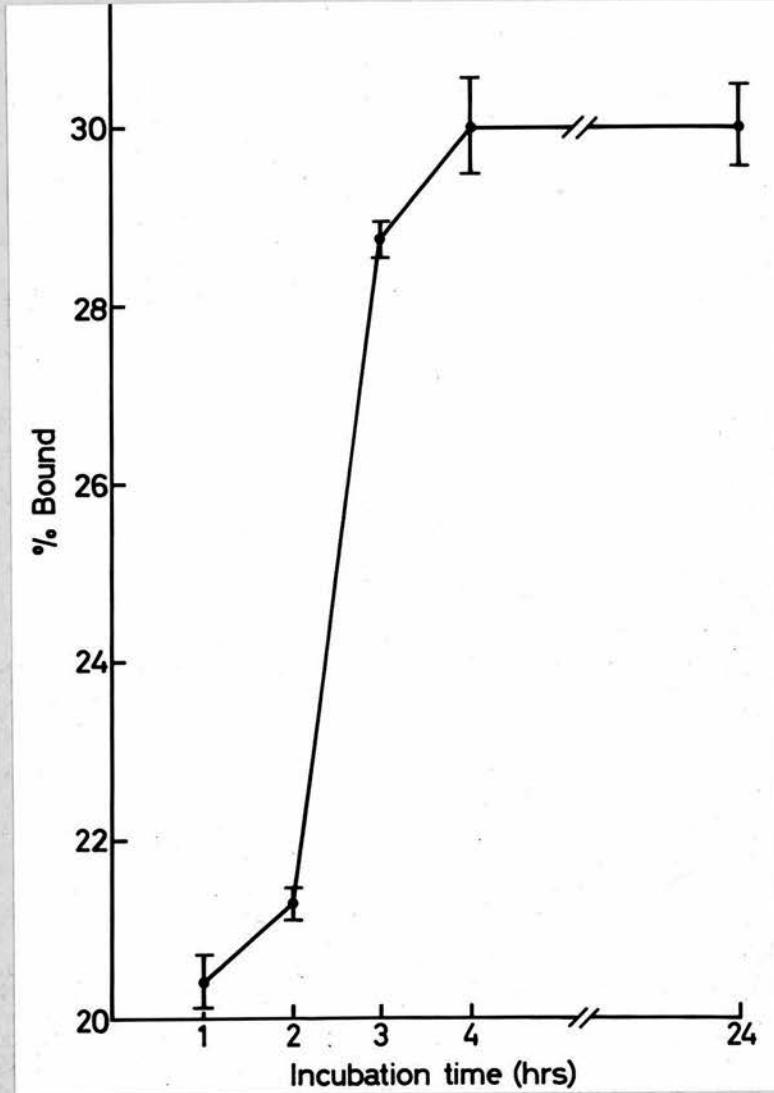


Fig. 3. Effect of time on the binding of 1, 2 - ^3H - progesterone to progesterone antiserum. Vertical bars = ± 1 S.E.M. Each point represents the mean of 10 observations.

(x) Sensitivity

For the standard curve, the percent counts/min (cpm) of bound progesterone was plotted against the logarithm of the dose of progesterone added (Fig. 4). The cpm bound when unlabelled progesterone was not added was defined as 100%. At the 95% confidence limit, 50 pg was significantly different from zero pg and the coefficient of variation at each point of the standard curve was less than 5%. The sensitivity varied from 50 to 80 pg of progesterone.

(xi) Effect of "stripping" by the dextran-charcoal (D/C) separation procedure.

This was evaluated by comparing the percent of radioactive progesterone remaining in the supernatant (% bound) with the delay in time between addition of the D/C mixture and centrifugation. This study was carried out using three different concentrations of charcoal (50, 100 & 250 mg respectively in 100 ml of buffer) while keeping the concentration of dextran (25 mg/100 ml) constant. (Fig.5). Concentrations of charcoal in excess of 250 mg/100 ml were not examined since some difficulty was found in pipetting this suspension freely. The 250 mg charcoal + 25 mg dextran/100 ml mixture proved to be the most suitable for the separation of the 'bound' and 'free' progesterone provided that the time delay between addition of the D/C and centrifugation was less than 8 min. Investigation of the time delay in decanting the supernatant following centrifugation revealed no significant alterations in the percent of progesterone bound provided the assay tubes remained at 4°C.

(xii) Purity of 1,2-³H - progesterone

The purity of the 1,2-³H - progesterone was checked on receipt of

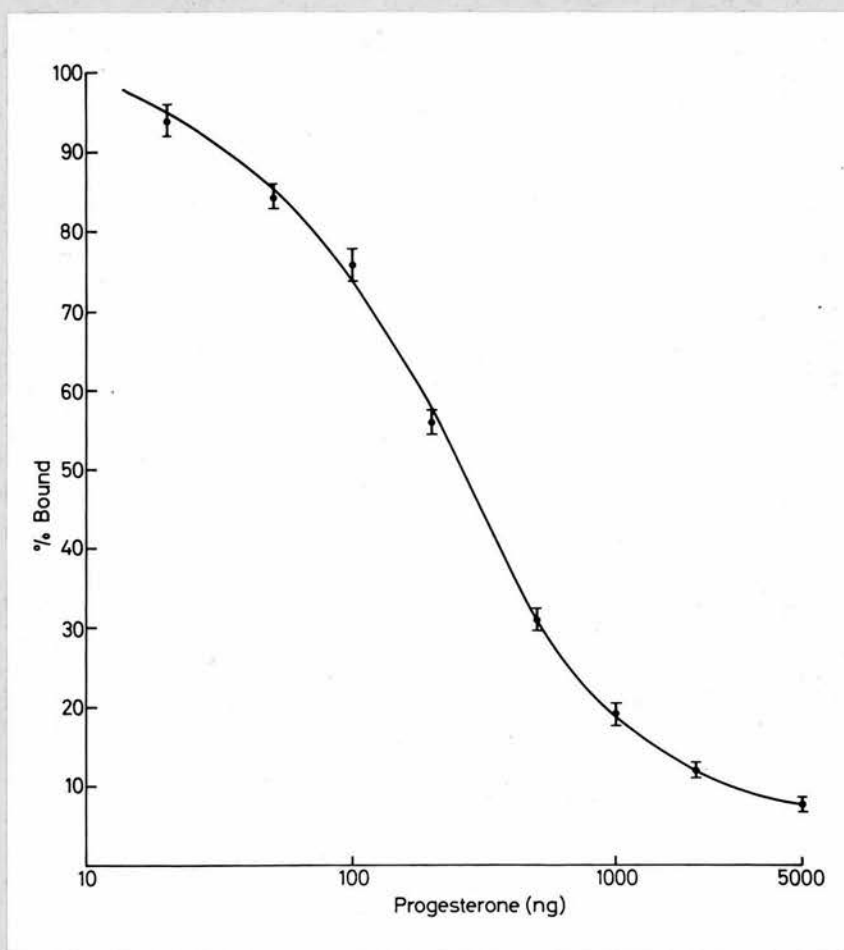


Fig. 4. Standard curve for progesterone. Vertical bars = ± 1 S.E.M.

Each point represents the mean of 6 observations.

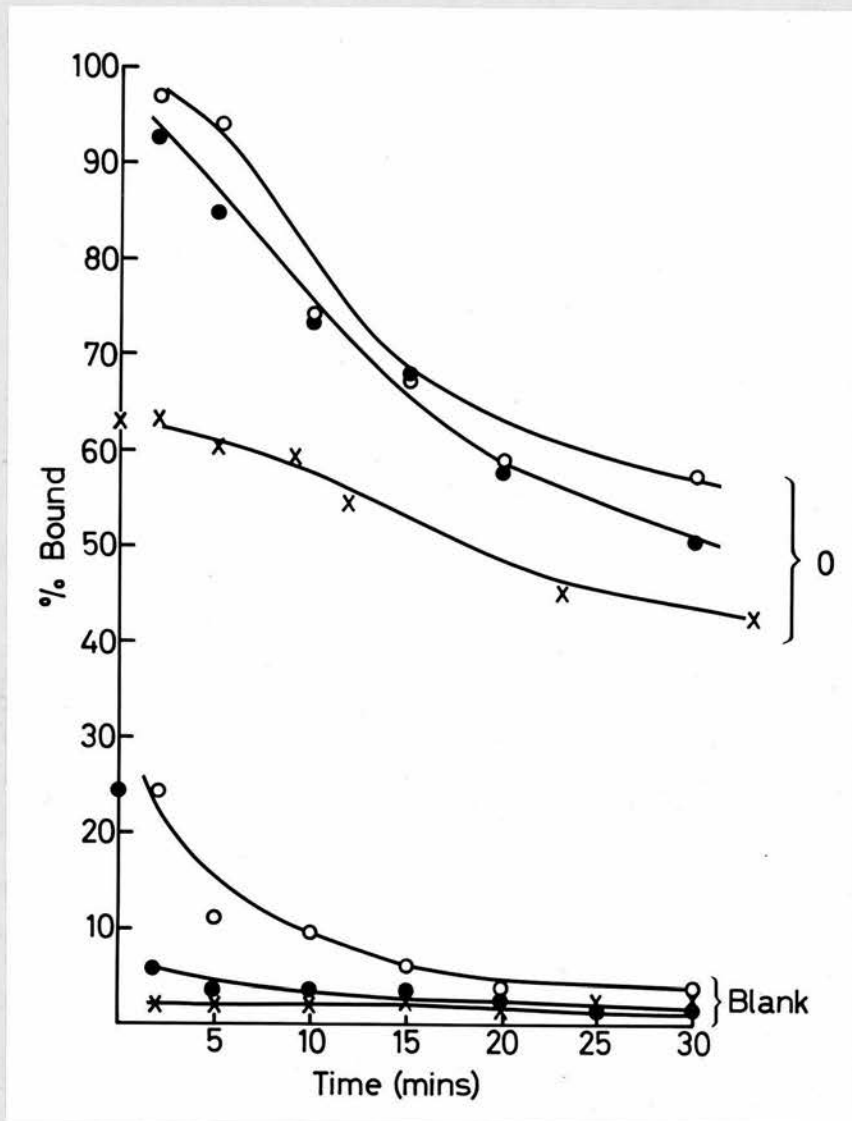


Fig. 5. Effect of 'stripping' with dextran-charcoal.

O—O = 50 mg charcoal

●—● = 100 mg charcoal

X—X = 250 mg charcoal

Blank refers to the amount of radioactivity remaining in the supernatants after dextran-charcoal treatment of assay tubes which previously contained 1, 2 - ^3H - progesterone + buffer but no antiserum to progesterone; O refers to the amount of radioactivity remaining in the supernatants of those assay tubes which previously contained 1, 2 - ^3H - progesterone + antiserum to progesterone. Each point represents the mean of triplicate experiments.

the sample and routinely at approximately 3 monthly intervals using column chromatography (Murphy, 1970, 1971). A 30 X 0.9 sephadex LH-20 column was prepared using a water-saturated chloroform-heptane-ethanol (50/50/0.25, v/v/v) mixture as solvent.

An aliquot of the radioactive progesterone in a solution of benzene and ethanol (9/1, v/v) was evaporated to dryness, redissolved in the chromatographic solvent (0.2 mls) and transferred to the top of the previously washed sephadex column. One ml eluates were collected directly into liquid scintillation vials and evaporated to dryness. The dried residue was redissolved in scintillant and the amount of radioactivity in each vial determined. A typical elution profile for radioactive progesterone is shown in Fig. 6. The progesterone isotope retained $\geq 93\%$ of its purity for at least 12 months when kept at 4°C in benzene and ethanol (9/1, v/v).

(xiii) Recovery

The recovery of progesterone from each plasma sample was assumed to be $> 90\%$ and in routine assay procedures the concentration of progesterone in peripheral plasma was not corrected for recovery. This assumption was made after testing the three factors which were thought to influence the quantitative recovery of progesterone:

- (1) time taken in mixing the plasma-petroleum ether layers;
- (2) mass of progesterone to be recovered and (3) loss of progesterone through adsorption to glass.

To evaluate the first two factors, $1,2\text{-}^3\text{H}$ - progesterone in $10\mu\text{l}$ of the buffer was added to a 0.2 aliquot of plasma mixed and subsequently extracted with petroleum ether as described above except that the petroleum ether was decanted directly into a scintillation vial and the radioactive counts recovered were compared to those added to the original plasma sample. The effect of the time taken in mixing the

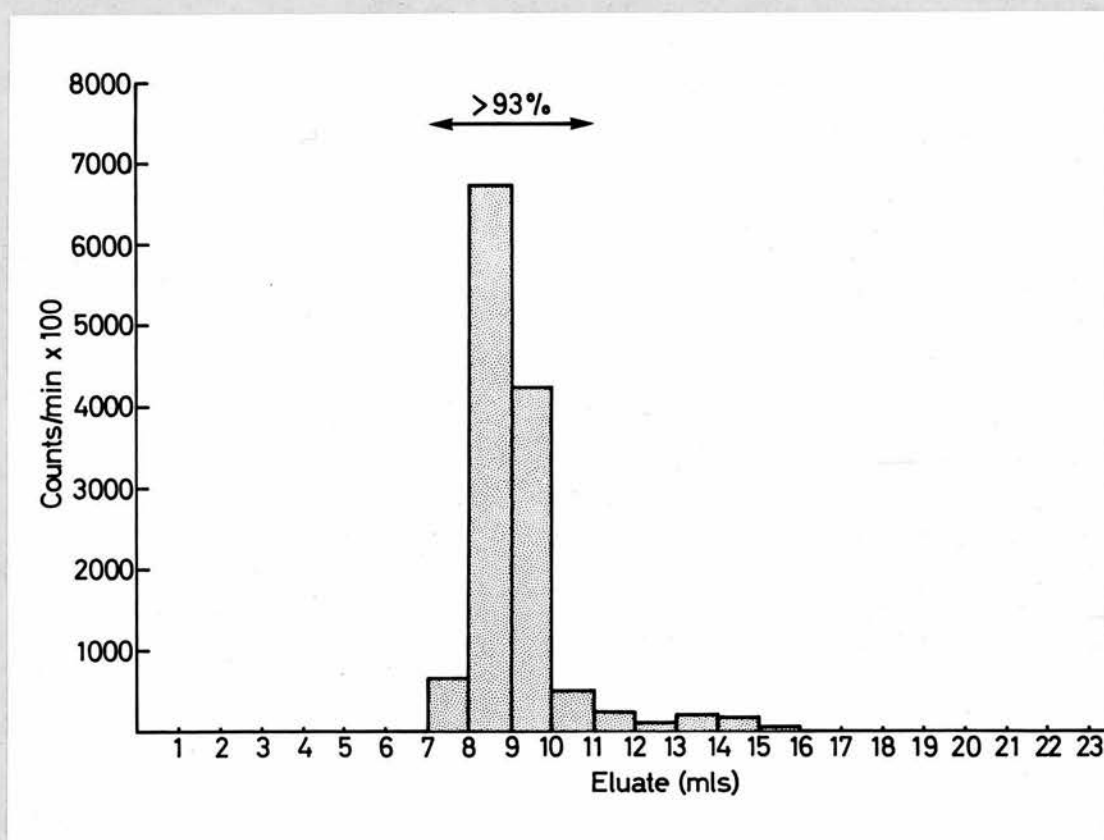


Fig. 6. Elution profile of radioactive progesterone. Progesterone was eluted through a sephadex LH-20 column (30 X 0.9 cm) using a water saturated, chloroform-heptane-ethanol mixture (50/50/0.25, w/v/v) as solvent.

plasma-petroleum ether layers on the recovery of varying amounts of progesterone (expressed as a percentage of the amount originally added to plasma) is shown in Table 3.

Table 3. Effect of mixing time on the mass of progesterone recovered.

Progesterone (pg)	<u>Percent progesterone recovered</u>				
	<u>Mixing Time (sec)</u>				
	(10)	(30)	(60)	(120)	(240)
(50)	73.9	87.8	96.7	97.8	96.3
(145)	80.4	90.1	98.6	98.4	17.9
(1450)	82.2	87.6	100.1	99.7	98.6

The mass of progesterone was assessed from the specific activity of the radioactive progesterone. Each result is the mean of triplicate experiments.

Provided the petroleum ether-plasma layers were mixed thoroughly ('vortex' mixed) for at least 60 sec. a recovery of $98.4 \pm 0.9\%$ (mean \pm s.e.m., n=9) was achieved, irrespective of the amount of progesterone in plasma.

To evaluate the third factor (adsorption of progesterone to glass) known amounts of radioactive progesterone 50, 145 or 1450 pg in 10 μ l of assay buffer were added to 0.2 ml plasma samples on six different occasions over an 18 months period. These samples were extracted as described above and incubated in 0.2 ml buffer and 0.1 ml of antiserum as described, but prior to the separation procedure, 1.0 ml of buffer was added to these samples which were then decanted into scintillation vials and counted to determine the amount of radioactive progesterone which remained in solution.

Their mean recoveries were $92.1 \pm 1.9\%$ (mean \pm s.e.m., n=18) for 50 pg radioactive progesterone added, $19.7 \pm 0.7\%$, n=18, for 145 pg added and 93.1 ± 1.6 , n=18 for 1450 pg added. The small loss ($\sim 6\%$) in recovery after proceeding through the assay until the separation step, when compared to recovering the radioactivity directly from plasma into a counting vial, was attributed to some progesterone being adsorbed to the walls of the test tube.

(xiv) Specificity

The specificity of an assay system relates to the ability of the antiserum to respond only to the compound which the assay is intended to quantify. A large number of C18, C19 and C21 steroids were tested for their ability to displace the radioactive progesterone from the antiserum.(Table 4). The cross-reactions were expressed as a percentage after dividing the mass of unlabelled progesterone displacing 50% of the radioactive progesterone by the mass of

unlabelled steroid displacing 50% of the radioactive progesterone. The cross-reaction of progesterone was taken as 100%. Apart from the hapten* components of the antigen used to raise the antiserum (i.e. 11α -hydroxyprogesterone and progesterone- 11α -hemisuccinate) only 3 other progestins showed significant cross-reaction (see Table 4). None of these polar cross-reacting steroids were likely to interfere significantly with the measurement of progesterone, since if they were present in plasma, they were unlikely to be recovered quantitatively using petroleum ether as a solvent (Dight & Hunter, 1974). The available evidence suggests that human granulosa cells in tissue culture secrete predominantly progesterone and pregnenolone but little if any of the potentially cross-reacting C18, C19 or C21 steroids (Channing, 1969a, and personal communication). Since pregnenolone has only a low cross-reaction with the antiserum which was used, the radioimmunoassay of unextracted aliquots of culture medium is probably specific for progesterone. The measurement of progesterone in human follicular fluid without prior extraction has not previously been reported. The C18, C19 and C21 steroids present in human follicular fluid in relatively high concentrations (other than progesterone) are 17α -hydroxyprogesterone, androstenedione, testosterone, oestradiol- 17β and oestrone, and none of these steroids significantly cross-reacts ($\leq 2\%$) with the antiserum used (Short & London, 1961; Short, 1962b, 1964b; Giorgi, 1963a, b, 1965; Edwards, Steptoe, Abraham, Walters, Purdy & Fotherby, 1972; Baird & Fraser, 1974b; de Jong, Baird & Van der Molen, 1974; Sanyal et al., 1974).

*Hapten: Steroids as such are not immunogenic; but when covalently coupled to protein carriers, they become immunogenic acting as 'haptens'.

Table 4.

Specificity of the rabbit antiserum to
progesterone-11 α - hemisuccinate-bovine serum albumin (R1-4)

<u>Compound</u>	<u>Cross-Reaction (%)</u>
Progesterone	100.0
Progesterone-11 α - hemisuccinate	85.20
11 α -Hydroxyprogesterone	28.62
11 β -Hydroxyprogesterone	10.00
11 - Oxoprogesterone	15.00
6 β -Hydroxyprogesterone	0.50
20 α -Hydroxypregn-4en-3-one	0.16
20 β -Hydroxypregn-4en-3-one	14.10
5 α -Pregnan-3,20 dione	7.00
5 β -Pregnan-3,20 dione	1.25
17 α -Hydroxyprogesterone	1.33
5 β -Pregnane-3 -ol-20-ONE	< 0.005
5 α -Pregnane-3 -20 -diol	< 0.005
Pregnenolone	0.02
11-Deoxcortisterone	0.66
11-Deoxycortisol	0.09
Cortisol	1.91
Androst-4-ene,3,17-dione (Androstenedione)	0.03
5 -pregnane-3 -20 -diol (Pregnanediol)	< 0.005
Testosterone	0.02
Oestradiol-17 β	< 0.005
Oestriol	< 0.005
Oestrone	< 0.005

(xv) Precision

The within-assay precision for the assay of progesterone for all of the biological fluids used were evaluated by duplicate measurements of the same samples in the same assay. The coefficient of variation (CV) of the results of duplicate determinations from their means was estimated by the following formulae (Snedecor, 1946):

$$CV = \sqrt{\frac{\sum d^2}{2n}}$$

where $d = \left[\frac{\text{highest value in each duplicate}}{\text{lowest value of same duplicate}} - 1 \right] \times 100$

n = number of duplicate determinations

From duplicate determinations of progesterone in peripheral plasma (n = 48, range 0.5 to 9.6 ng/ml), follicular fluid (n = 50, range 8 to 2,200 ng/ml), or culture medium containing 20% calf serum (n = 64, range 50 - 6000 ng/ml) the CV was 6.6%, 8.3% and 5.0% respectively.

The between-assay variance was evaluated by duplicate measurements of the same sample in two different assays. The CV in 30 duplicate determinations of either plasma, follicular fluid or culture medium containing 20% calf serum was 14.8%, 16.9% or 6.8% respectively.

(xvi) Validation of the assay for follicular fluid

The accuracy of the assay was determined by the addition of known amounts of progesterone to a pool of follicular fluid so that the final concentrations were 60, 100, 200, 550, 1000 & 2500 ng/ml. The mean results of assays conducted in quadruplicate at two different dilutions (1/100 and 1/1000) and corrected for the dilution factor are shown in Fig. 7. The calculated linear regression equation is $Y = 0.92 X + 23$ (X = concentration of progesterone added to the medium; Y = estimated value by the assay; n = 10).

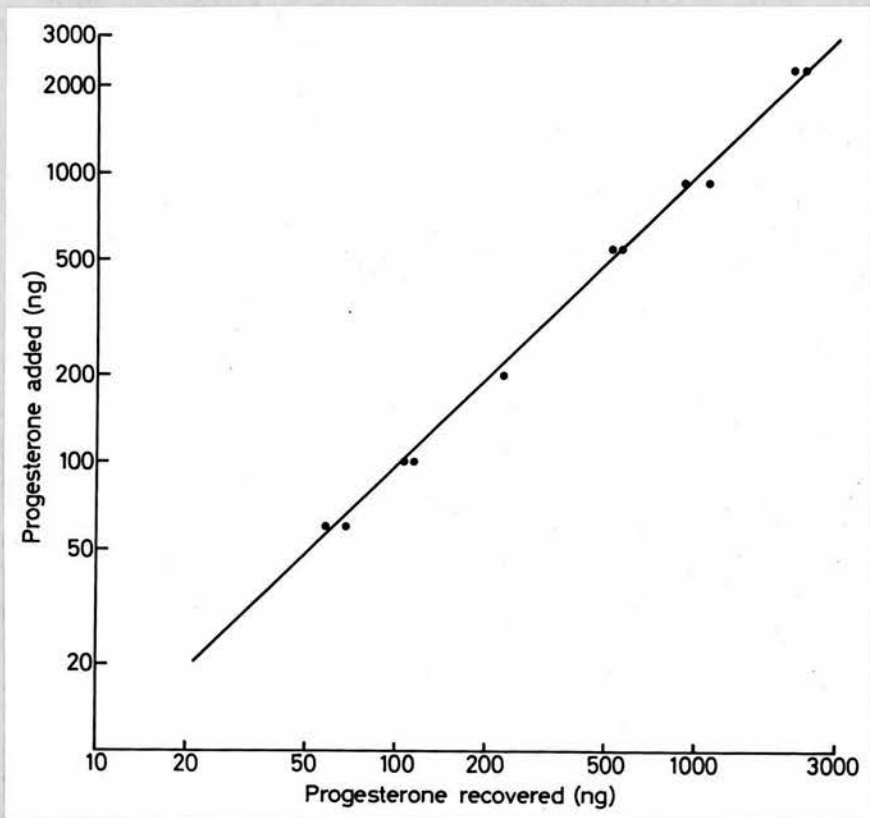


Fig. 7. Accuracy of the progesterone assay on unextracted samples of follicular fluid containing known amounts of progesterone. Each point is the mean of quadruplicate determinations.

Table 5. Comparison of the results of radioimmunoassay for progesterone in extracted versus unextracted follicular fluid.

<u>Progesterone (ng/ml)</u>				
<u>Assay (without extraction)</u>	<u>Progesterone added</u>	<u>Expect</u>	<u>Extracted</u>	<u>Unextracted</u>
55	100	155	132	165
74	50	124	110	125
520	50	570	545	545
1,600	1,000	2,600	2,700	2,300
17	100	117	117	112
5	50	55	52	68
66	250	316	324	311

Each result is the mean of triplicate determinations

A comparison of the results of assays for follicular fluid extracted with petroleum ether (20/v, v/v) and unextracted is shown in Table 5.

These results confirm the accuracy, specificity and precision of the radioimmunoassay technique for the measurement of progesterone in unextracted aliquots of follicular fluid.

(xvii) Validation of the assay for culture medium

The assay of progesterone in culture medium containing 20% human serum was identical to that described for human peripheral plasma. The within-assay and between-assay variance was also similar to that achieved for peripheral plasma. However, the majority of assays of progesterone, in culture media were measured in the presence of 20% calf serum (v/v) (batch 40498, Flow Laboratories, Irvine, Scotland) and were estimated without prior extraction of the media. The accuracy of the assay was determined by the addition of known amounts of progesterone to a pool of culture medium so that the final concentrations were 100, 200, 500, 1000, 2000 and 5000 pg/ml. The results of assays at these dilutions are shown in Fig. 8 for which the calculated linear regression equation is $Y = 0.99 X + 10.90$ where X = concentration of progesterone added to the medium; Y = estimated value by the assay (n = 40).

A comparison of the results of assays for medium extracted with petroleum ether (20/1, v/v) and unextracted medium is shown in Fig. 9. Regression analysis of the results gave the equation $Y = 0.99 X + 0.75$ where X = concentration of progesterone in unextracted medium and Y = the value obtained after extraction (n = 18). Assays were also carried out on extracted and unextracted medium after the addition of known amounts of progesterone. The

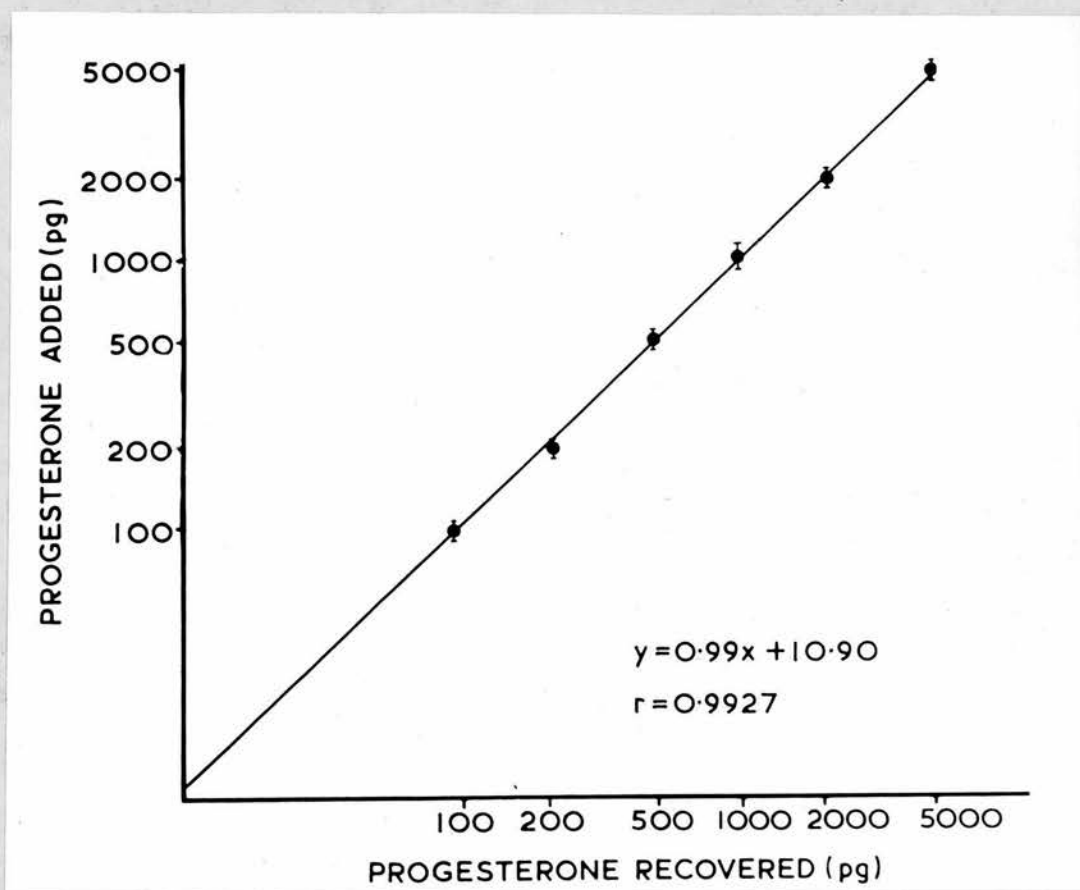


Fig. 8. Accuracy of the progesterone assay on unextracted samples of culture medium containing 20% calf serum (v/v) and known amounts of progesterone. Vertical bars = ± 1 standard deviation. Each point is the mean of at least 6 determinations.

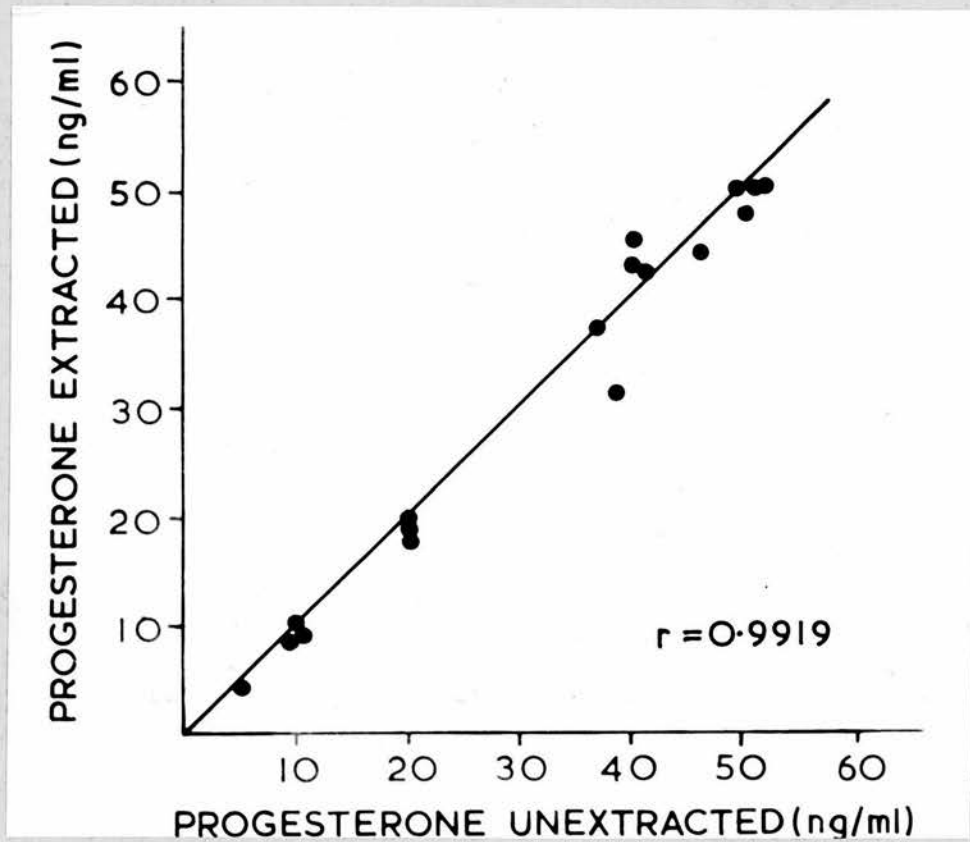


Fig. 9. Comparison of the results of progesterone assays for culture medium extracted with petroleum ether (20/1, v/v) and unextracted culture medium.

results which are shown in Table 6 indicate that either method is acceptable.

The assay of culture medium without extraction appears to be applicable only when the culture medium contains calf serum since a similar analysis of the assay procedure for culture medium containing either human or lamb serum produced spurious results (see Table 7).

Androstenedione

Antiserum for the assay of androstenedione was raised in rabbits immunised with 11α -hydroxy-4-androstene-3,17 dione-11-hemisuccinate coupled to bovine serum albumin (supplied by Dr. W. Schopman, Rotterdam, Holland). Cross-reactions of other steroids in the assay were : 11α -hydroxyandrostenedione (36%), andrenosterone (43%) and testosterone (0.3%).

The assay for androstenedione in plasma and follicular fluid differed from that described by Baird, Burger, Heaven-Jones & Scaramuzzi (1974) only in the method of extraction. For estimation of androstenedione in plasma, 0.1 ml aliquots containing a tracer amounts of $1,2\text{-}^3\text{H}$ androstenedione (2000 dpm \approx 5 pg) was extracted with 16 volumes of hexane and ether, 4/1 (v/v). The organic layer which was separated from the aqueous layer by quick-freezing in dry ice was transferred to a 28 X 8 mm alumina column previously washed sequentially with 3.2 ml ethanol, 6.4 ml methanol, 4.8 ml dichloromethane and methanol (1/1, v/v) and 4.8 ml dichloromethane. The sample was allowed to run through the column which was then washed with a further 2.4 ml of hexane and ether (4/1, v/v) and also 6.4 ml of 0.1% ethanol in hexane. The androstenedione fraction was then collected after adding 3.2 ml of 1.0% ethanol in hexane to the

Table 6. Comparison of the results of radioimmunoassay for progesterone in extracted versus unextracted culture medium.

<u>Progesterone (pg/ml)</u>		
<u>Unextracted medium</u>	<u>Extracted medium</u>	<u>Result expected</u>
10	10	0*
98	92	100
200	194	200
476	560	500
980	940	1000
2000	1810	2000
5000	4760	5000

*Blank culture medium. Each result is the mean of triplicate determinations.

Table 7. Comparison of the results of radioimmunoassay for progesterone in unextracted culture medium containing either 20% human, lamb or calf serum.

<u>Progesterone added (ng)</u>	<u>Progesterone measured (ng)</u>		
	<u>Human</u>	<u>Lamb</u>	<u>Calf</u>
1000	250	500	900
500	200	300	450
250	100	160	230
100	40	70	110
50	21	50	50
25	7	21	27
10	-	8	11
5	-	2	4

Each result is the mean of triplicate determinations

column. The sample was evaporated to dryness and redissolved in 0.5 ml phosphate buffer (0.1M, pH 7.0) containing 0.1% gelatin. An aliquot (0.1 ml) was removed for estimating the recovery of 1,2-³H androstenedione through the extraction procedure and aliquots of the remainder were assayed by the radioimmunoassay technique reported elsewhere (Baird *et al.*, 1974).

After it had been established that both the extraction and chromatographic procedures were unnecessary, androstenedione in follicular fluid were assayed directly on 0.1 ml aliquots of diluted fluid (1/50 to 1/100, v/v) in buffer.

The assays in plasma were carried out in duplicate while those for follicular fluid were carried out in triplicate. The minimum detectable level of androstenedione in plasma was 150 pg/ml and in follicular fluid was 8-15 ng/ml.

Testosterone

The antiserum (E01, supplied by Dr. S. A. Tillson, Aliza Corporation, Palo Alto, U.S.A.) for the assay of testosterone was raised in a goat immunised with testosterone-3-oxime coupled to bovine serum albumin. Cross-reactions of other steroids were: 5 α - dihydrotestosterone (25%), oestradiol (0.20%) and androstenedione (0.08%).

The assay for testosterone in follicular fluid was similar to that described by Furuyama, Mayes & Nugent (1970) except that the chromatographic step in the extraction procedure was found to be unnecessary. The concentration of testosterone in follicular fluid was relatively low when compared to the other steroids measured and consequently sufficient volumes of fluid

(after previously assaying other hormones on the same sample) were only available from large follicles (≥ 8 mm). Undiluted extracts of follicular fluid containing tracer amounts of testosterone (3000 dpm \equiv 20 pg) were extracted into 10 volumes of hexane and ether (4/1, v/v) which was evaporated to dryness and the residue dissolved in phosphate buffer (0.1 M, pH 7.0) + 0.1% gelatin for assessment of recovery and the unknown amount by radioimmunoassay (Furuyama et al., 1970). The minimum detectable level of testosterone in follicular fluid was 2 ng/ml. Testosterone was not measured in peripheral plasma.

CHAPTER 3

**CHANGES IN THE CONCENTRATION OF PITUITARY AND
STEROID HORMONES IN FOLLICULAR FLUID OF
HUMAN GRAAFIAN FOLLICLES THROUGHOUT THE MENSTRUAL CYCLE**

INTRODUCTION

There is considerable evidence to suggest that pituitary hormones are obligatory for the later stages of follicular growth, maturation of the oocyte and ovulation (see reviews by Mauleon, 1969; Baker, 1972; see also General Introduction). Although it is possible to correlate changes in gonadotrophins in peripheral plasma with the production of steroids from the ovary, the actions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on the follicle remain obscure. It has been suggested that the gonadotrophins within the follicular fluid itself may have important consequences for follicular steroidogenesis (Channing, 1970a, 1972; Edwards, 1974), growth of the follicle, oocyte maturation and ovulation (Edwards, 1974).

In the present study the concentrations of LH, FSH, prolactin, oestradiol- 17β , progesterone and androstenedione were measured in samples of peripheral plasma and follicular fluid collected from women at varying stages of the menstrual cycle. In addition, testosterone was measured in follicular fluid. It was hoped in this way to investigate the relationships between the concentrations of pituitary and steroid hormones in follicular fluid and to relate them to concentrations in plasma, follicle size and the stage of the menstrual cycle.

RESULTS

The concentrations of hormones in plasma and follicular fluid in subjects with stage 0 carcinoma of the cervix were compared with those of patients with menorrhagia and were not

found to be significantly different. The results from all patients have therefore been pooled.

Concentrations of FSH, LH, oestradiol, progesterone, androstenedione and testosterone in the antral fluid of follicles aspirated in situ were not significantly different from those aspirated after ovariectomy ($P > 0.2$, Student's t test). The results obtained for each hormone from the two methods of collection have therefore been pooled.

Concentrations of prolactin in the antral fluid of follicles aspirated in situ are not reported, since these samples were stored at -20°C for at least 12 months before being assayed and the immunological and biological activity of prolactin in physiological fluids declines with prolonged storage (H. G. Friesen, personal communication). All results were compared using the Student's t test.

Concentration of luteinizing hormone, follicle-stimulating hormone, prolactin, progesterone, oestradiol and androstenedione in peripheral plasma

Mean values (\pm S.E.M.) obtained for the concentrations of LH, FSH, prolactin, progesterone, oestradiol and androstenedione in peripheral plasma with respect to the stage of the menstrual cycle are shown in Fig. 10.

Distribution of the size of excised follicles in relation to the stage of the menstrual cycle

A scatter plot of follicle size in relation to the stage of the menstrual cycle is shown in Fig. 11. The greatest range in follicle sizes (4-20 mm) was found in the late follicular and late luteal phases. The greatest number of 'cystic'

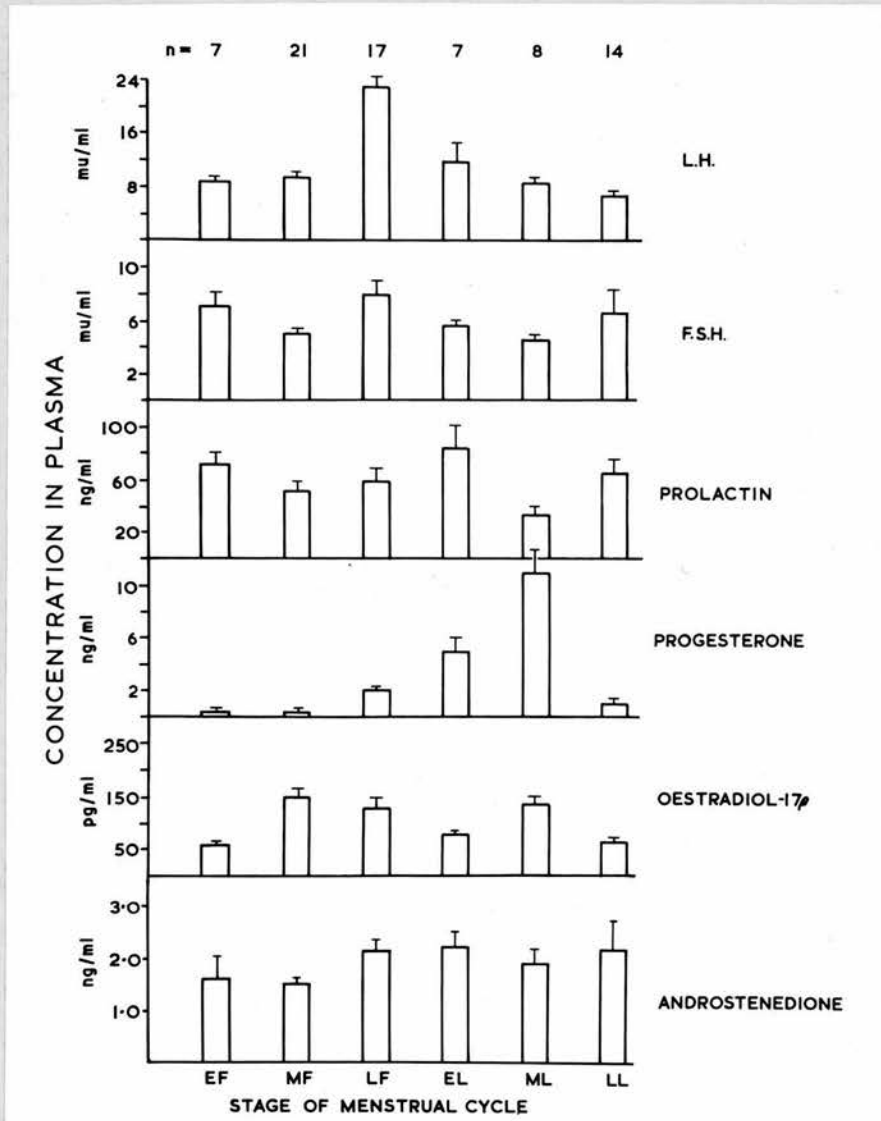


Fig. 10. Mean concentrations of LH, FSH, prolactin, progesterone, oestradiol and androstenedione in peripheral plasma during the menstrual cycle. Vertical lines represent + 1 standard error of the mean (+ 1 S.E.M.). EF, MF, and LF refer to early (still menstruating), mid- and late-follicular phases respectively, while EL, ML, and LL refer to early-, mid- and late-luteal phases respectively. Numbers of observations are given at the top of the figure.

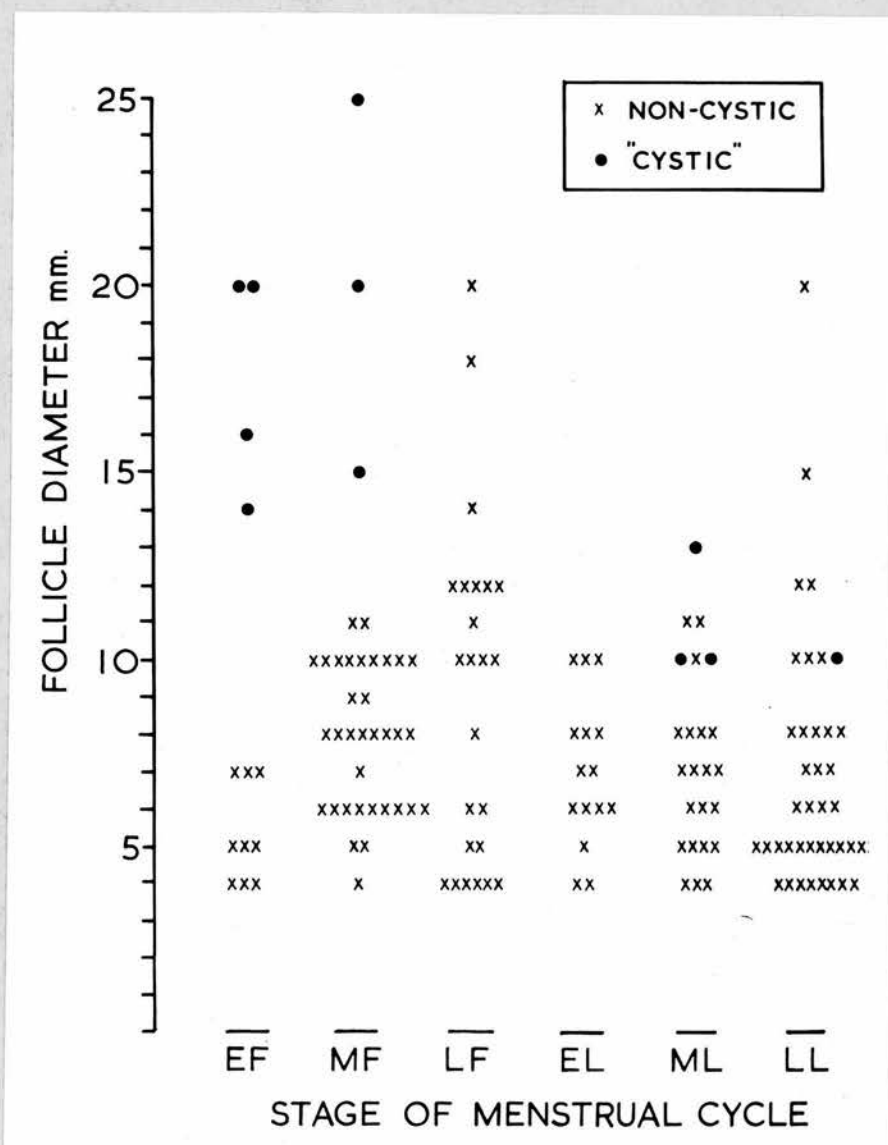


Fig. 11. Distribution of size of excised follicles in relation to the stage of the menstrual cycle. Follicles 4 mm were not collected; •, 'cystic' follicles; x, 'non-cystic' follicles. See Fig. 10 for abbreviations.

follicles (45%) was found in the early follicular phase whereas none was found during the late follicular or early luteal phase.

The concentrations of pituitary and steroid hormones in the follicular fluid of individual Graafian follicles

(i) Follicle-stimulating hormone

The mean concentration of FSH in all antral fluids in relation to size and the stage of the menstrual cycle is shown in Fig. 12. The minimum detectable level of FSH in the smallest follicles (4 mm) was 1.3 mu/ml, which was therefore chosen as the limit of detection for all follicles. The percentage of follicles with detectable FSH at each stage of the cycle is shown in Table 8. In general, the greatest proportion of follicles with detectable FSH was found either during or immediately after the increase in levels in the plasma (Fig. 10 and Table 8). In contrast, the concentration of FSH in some large follicles was high when the plasma concentrations were low (Fig. 10 and Fig. 12). Follicle-stimulating hormone was detectable in a proportion of the smallest follicles examined (see Table 8 and 9). However, at no time during the cycle was the concentration of FSH in follicular fluid more than 60% of the levels found in plasma.

(ii) Luteinizing hormone

The mean concentration of LH in all antral fluids examined with respect to follicle size and the stage of the menstrual cycle is shown in Fig. 13. The percentage of follicles containing detectable concentrations of LH at various stages of the cycle is shown in Table 8; the lowest detectable level

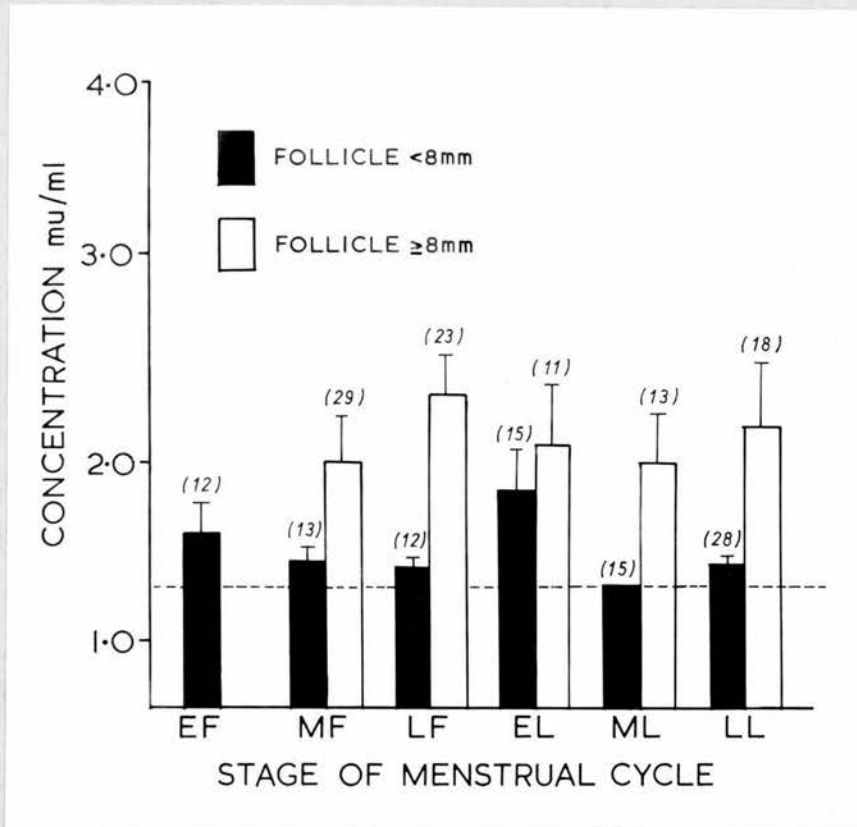


Fig. 12. Concentration of FSH in antral fluid in relation to follicle size and phase of the menstrual cycle. The vertical bars represent + 1 S.E.M. Broken line = minimal detectable level of FSH (1.3 mu./ml). See Fig. 10 for abbreviations. Number of observations in parentheses.

Table 8. Percentage of follicles with detectable levels of luteinizing hormone and follicle-stimulating hormone in relation to size at each phase of the menstrual cycle.

Pituitary hormone (limit of sensitivity)	Follicle size (mm)	EF	MF	LF	EL	ML	LL
FSH (1.3 mu./ml)	≥ 8	*	38.0	82.5	45.5	38.5	28.0
	< 8	33.0	15.5	25.0	46.5	0.0	3.5
LH (2.8 mu./ml)	≥ 8	*	11.0	69.5	25.0	14.0	22.0
	< 8	0.0	0.0	16.5	16.5	0.0	0.0
LH and FSH both present	≥ 8	0.0	11.0	69.5	25.0	14.0	22.0
	< 8	0.0	0.0	16.5	16.5	0.0	0.0

EF, MF and LH refer to early-still menstruating, mid- and late follicular phase respectively, while EL, ML and LL refer to early, mid- and late luteal phase respectively. * = no follicles present.

Table 9. Concentration of FSH in fluid from different sized follicles at each phase of the human menstrual cycle.

(Values are means \pm S.E.M. in mu./ml. Samples less than the detection limit were assumed to have a concentration of 1.3 mu./ml for the purposes of the group mean.)

Stage of menstrual cycle*	Follicle diameter (mm)			
	6	6-8	9-12	12
BF	1.50 \pm 0.09 (6)	2.30 (3)	-	-
MF	1.30 (3)	1.40 \pm 0.05 (18)	2.30 \pm 0.40 (13)	-
LF	1.76 \pm 0.26 (8)	1.68 (3)	2.20 \pm 0.26 (10)	3.20 (3)
EL	1.66 \pm 0.23 (8)	2.10 \pm 0.50 (9)	2.10 (3)	+
ML	1.30 (7)	1.30 (11)	1.30 (3)	-
LL	1.46 \pm 0.13 (20)	1.30 (12)	3.00 \pm 1.10 (8)	3.28 (2)

*For abbreviations see Table 8. Number of observations in parentheses.

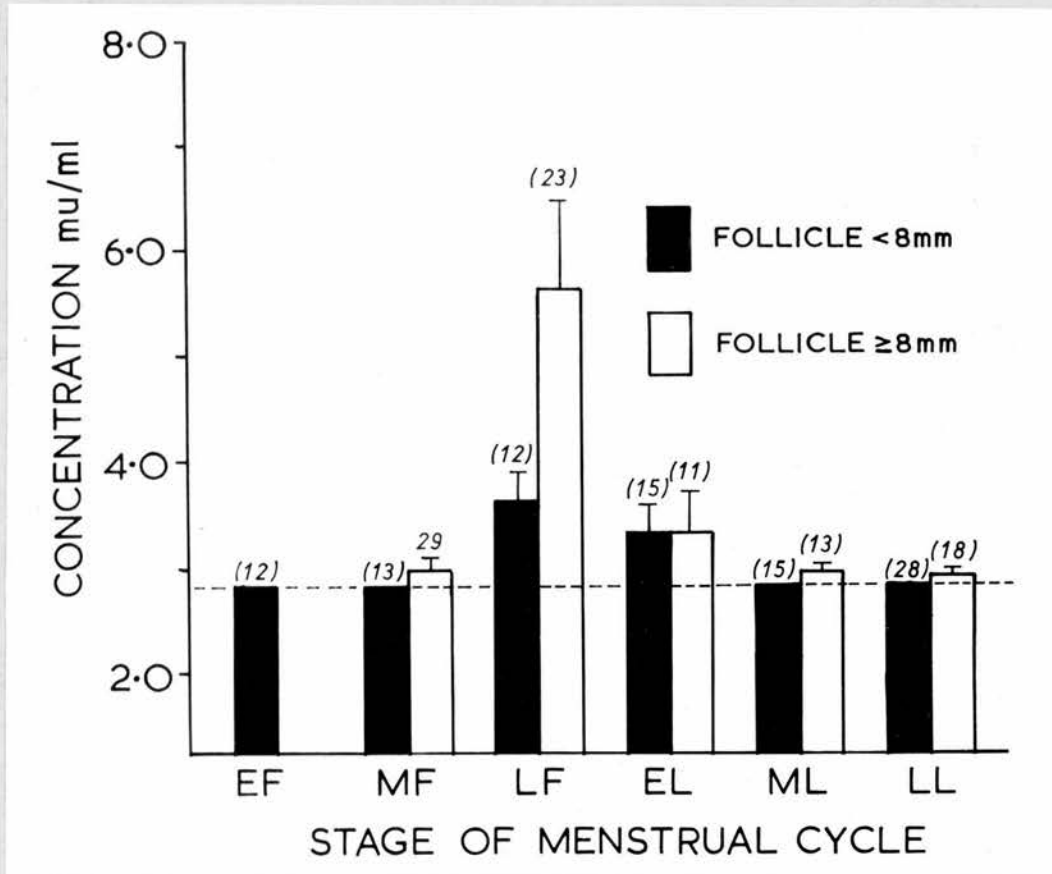


Fig. 13. Concentration of LH in antral fluid in relation to follicle size and phase of the menstrual cycle. The vertical bars represent + 1 S.E.M. Broken line = minimal detectable level of LH (2.8 mu./ml). See Fig. 10 for abbreviations. Number of observations in parentheses.

in the smallest follicles examined (4 mm) was 2.8 mu./ml, which was therefore chosen as the limit of sensitivity. There was a greater proportion of large follicles with detectable LH during the late follicular phase (Table 8), when the concentrations were higher than at any other stage of the cycle (Fig. 13). Luteinizing hormone was not detectable in any follicle of < 8 mm during the early and mid-follicular phases and mid- and late luteal phases. Fluids from 20 early and mid-follicular phase follicles were pooled to give a total volume of 50 μ l, and the concentration of LH was 0.96 mu./ml (detection limit 0.8 mu./ml). Throughout all stages of the cycle the fluid concentration of LH was \leq 30% of that found in peripheral plasma. Furthermore, LH was only found in those follicles that also contained FSH (Table 8).

(iii) Prolactin

Unlike LH and FSH, prolactin was detectable in almost all the follicles examined. The minimum detectable concentration was 1.5 ng/ml. The concentration of prolactin was significantly lower in the large follicles during the late follicular phase than in any other stage of the menstrual cycle ($P < 0.05$) (Fig. 14) with the exception of the large follicles during the early luteal phase. The general pattern of prolactin in antral fluid indicates a progressive fall in concentration during the follicular phase followed by a rise in concentration during the luteal phase.

(iv) Oestradiol

The concentration of oestradiol with respect to size of follicle and stage of the cycle is shown in Fig. 15. The levels of

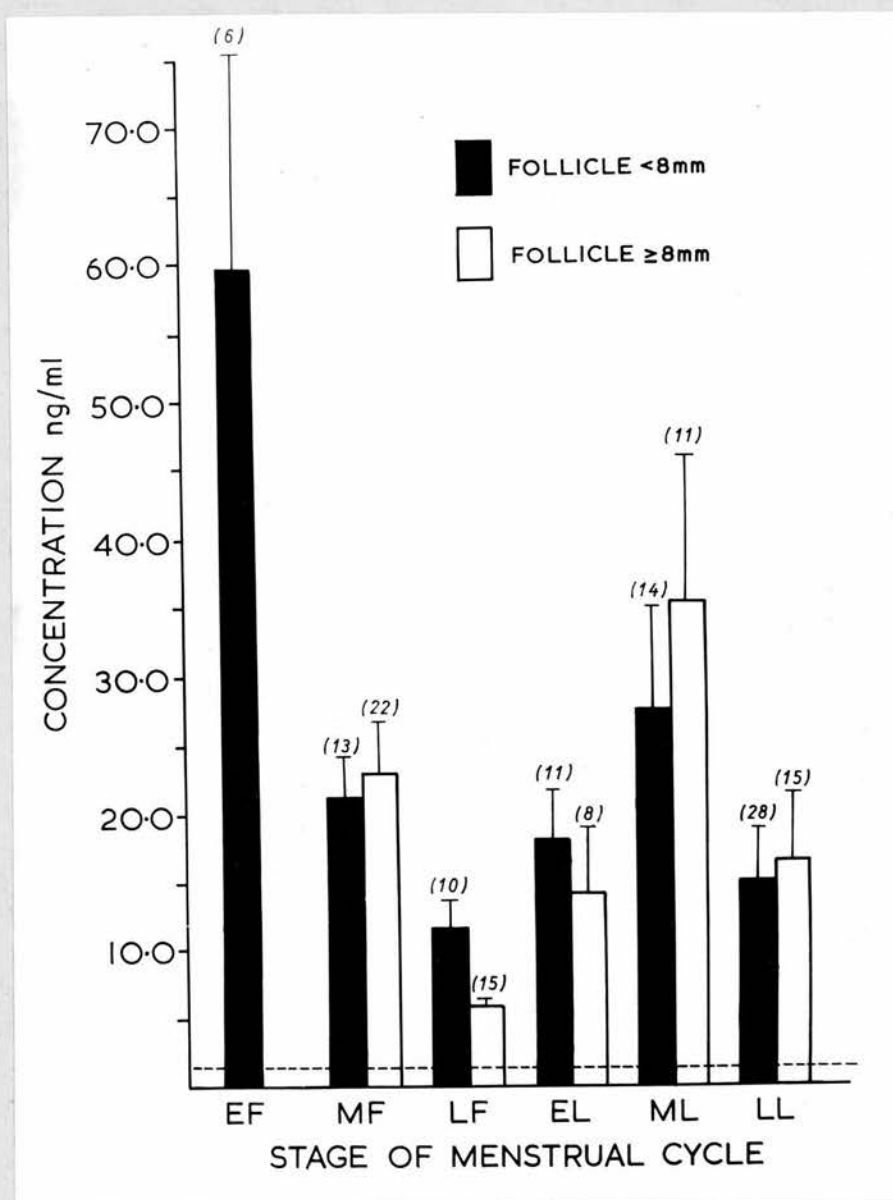


Fig. 14. Concentration of prolactin in follicular fluid in relation to follicle size and stage of the cycle. Vertical bars represent + 1 S.E.M. Minimal detectable level of prolactin (1.5 ng/ml) is indicated by the broken line. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

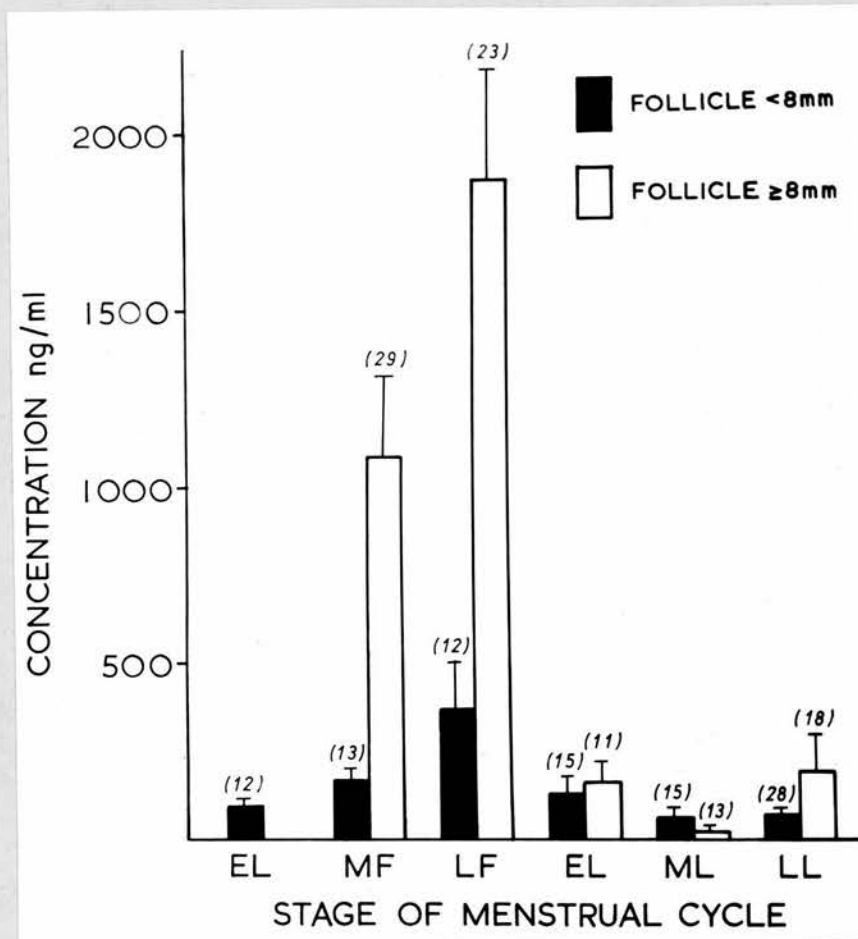


Fig. 15. Concentration of oestradiol in follicular fluid in relation to follicle size and stage of the cycle. Vertical bars represent + 1 S.E.M. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

oestradiol in follicles at all stages of the cycle are between 40 and 40000 times higher than those in peripheral plasma. During the mid and late follicular phases the large follicles contained a significantly higher concentration than that in small follicles at the same phase ($P < 0.001$). During the luteal phase there were no significant differences in the concentration of oestradiol between small and large follicles ($P > 0.05$), and the levels did not exceed those found in follicles during the early follicular phase.

(v) Progesterone

The concentration of progesterone with respect to size of follicle and stage of menstrual cycle is shown in Fig. 16. Levels of progesterone in large follicles during the proliferative phase were significantly higher than those found in the corresponding small follicles ($P < 0.001$). The most dramatic increase was found in the large follicles during the late follicular phase, where the levels were up to 20 times higher than in any other follicle throughout the cycle. By contrast, the small follicles (< 8 mm) during the early luteal phase had a significantly higher concentration of progesterone than any other follicle during the luteal phase ($P < 0.001$).

(vi) Androstenedione

The concentration of androstenedione with respect to size of follicle and stage of menstrual cycle is shown in Fig. 17. The levels of androstenedione in all follicles throughout the menstrual cycle were between 100 to 500 times higher than those in peripheral plasma. Unlike all the other steroids measured, the concentration of androstenedione in follicular fluid was relatively constant throughout the menstrual cycle and not significantly different between

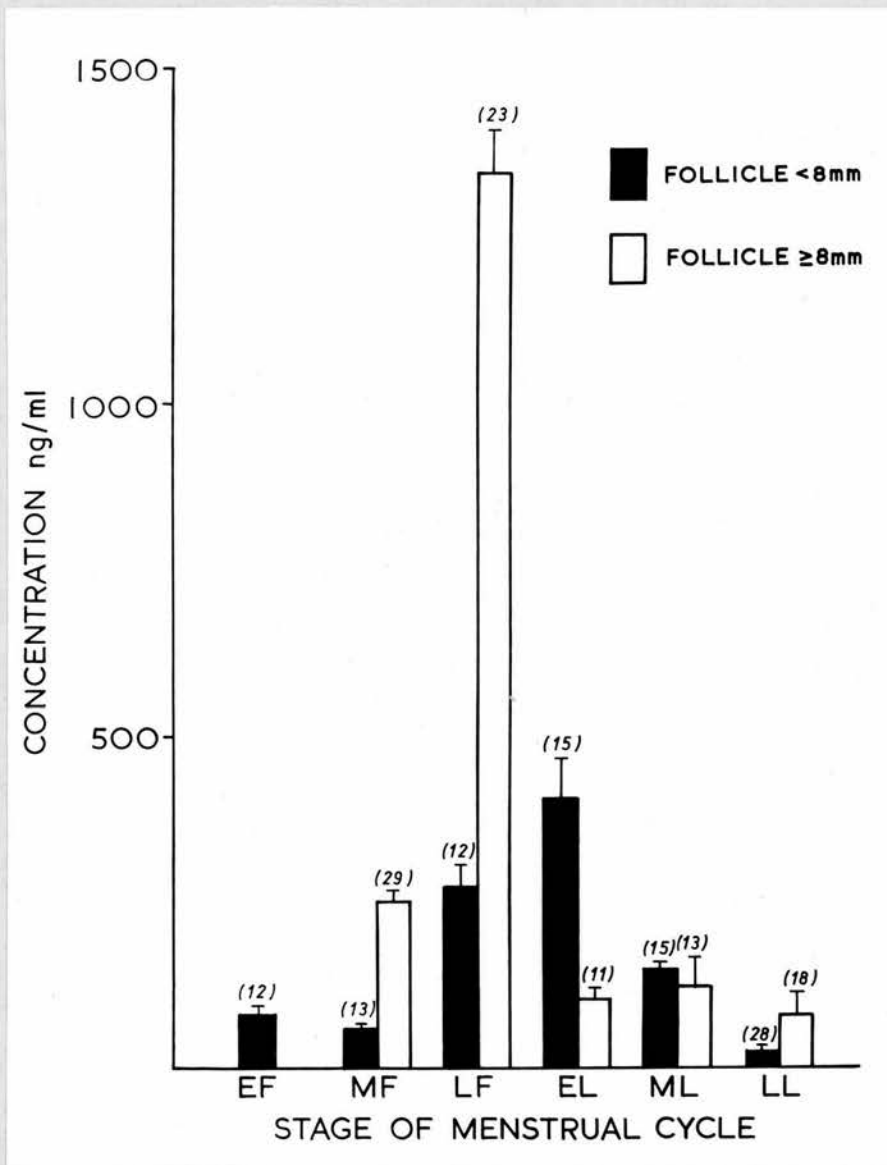


Fig. 16. Concentration of progesterone in follicular fluid in relation to follicle size and stage of the cycle. Vertical bars represent + 1 S.E.M. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

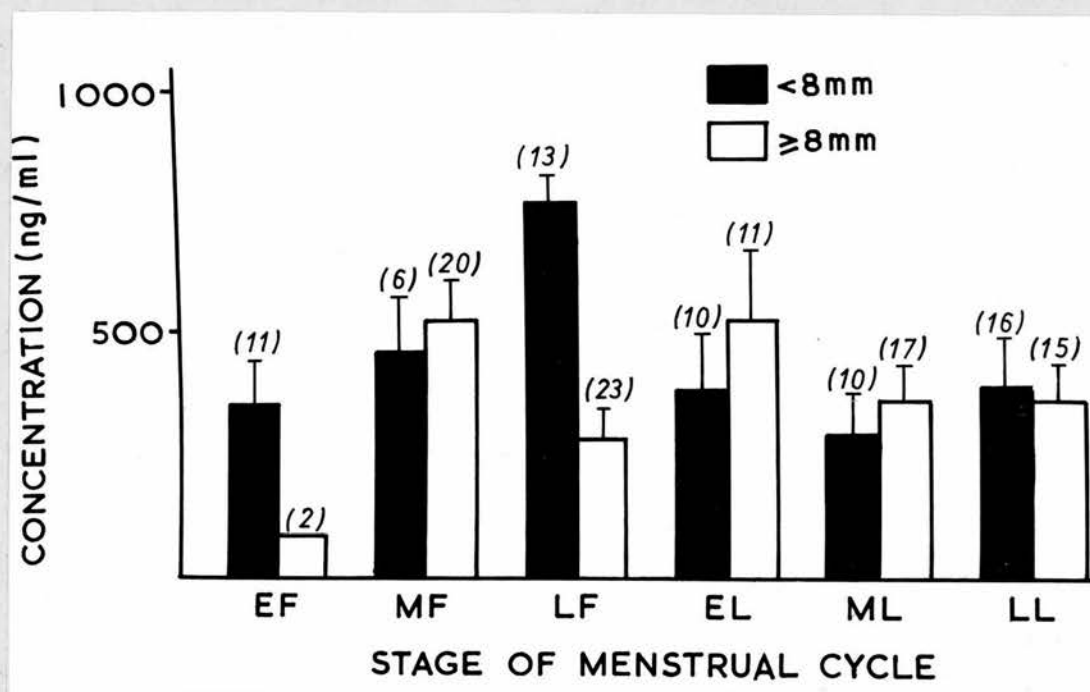


Fig. 17. Concentration of androstenedione in follicular fluid in relation to follicle size and stage of the cycle. Vertical bars represent + 1 S.E.M. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

the large (≥ 8 mm) and small (< 8 mm) follicles. The single exception to this was found during the late follicular phase when the concentration of androstenedione in the large follicles was significantly lower than in the small follicles at this time ($P < 0.05$).

(vii) Testosterone

The concentration of testosterone in large follicles (≥ 8 mm) with respect to stage of menstrual cycle is shown in Fig. 18. The concentration of testosterone in follicular fluid throughout the menstrual cycle was between 10 and 3000 times higher than those in peripheral plasma (Mayes & Nugent, 1968; Vermeulen & Verdonck, 1970). During the early and mid-follicular and mid- and late-luteal phases the mean concentration of testosterone was relatively constant. By contrast during the late follicular phase the mean concentration of testosterone was 2 times higher than at any other stage of the menstrual cycle.

Hormones in the fluid of recently ruptured and 'cystic' follicles

Haemorrhagic fluid was aspirated in vitro from two recently ruptured follicles in subjects whose endometria still showed very late proliferative changes. The concentrations of hormones in the fluids were: progesterone, 8,600 - 10,800 ng/ml; oestradiol, 335 - 210 ng/ml; LH, 9.6 - 7.6 mu./ml; FSH, 7.9 - 6.8 mu./ml; prolactin, 14.6 - 12.1 ng/ml. The levels of LH and FSH were similar to those in peripheral plasma whereas the concentrations of prolactin were about half the peripheral plasma concentrations. The oestradiol and progesterone concentrations were 100 to 1000-fold higher than the concentrations in peripheral plasma. The concentration of progesterone in the fluid was also four to eight times higher than in late follicular phase fluid, but the concentration of oestradiol was at least ten times

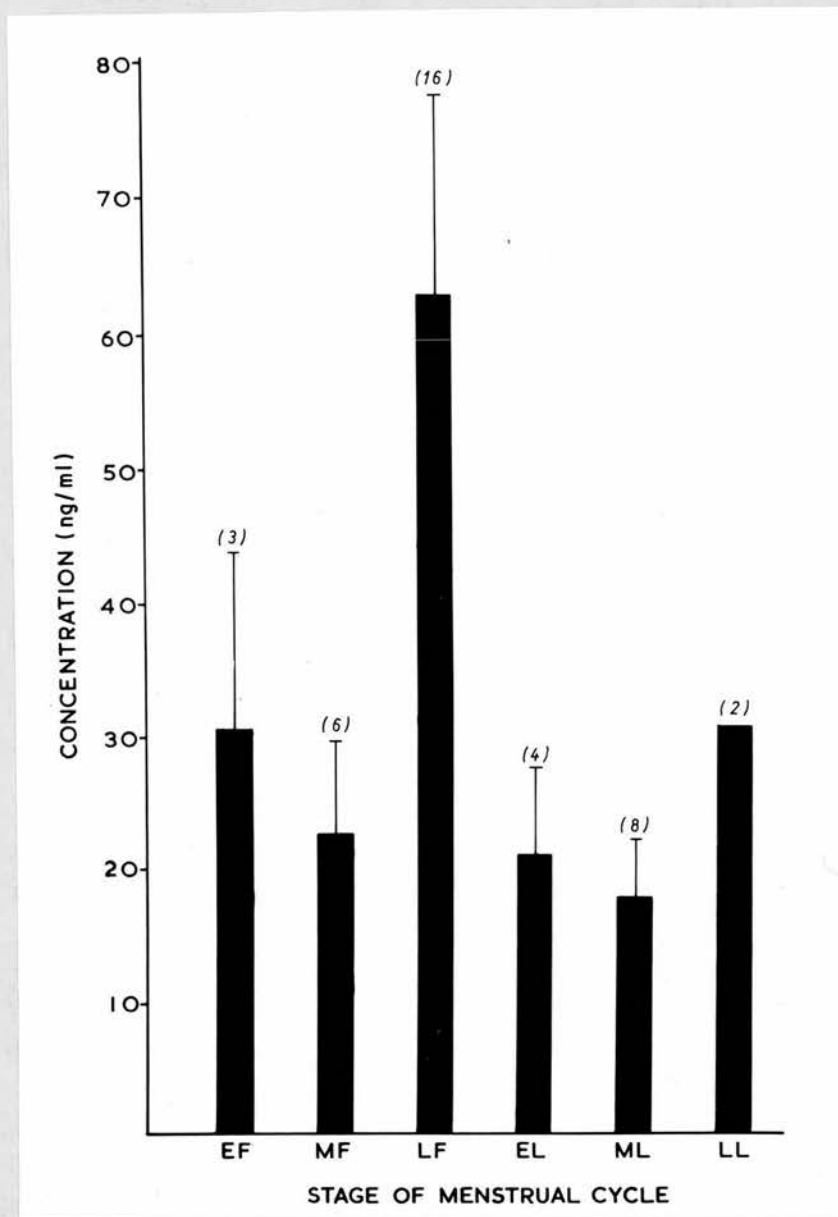


Fig. 18. Concentration of testosterone in follicular fluid of large follicles (≥ 8 mm) with respect to stage of the menstrual cycle. The vertical bars represent $+ 1$ S.E.M. See Fig. 10 for abbreviations. Number of observations in parentheses.

lower.

Data relating to the hormone levels in cystic follicles are shown in Table 10. All these follicles contained low levels of oestradiol and progesterone irrespective of the stage of the cycle, suggesting that they were inactive (Baird & Fraser, 1974b).

Relationship between follicular concentration of hormones

Those follicles which had detectable levels of FSH during the follicular phase without exception contained significantly higher concentrations of oestradiol than those with undetectable FSH (Table 11) (EF, MF, LF; $P < 0.01$, $P < 0.001$, $P < 0.001$, respectively). Conversely, follicles with undetectable FSH contained low levels of oestradiol irrespective of the stage of the cycle. During the luteal phase these correlations no longer held, since follicles with or without FSH had consistently low levels of oestradiol.

A significantly higher concentration of progesterone was found in follicles containing LH (Table 11) (MF, LF, EL, ML, LL; $P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.01$, respectively). All the follicles containing LH and a high concentration of progesterone were ≥ 8 mm diameter and also contained FSH.

During the mid-follicular, late-follicular and the late luteal phase of the cycle the concentration of oestradiol in follicular fluid was correlated with that of progesterone, ($r = 0.44$, $n = 43$, $P < 0.01$; $r = 0.88$, $n = 23$, $P < 0.001$; and $r = 0.49$, $n = 37$, $P < 0.01$, respectively).

The concentrations of oestradiol- 17β and androstenedione measured in the same samples of follicular fluid with respect to follicular size and stage of the menstrual cycle are shown in Fig. 19 and Fig. 20. In small follicles (< 8 mm) throughout the

Table 10. Hormone levels in human cystic follicles.

(Samples less than the detection limit were assumed to have a concentration of 1.3 mu./ml, 2.8 mu./ml, 1.5 ng/ml for FSH, LH and prolactin respectively for the purposes of the group means.)

Stage of cycle*	Follicle size (mm)	LH (mu./ml)	FSH (mu./ml)	Prolactin (ng/ml)	Oestradiol (ng/ml)	Progesterone (ng/ml)
EF	20	2.8	2.9	13.8	9	55
	16	6.02	1.7	39.0	205	20
	14	2.8	1.4	25.2	17	20
	20	2.8	1.2	47.0	40	15
MF	20	2.8	6.6	19.9	18	90
	15	12.0	1.3	23.0	230	17
	25	2.8	2.2	28.2	36	58
ML	10	2.8	1.3	23.0	150	90
	13	10.0	1.3	21.0	104	70
	10	2.8	1.3	21.0	30	80
LL	10	14.0	2.4	3.0	19	48

*For abbreviations see Table 8.

Table 11. Concentrations (a) of oestradiol in the follicular fluid with or without FSH and (b) of progesterone in follicular fluid with or without LH (values are means \pm S.E.M. in ng/ml).

	<u>Stage of menstrual cycle*</u>					
	EF	MF	LF	EL	ML	LL
(a) Oestradiol concentrations						
FSH (> 1.3 mu./ml)	300 \pm 34 (4)	2120 \pm 410 (13)	2180 \pm 310 (22)	135 \pm 40 (12)	33 \pm 26 (5)	211 \pm 200 (5)
FSH (≤ 1.3 mu./ml)	103 \pm 22 (8)	196 \pm 60 (29)	140 \pm 55 (13)	102 \pm 58 (14)	24 \pm 1 (23)	60 \pm 38 (41)
(b) Progesterone concentrations						
LH (> 2.8 mu./ml)	760 (2)	1720 \pm 280 (18)	715 \pm 60 (4)	196 (2)	320 \pm 40 (4)	
LH (≤ 2.8 mu./ml)	110 \pm 31 (12)	96 \pm 33 (40)	352 \pm 84 (17)	66 \pm 11 (22)	111 \pm 15 (26)	100 \pm 20 \dagger (42)

*For abbreviations see Table 8. Number of observations in parentheses. Limit of detection for FSH is 1.3 mu./ml and for LH is 2.8 mu./ml.

\dagger No follicles containing LH.

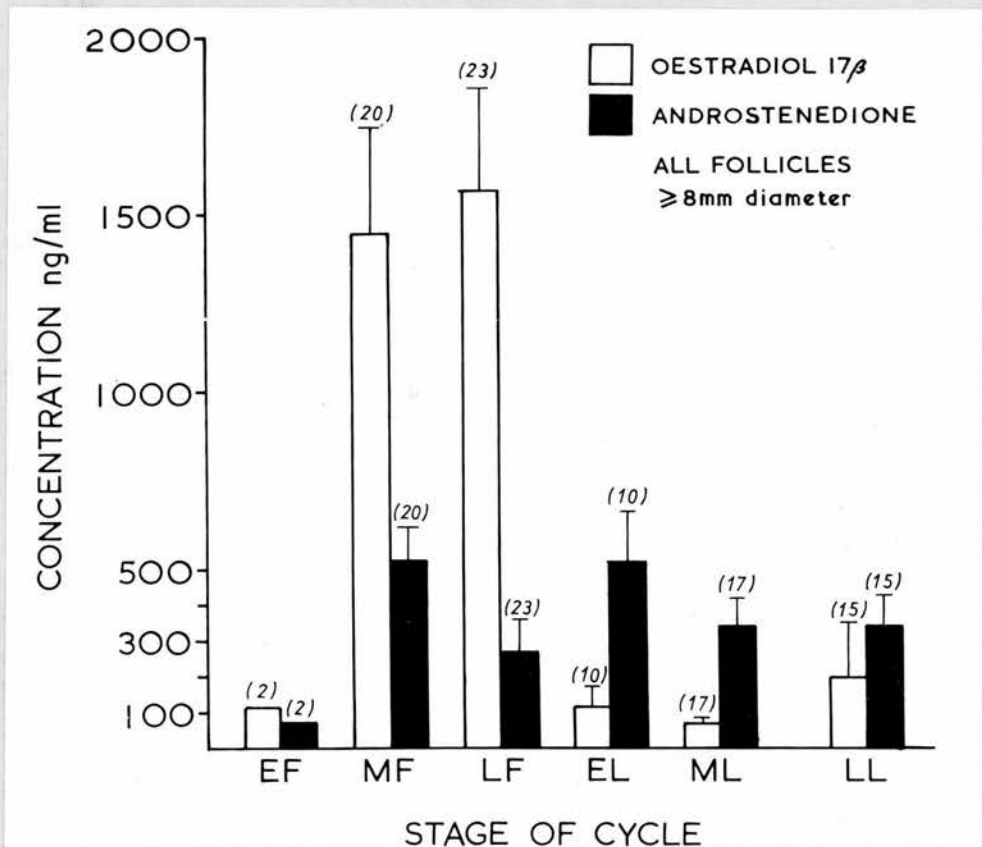


Fig. 19. Concentration of androstenedione and oestradiol in large follicles (≥ 8 mm) with respect to stage of the cycle. Vertical bars represent + 1 S.E.M. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

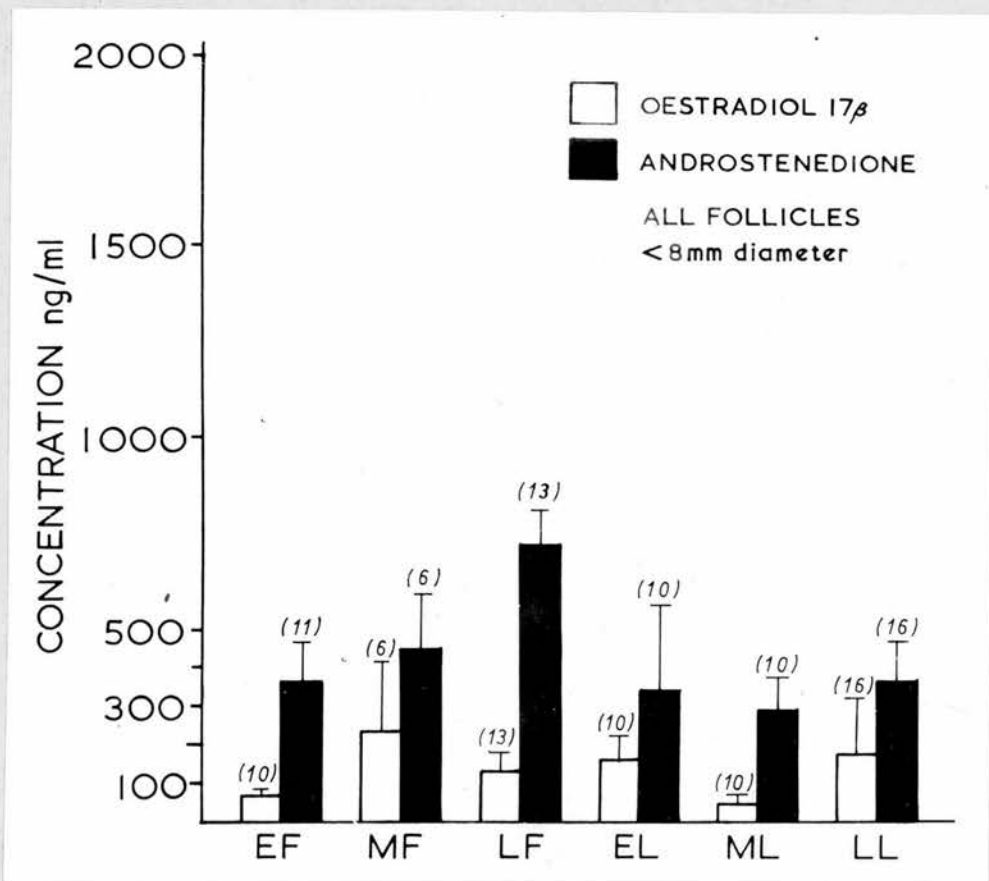


Fig. 20. Concentration of androstenedione and oestradiol in follicular fluid of small follicles (< 8 mm) with respect to stage of the cycle. Vertical bars represent + 1 S.E.M. See Fig. 10 for abbreviations. Numbers of observations in parentheses.

menstrual cycle and in large follicles (> 8 mm) throughout the luteal phase, the ratio of androstenedione (A_4) to oestradiol (O_2) was always > 1 and varied from 2 to 7. The highest ratio was found in the small follicles during the late follicular phase. By contrast the ratio in large follicles during the follicular phase was always < 1 and ranged from 0.05 to 0.6, the lowest ratio being found in the large follicles during the late follicular phase.

DISCUSSION

There was no difference between the concentration of hormones in fluid collected from follicles after ovariectomy and those collected in vivo, suggesting that there was little if any production or metabolism of hormones in the time between ovariectomy and aspiration of fluid (cf Giorgi, Addis & Colombo, 1969; Sanyal et al., 1974). Although patients were undergoing surgery for a variety of reasons, the general pattern of hormones in peripheral plasma (Fig. 10) was similar to that described in normal women during the menstrual cycle (e.g. Robyn et al., 1973; Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). However, the concentration of prolactin in peripheral plasma was up to sixfold higher than that in normal women (McNeilly et al., 1973; McNeilly & Chard, 1974). This is probably because the samples were collected under the stress of anaesthesia and surgery, which is known to stimulate the release of prolactin (Robyn et al., 1973). The overall mean concentration of prolactin in follicular fluid (20^+ ng/ml, $n = 189$) was much lower than that in peripheral plasma. In the absence of information about the rate of exchange of prolactin between plasma and follicular fluid in vivo it is impossible to determine whether this difference is of physiological importance or whether it is an

artefact due to the elevated prolactin concentration in peripheral plasma as a result of stress. Since all the samples were collected under similar conditions, the striking change in concentration of prolactin in follicular fluid at different stages of the cycle (Fig. 14) is probably not an artefact.

The distribution of follicle size in relation to the stage of the cycle is similar to that previously described in women (Block, 1951a) and rhesus monkeys (Koering, 1969). Large follicles (≥ 8 mm) were much commoner during the mid- and late follicular and late luteal phases than at any other stages of the cycle. Although it is difficult to draw conclusions about the dynamics of follicle growth from these cross-sectional observations, it is tempting to speculate that these large follicles represent the end result of two waves of follicular development initiated by the peaks of secretion of FSH occurring at the onset of menses and again at mid-cycle. The minimum time necessary to develop a follicle from the antral to the mature preovulatory stage in women is probably 6 - 10 days (Brown *et al.*, 1969; Gemzell & Johansson, 1971; Bertrand, Coleman, Crooke, Macnaughton & Mills, 1972). The concentration of FSH in small follicles reaches a maximum during the early follicular, late follicular or early luteal phases of the cycle (Table 9) which is either during or just after the peak concentration of FSH in peripheral blood. Furthermore, the concentration of FSH in these follicles apparently increases as they develop into mature preovulatory follicles in spite of the falling levels of FSH in peripheral blood (cf Table 9 and Fig. 10). Some factor may increase the affinity of these follicles for FSH.

Oestradiol, the concentration of which is extremely high in the mid-and late proliferative phase follicles, is known to increase the sensitivity of the ovary to FSH (Goldenberg et al., 1972a). The high levels of oestradiol in the large follicles during the mid-and late follicular phase are similar to those previously reported by other workers in normal (Smith, 1960; Short & London, 1961; Baird & Fraser, 1974b; Sanyal et al., 1974) and gonadotrophin-stimulated ovaries (Short, 1964b; Edwards et al., 1972). Follicles which have detectable levels of FSH during the follicular phase also have a high concentration of oestradiol and an androstenedione to oestradiol ratio of <1 whereas those follicles with undetectable levels of FSH have significantly lower levels of oestradiol and an androstenedione to oestradiol ratio >1 (see Fig. 12 and Table 10).

When the concentration of FSH in peripheral plasma was high (early follicular phase and at mid-cycle) FSH was detectable in only a minority of small follicles (diameter < 8 mm) (Tables 8 and 9). Presumably follicles in which the concentration of FSH is high are those which are stimulated to further development (Table 11). It is not known how this minority of small antral follicles are selected although it has been suggested that oocytes are 'programmed' for development in the order in which they enter meiosis during fetal life (Henderson & Edwards, 1968).

About 17% of the small follicles (diameter < 8 mm) in the early luteal phase of the cycle had measurable amounts of FSH and LH (Table 8), presumably as a result of the preovulatory peaks of the gonadotrophins. In contrast to the follicular phase of the cycle, none of the follicles in the luteal phase were functionally 'active'

as indicated by the persistently low concentrations of oestradiol and an androstenedione to oestradiol ratio of >1 . From the time of the LH 'surge' there is an abrupt fall in the mitotic activity of granulosa cells (Delforge et al., 1972). Thus the presence of LH in some of these small follicles (Table 8) may interfere with their normal orderly development and consequently their steroidogenic potential (Table 11). The marked increase in cystic follicles (Fig. 11) from the mid-luteal phase is probably a consequence of this. It may be that an ordered sequence of gonadotrophins, e.g. FSH alone followed by FSH and LH, is necessary for normal follicular development.

Luteinization of the granulosa cells of the preovulatory follicle commences some 24-36h before ovulation in response to the mid-cycle LH surge (Hertig, 1967; Delforge et al., 1972). After exposure to LH, the preovulatory follicle secretes increasing amounts of progesterone as indicated by the rise in concentration in the ovarian vein (Mikhail, 1970; Lloyd et al., 1971), and in peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970). The relatively high concentration of progesterone in follicular fluid of preovulatory follicles is similar to that found by other workers in normal (Sanyal et al., 1974) and gonadotrophin-stimulated ovaries (Short, 1964b; Edwards et al., 1972) and it is likely that the luteinizing granulosa cells are the source of this steroid (Channing, 1969a, 1970a; Edwards et al., 1972). The ovary containing the preovulatory follicle secretes significantly more androstenedione than the contralateral ovary, presumably due to increased secretion from the preovulatory follicle since there is a significant correlation between androstenedione and oestradiol in ovarian and

peripheral venous plasma (Baird et al., 1974; de Jong et al., 1974) By contrast the follicular concentration of androstenedione in the preovulatory follicle is significantly lower than in smaller follicles at the same stage of the cycle. It is unlikely that luteinising granulosa cells have the capacity to convert androstenedione to oestradiol in vivo since they have an extremely weak aromatizing ability (Ryan et al., 1968; Younglai & Short, 1970). The low concentrations of androstenedione in the preovulatory follicle might be attributable to both an increased permeability of the follicle (Edwards, 1974) and a lack of a binding protein for this hormone in contrast to oestradiol (Takikawa, 1966; Takikawa & Yoshinaga, 1968). The concentrations of testosterone in follicular fluid indicate that the source of this steroid is ovarian and probably follicular in origin since it is present at levels which are 100 times higher than in peripheral plasma (Tagatz & Gurpide, 1973) and 7 to 10 times higher than in ovarian venous plasma (de Jong et al., 1974). Although the highest concentrations were present in the large preovulatory follicle they were variable and not significantly different from those at other stages of the cycle. Presumably testosterone binding globulin is present in follicular fluid since the electrophoretic pattern of follicular fluid is similar to that of serum (Edwards, 1974) and this may account for the increased accumulation of testosterone in follicular fluid during the late follicular phase.

The large preovulatory follicles were characterised by a highly vascular appearance (Short, 1964a). The granulosa cells were very loosely attached to one another; the walls of the follicle were slimy and mucoid, and the follicular fluid was bright yellow and

viscous. When the granulosa cells from large follicles collected during the preovulatory phase were cultured in vitro they secreted maximal amounts of progesterone in response to minimal physiological concentration of gonadotrophins in the medium (Channing, 1970a; see also results, Chapter 5). The granulosa cells are stimulated in vivo by the relatively high concentrations of LH in blood and follicular fluid. Presumably the metabolic requirement of these actively secreting cells is high and may account for the relatively low oxygen tension within the follicle (Fraser, Baird & Cockburn, 1973). The relatively low concentration of prolactin may also reflect utilization or metabolism of this hormone by the follicle. It may also play a key role in controlling steroid synthesis, for the production of progesterone by human granulosa cells in vitro is inhibited when the concentration of prolactin in the medium exceeds 20 ng/ml (see results, Chapter 6).

It is apparent that despite the presence of FSH in antral fluid the granulosa cells do not secrete progesterone in the absence of LH. Furthermore, the concentration of progesterone in follicular fluid of small follicles during the luteal phase is significantly lower than in preovulatory follicles, even though they contain both FSH and LH. The failure of the granulosa cells in small follicles to secrete substantial amounts of progesterone when exposed to LH suggests that these cells require time before they are capable of responding fully. The latter finding is consistent with the observation that granulosa cells harvested from preovulatory follicles in the pig have a significantly greater number of receptors for LH when compared with the cells harvested at other stages of the

oestrous cycle (Channing & Kammerman, 1973a; C. P. Channing, personal communication).

The concentration of oestradiol in the haemorrhagic fluid of recently ruptured follicles was very much lower than that found in the large preovulatory follicles. Although most of the fluid is lost at ovulation the low concentration of oestradiol in haemorrhagic fluid is consistent with the low plasma levels during the immediate post-ovulatory period (Fig. 10) (Moor, 1974). In contrast however, the levels of progesterone were higher than those found in the preovulatory follicle and this is consistent with the increased plasma levels, indicating the increasing secretory capacity of the luteinizing granulosa cells.

Steroid levels in the 'cystic' follicles were low irrespective of the stage of the cycle and in many the pituitary gonadotrophin concentrations were high and similar to those found in plasma. These findings suggest that 'cystic' follicles are relatively inactive and are incapable of secreting much oestradiol or progesterone even in the presence of gonadotrophins.

These data suggest that a precise sequence of hormonal changes occurs within the microenvironment of the developing Graafian follicle; the order in which the changes occur may well be of considerable importance for the growth of that follicle and the secretory activity of the granulosa cells both before and after ovulation. Figure 21 shows the possible inter-relationships between the concentrations of pituitary hormones in plasma and follicular fluid with respect to the steroidogenic activity and growth of a follicle during the follicular and luteal phase of the menstrual cycle. The changes in the concentrations of pituitary hormones in follicular fluid throughout the menstrual cycle are related to those which occur in

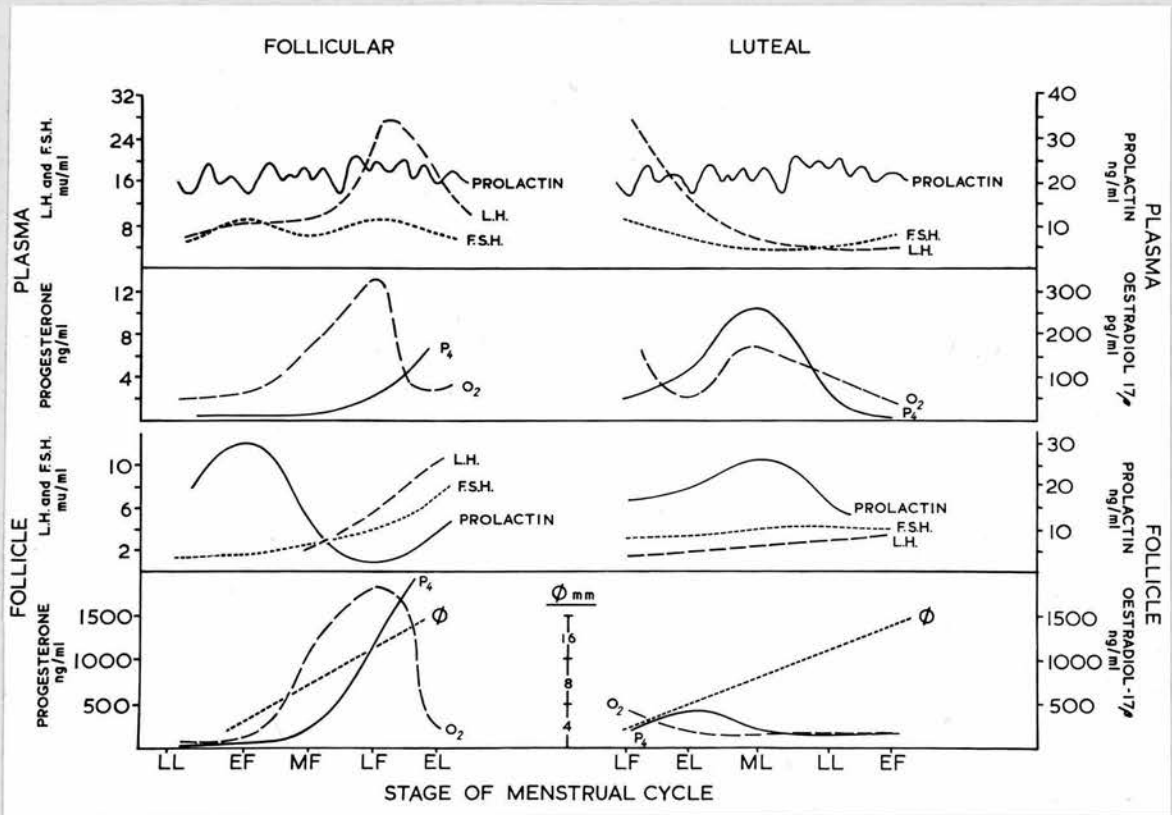


Fig. 21. The postulated relationships between the concentrations of pituitary and steroid hormones in peripheral plasma and in the follicular fluid of a developing follicle during the follicular or luteal phase of the menstrual cycle (see text). O_2 = oestradiol, P_4 = progesterone, \emptyset = follicle diameter. See Fig. 10 for abbreviations.

peripheral plasma. The presence of FSH in the smallest follicles appears to be important for their development. The presence of FSH and oestradiol in the follicular phase follicles prepares them for the preovulatory production of progesterone under the influence of LH. The very low concentrations of prolactin in these follicles may be related to the upsurge in metabolic activity within the follicle during this time. The antral fluid of the preovulatory follicle contains relatively large amounts of oestradiol and progesterone, low physiological levels of prolactin, and concentrations of LH and FSH approaching 30 and 60% respectively of those found in plasma. These changes in the growth of a follicle destined to ovulate are different from those proposed for the pig (Channing, 1972). Such differences that exist will possibly depend on the permeability of a Graafian follicle in different species to protein hormones (see Edwards, 1974, for review).

Furthermore it is suggested that the large inactive follicles found during the luteal phase are a consequence of LH interference during the growth of these follicles under the influence of FSH. The consequence of these findings may prove to be important in the treatment of amenorrhoeic or anovulatory patients with exogenous gonadotrophins.

SUMMARY

The concentration of FSH, LH, prolactin, oestradiol, progesterone and androstenedione were measured in peripheral plasma and follicular fluid of women throughout the menstrual cycle. In addition testosterone was also measured in follicular fluid. With the exception of prolactin and androstenedione, concentrations of pituitary and steroid hormones in follicular fluid could be correlated with those in peripheral plasma.

Follicle-stimulating hormone was present in a greater number of small follicles (< 8 mm) during or just after the peaks of FSH in peripheral plasma. During the mid-follicular phase the concentration of both FSH and oestradiol was high in fluid from large follicles (≥ 8 mm). During the late follicular phase the large follicles (≥ 8 mm) contained high levels of progesterone and testosterone in addition to oestradiol, low levels of prolactin and androstenedione and concentrations of LH and FSH of about 30 and 60% respectively of those found in plasma. By contrast no large 'active' follicles (≥ 8 mm) were found during the luteal phase although many contained both LH and FSH: the concentration of androstenedione in these follicles always exceeded that of oestradiol. Luteinizing hormone was present in a proportion of small follicles (< 8 mm) during the late follicular and early luteal phases but not at other stages of the menstrual cycle.

CHAPTER 4

CHANGES IN THE MORPHOLOGY OF HUMAN GRANULOSA CELLS IN VIVO
AND IN VITRO AFTER EXPOSURE TO DIFFERING HORMONAL ENVIRONMENTS.

INTRODUCTION

Histological sections were prepared from large follicles from corpora lutea and from granulosa cells which had been cultured in vitro for ten days. In addition histological sections of the endometrium from each patient were prepared. The pituitary and steroid hormones in plasma and follicular fluid were measured. The morphology of both the theca and granulosa cells in vivo were related to the hormone concentrations in plasma and follicular fluid. Similarly the morphology of the endometrium was related to the hormonal status of each patient. In addition the morphology of human granulosa cells after in vitro culture were compared to the luteinised granulosa cells of the corpus luteum.

RESULTS

The Graafian follicle during the mid-to late follicular phase

The morphological changes occurring in the wall of the large Graafian follicle (12 mm diameter) and those occurring simultaneously in the endometrium are shown in Plates 6 and 7 respectively. The follicle and endometrium were obtained on Day 11 of the menstrual cycle from a patient (E.A. age 32 yrs.) whose previous menstrual cycles were normal ($\frac{5}{28}$ days). The hormone levels in plasma (Table 2) indicate that the specimens were obtained during peak concentrations of oestradiol- 17β but immediately prior to the mid-cycle peak of LH, at which time the levels would normally exceed 30 $\mu\text{g./ml.}$ The endometrium displays an oedematous stroma and the glands are lined with cuboidal epithelial cells showing a degree of pseudostratification. Some mitotic figures are present; however the general picture is of an endometrium undergoing mid-to late proliferative changes (Noyes, Hertig & Rock, 1950).

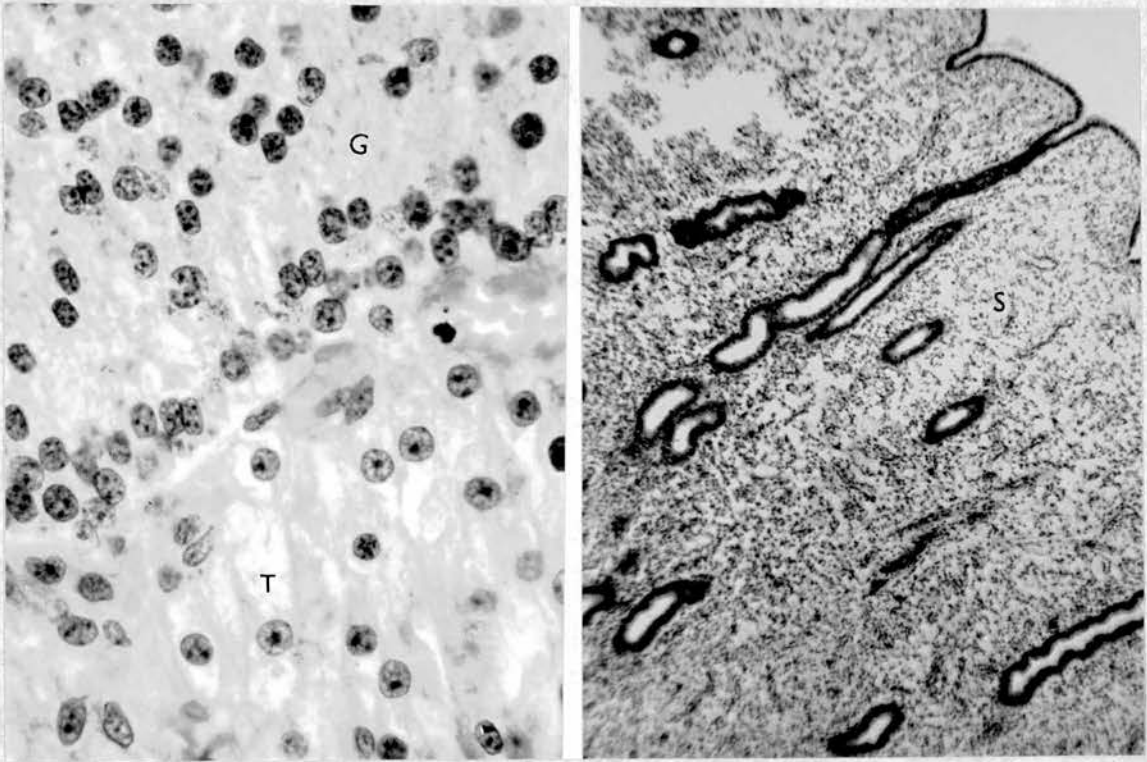


Plate 6

Plate 7

Plate 6. 12 mm Graafian follicle removed from a woman (E.A. aged 32 yrs.) on Day 11. Previous cycle record $\frac{5}{28}$. The cytoplasm of the theca cells (T) is abundant and pale staining in contrast to that of the granulosa cells (G). Mag. X 580.

Plate 7. Mid-late proliferative phase endometrium from the same woman (E.A.). Note the oedematous stroma (S) and the complete absence of glandular secretions. Mag. X 30.

Table 12. Concentration of pituitary and steroid hormones in plasma and follicular fluid.

Patient's Initials	Phase of menstrual cycle	Plasma				Follicular fluid			
		LH mu./ml	FSH mu./ml	O ₂ ng/ml	P ₄ ng/ml	LH mu./ml	FSH mu./ml	O ₂ ng/ml	P ₄ ng/ml
E.A.	M-LF	14.0	5.1	0.15	0.2	0.8	3.8	320	36
M.B.	L-F	35.2	10.5	0.13	0.2	5.6	6.1	3500	2460
J.C.	V-EL	8.6	7.6	0.05	3.0	7.4	8.4	240	10200
A.S.	ML	4.6	6.3	0.110	14.0	-	-	-	-

For abbreviations see Fig. 10

The cytoplasm of the theca cells (T: Plate 6) is abundant and pale staining in contrast to that of the granulosa cells (G). The theca cells appear to be actively secreting steroid, presumably oestradiol (Table 12). By contrast the granulosa cells which are smaller and more compact show no sign of secretory activity.

The Graaffian follicle during the late-follicular phase

The morphological changes occurring in the wall of the large Graafian follicle (18 mm) about 24-48 hours prior to rupture and those occurring simultaneously in the endometrium are shown in Plates 8 and 9 respectively. The follicle and endometrium were obtained on Day 15 of the menstrual cycle from a patient (M.B. aged 38 yrs.) whose previous menstrual cycles were normal ($\frac{3}{28}$ days). The hormone levels in plasma (Table 12) indicate that the specimens were obtained during peak levels of LH and FSH in plasma. The endometrium shows enlarged and tortuous glands, the epithelial cells of which show evidence of mitotic activity and pseudostratification is more pronounced than that shown in Plate 7. This is a picture of an endometrium undergoing late proliferative changes.

The theca cells (T) resemble those in Plate 6, but the granulosa cells closest to the basement membrane and the blood vascular system, are showing signs of early luteinization. The cytoplasm of the granulosa cells is enlarged and granular in appearance. The concentration of progesterone in follicular fluid has increased 70-fold compared to that in the mid-to-late proliferative phase follicle.

The newly-formed corpus luteum

The morphological changes occurring within the recently ruptured 18 mm haemorrhagic follicle (or newly formed CL) and those occurring

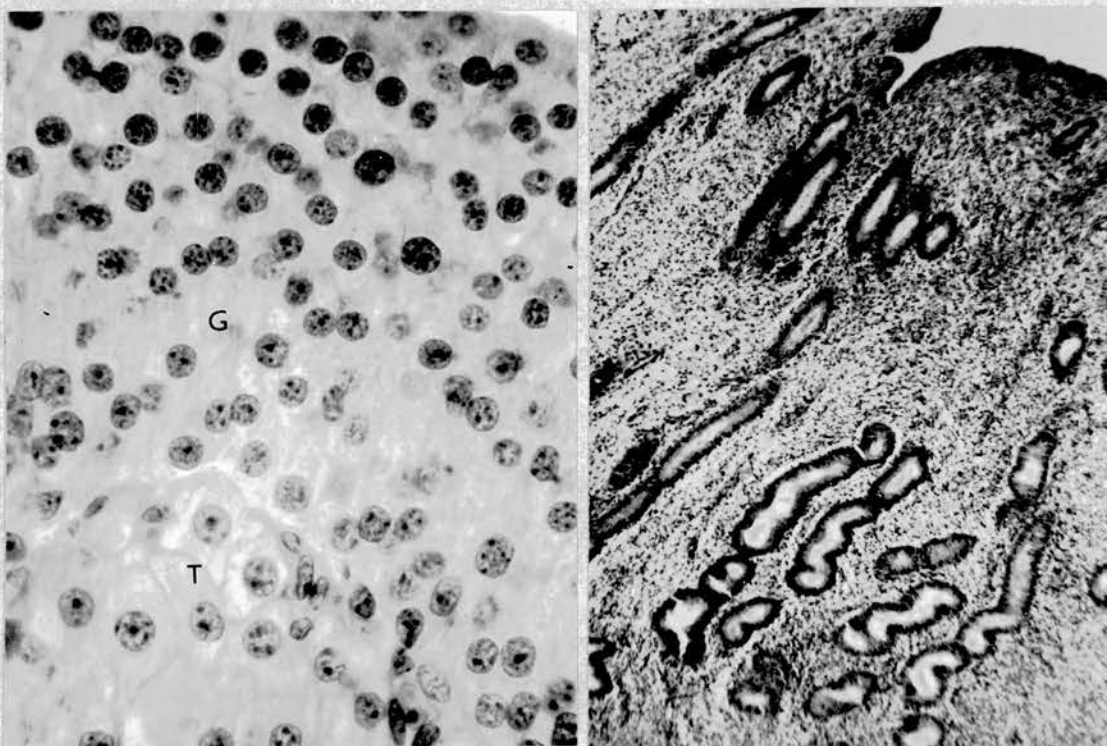
Plate 8Plate 9

Plate 8. 18 mm Graafian follicle removed from a woman (M.B.) aged 38 years on Day 15. Previous cycle record: $\frac{3}{28}$. The theca cells (T) resemble those in Plate 6, while the cells of the membrana granulosa (G) are well dispersed and display a more abundant cytoplasm. Mag. X 580.

Plate 9. Late proliferative phase endometrium from the same patient (M.B.). Note the marked reduction in oedema compared to that shown by the mid- to late proliferative phase endometrium (see Plate 7). Mag. X 30.

simultaneously in the endometrium are shown in Plate 10 and 11 respectively. The CL and endometrium were obtained on Day 15 of the menstrual cycle from a patient (J.C., aged 34 years) whose previous menstrual cycles were normal ($\frac{4}{28}$ days). The hormone levels in plasma (Table 12) indicate that the specimens were obtained during the early progestational phase at a time when the gonadotrophins were still elevated. The endometrium shows epithelial cells with marked mitotic activity, with a stroma that is much less oedematous than is the case during the proliferative phase, moreover many of the glands are displaying basal vacuolation of the epithelium. The endometrium thus shows changes characteristic of both very late proliferative as well as early secretory stages and the changes in the endometrium are consistent with the hormonal status of the patient.

Histologically the theca cells appear less active than those shown in Plates 6 and 8. The concentration of oestradiol in plasma and in the haemorrhagic fluid is very low (Table 12) indicating that this gland is secreting little if any oestradiol at this time.

The corpus luteum during the mid-luteal phase

The morphological appearance of a fully functional CL (2.42 gms, 19 mm diameter) and that of the endometrium are shown in Plates 12 and 13 respectively. The CL and endometrium were obtained on Day 18 of the menstrual cycle from a patient (A.S., aged 43 years) whose previous menstrual cycles were normal ($\frac{5}{30}$ days). The hormone levels (Table 12) in plasma indicate that the specimens were obtained during the mid-progestational phase. The endometrium has a very oedematous stroma with tortuous glands. The eosinophilic of the luminal cells within the glands is indicative of secretion. These changes are consistent with an endometrium showing early to mid-

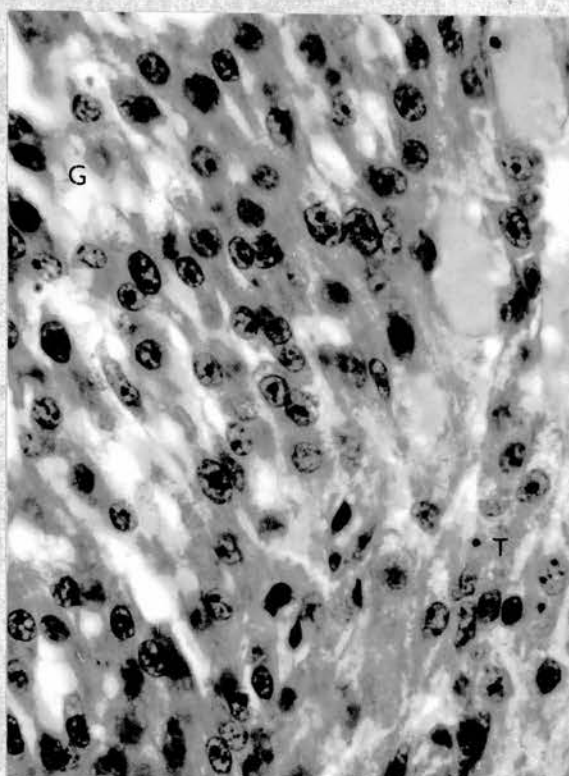


Plate 10.

Plate 10. Recently ruptured Graafian follicle removed from a woman (J.C.) aged 34 years on Day 15. Previous cycle record: $\frac{4}{28}$. The granulosa cells (G) are well dispersed while the cytoplasm of the theca cells (T) is much less abundant than those in the late follicular phase follicle (see Plate 8). Mag. X 580.

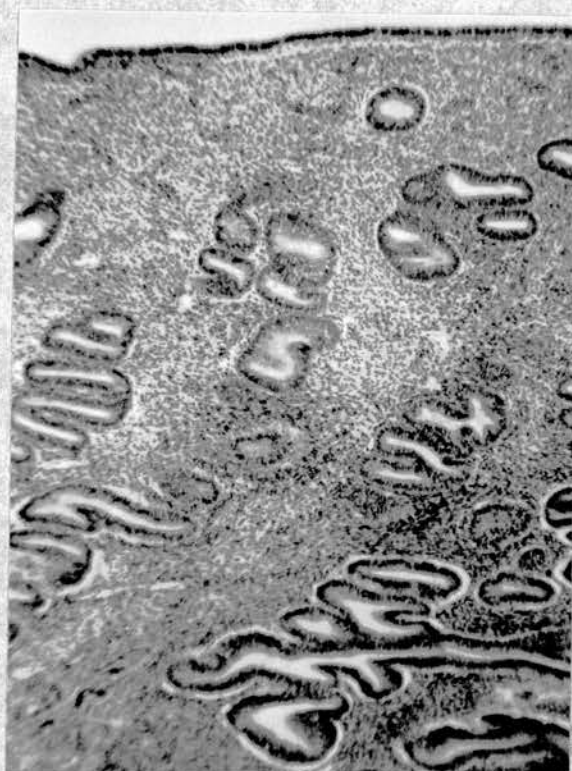


Plate 11.

Plate 11. Very late proliferative-early secretory phase endometrium from the same patient (J.C.). Note the 'tortuous' appearance of the glands compared to those during the late-proliferative phase (see Plate 9). Mag. X 30.

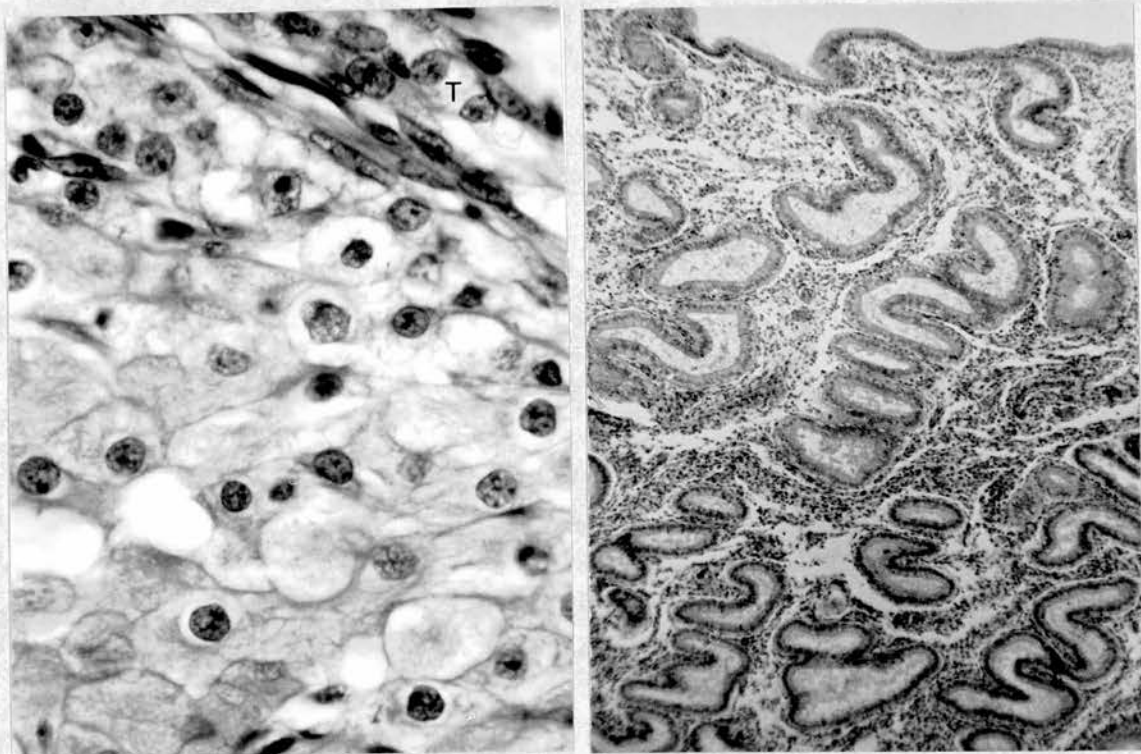
Plate 12Plate 13

Plate 12. Corpus luteum of a woman (A.S.) aged 43 years on Day 18. Previous cycle record: $\frac{5}{30}$. Granulosa cells have fully luteinized and are well dispersed. The theca cells (T) appear large and show an abundant cytoplasm. Mag. X 580.

Plate 13. Mid-secretory phase endometrium from the same patient (A.S.) Note the oedematous stroma and the 'tortuous' appearance of the glands. Mag. X 30.

secretory changes.

The theca cells (T) appear in strands of tissue between the granulosa cells (G) which have fully luteinized. The theca cells unlike those in the newly formed CL, are apparently secreting oestradiol since the plasma levels of this hormone are elevated.

Granulosa cells in culture

Granulosa cells harvested from either the mid to late or late proliferative phase follicles described earlier (Plates 6 and 8), after 10 days culture in vitro, are shown in Plates 14 and 15 respectively. In both cases the cells display a large prominent nucleus and extensive cytoplasmic mass, and are similar in appearance to the cells of the CL. The cytoplasm of the granulosa cells in culture appears granular and vacuolated which suggests that they are actively secreting progesterone. Although the cells in both cultures are similar in appearance there is a 5-fold difference in their daily output of progesterone. By contrast there were no detectable levels of oestradiol in either culture medium. Histologically the difference appears to be that the cells secreting more progesterone are much more eosinophilic (Plate 15).

DISCUSSION

These studies have described the temporal relationships between the histological and endocrine changes within the human Graafian follicle, and corpus luteum. They strongly suggest that oestradiol is secreted mainly by the theca cells of the follicle and that the dramatic rise in progesterone concentration in the preovulatory follicle is a consequence of early luteinization by the membrana granulosa. Furthermore they also suggest that the newly formed CL (or haemorrhagic follicle) is histologically similar to the mature CL since the granulosa cells are well dispersed and the theca cells which are

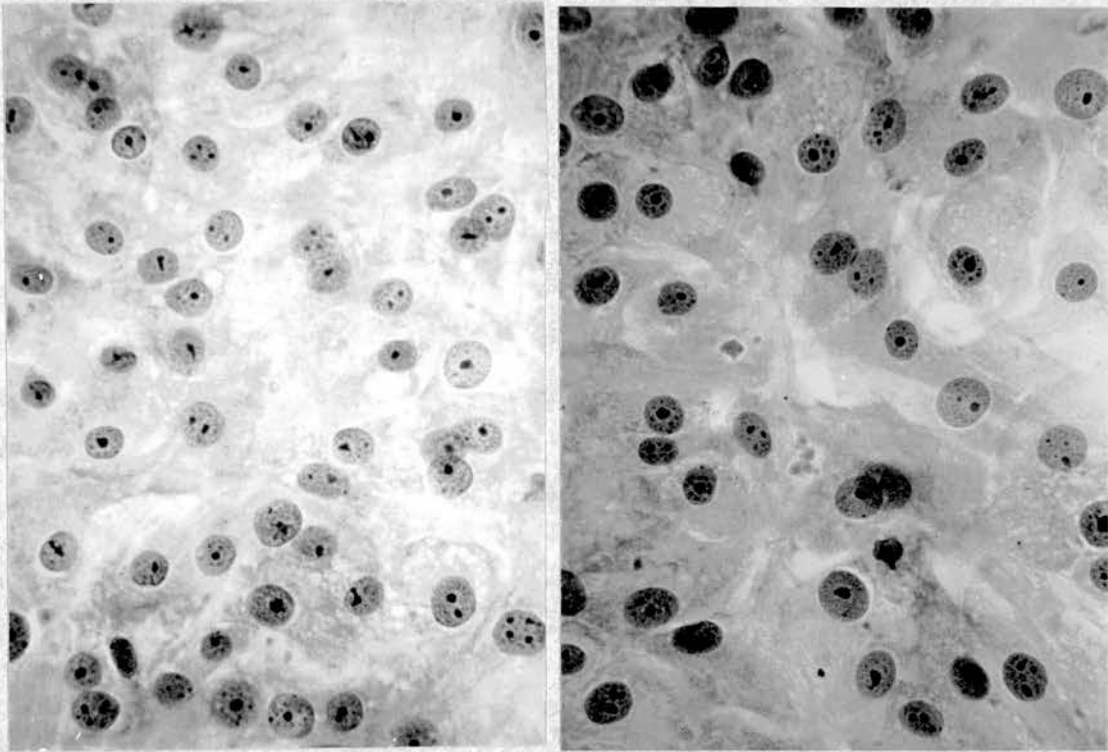


Plate 14

Plate 15

Plate 14. Granulosa cells after 10 days in culture. Cells were harvested from the same follicle described in Plate 6. Note that these cells are similar in appearance to those of the corpus luteum (see Plate 12). Mag. X 580.

Plate 15. Granulosa cells after 10 days culture. Cells were harvested from the same follicle described in Plate 8. Note that these cells appear more darkly stained than those in Plate 14, although they appear similar in appearance overall. Mag. X 580.

localized in streaks of tissue have penetrated deeply into the gland. However the cytoplasm of the theca cells in the newly formed CL is less extensive than in the mature CL and non-secretory in appearance. Since most of the antral fluid is lost during follicular rupture the levels of steroid in the haemorrhagic fluid probably reflects the secretory status of this gland since it contains high concentrations of progesterone and low concentrations of oestradiol. The levels of gonadotrophin in the fluid are similar to those found in plasma, highlighting the marked increase in vascularisation which has taken place. Although there are probably qualitative differences in the secretory products of the CL when compared to the secretions from the follicle (Ryan et al., 1968) it is of interest to note that in women (Lloyd et al., 1971) and rhesus monkeys (Weiss et al., 1973; Baird, Hearn & Short, unpublished observations) where there are distinct populations of theca lutein cells (Corner, 1956; Gillim, Christensen & McLennan, 1969; Koering, 1969, and Plate 12) there are higher concentrations of oestradiol in the vein draining the ovary containing the CL than on the contralateral side. By contrast the theca lutein cells of the ewe and cow are barely identifiable (Harrison, 1948; Hansel et al., 1973) and the CL in both species are unable to synthesize oestrogen in vitro (Savard et al., 1965; Kaltenbach et al., 1967). Furthermore the sheep CL secretes only progesterone and 20 -dihydro progesterone in vivo (Short, 1964b; Baird et al., 1973). It seems reasonable therefore to assume that the luteinized granulosa cells of the mature human CL secrete mainly progestins and that the theca lutein cells secrete oestradiol although both cell types may secrete significant amounts of androgens (Lloyd et al., 1971).

Histologically granulosa cells in in vitro culture are similar in appearance to the granulosa lutein cells of the corpus luteum. Cells harvested from preovulatory follicles when cultured in vitro are markedly eosinophilic and they exhibit most of the fine structural features of human luteal cells in situ. (Channing & Crisp, 1972; T. M. Crisp, personal communication). By contrast cells harvested from follicles other than preovulatory and cultured in vitro secrete significantly less progesterone (Fig. 16) and are less eosinophilic (Plate 14) than the preovulatory cultured cells and show some fine structural characteristics similar to those of follicular granulosa cells in situ (Channing & Crisp, 1972). Human granulosa cells cultured in vitro therefore, provide a convenient biochemical model system for studying a number of factors associated with the formation and function of the corpus luteum.

SUMMARY

The morphology of Graafian follicles, corpora lutea and granulosa cells after in vitro culture was examined in relation to the stage of the menstrual cycle, hormone levels in plasma and follicular or haemorrhagic fluid. In addition, the morphology of the endometrium from each patient was described and related to the hormone levels in plasma.

Histologically the granulosa or theca cells of the follicle or corpus luteum show changes which correlate with the hormone levels in both plasma and follicular or haemorrhagic fluid. In general the granulosa and theca cells have an enlarged cytoplasm which is granular and vacuolated if there are high concentrations of progesterone and oestradiol in plasma and fluid. In vitro however, granulosa cells regardless of their daily rate of progesterone secretion were similar in appearance to those in the corpus luteum.

CHAPTER 5

**RELATIONSHIP BETWEEN THE ENDOCRINE ENVIRONMENT WITHIN THE GRAAFIAN
FOLLICLE AND THE SUBSEQUENT RATE OF PROGESTERONE SECRETION BY
HUMAN GRANULOSA CELLS IN VITRO**

INTRODUCTION

The granulosa cells within the Graafian follicle do not have direct access to a blood supply but are bathed in a follicular fluid which contains most serum proteins, variable amounts of gonadotrophins and high concentrations of steroids (Edwards, 1974; see also Chapter 3). This hormonal microenvironment may be of importance in determining the subsequent function of these cells in the corpus luteum.

There is considerable evidence in mammals to suggest that the steroidogenic potential of granulosa cells in vitro is related to the stage of the ovarian cycle at which they are harvested (Channing, 1969, 1970a). It has been shown that a precise sequence of hormonal changes occurs in the follicular fluid of the developing human Graafian follicle, and it has been suggested that the sequence in which these changes occur is of considerable importance for the growth of that follicle and the secretory activity of its granulosa cells both before and after ovulation (see Chapter 3). In order to obtain direct evidence on this point, follicular fluid and granulosa cells were obtained from human Graafian follicles of varying sizes throughout the menstrual cycle. The concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestradiol and progesterone were measured in follicular fluid and cells from each follicle were cultured in vitro to assess their steroidogenic potential.

RESULTS

Viability of granulosa cells prior to and during in vitro culture

The mean viability of human granulosa cells at harvesting was 37.0% (range 4.4-100.0%, n = 138). There were no apparent relationships between cell viability and stage of the cycle, follicle

size, or the hormone concentrations in follicular fluid at the time of harvest. The greatest proportion of live cells were found in follicles harvested soonest after oophorectomy. There were no significant differences in the assessment of viability when either nigrosin or lissamine green vital stains were used ($P > 0.2$).

Granulosa cells remained viable in culture for at least 16 days, although after 20 days the numbers remaining on the coverslips declined rapidly. The mitotic index (number of cells present after ten days, divided by the number of live cells at the commencement of culture) was related to the environment that they had been subjected to in the follicle in vivo (Table 13). Details of the hormone concentrations in follicular fluid are shown in Table 14. When granulosa cells from any stage of the cycle were grown in culture medium containing < 1 mu. LH/ml, mitosis only occurred in the presence of added FSH (30 mu./ml) and oestradiol ($1 \mu\text{g/ml}$), when the mitotic index was $6.6^{+1.9}$, $n = 10$).

Biological activity of the hormones

The calf serum which was added to the culture medium contained immunological LH activity equivalent to 0.7 ng/ml NIH-LH-S14, or < 0.4 mu./ml MRC 68/40 (FSH was not determined). However, this calf serum stimulated progesterone production by human granulosa cells in vitro in a manner comparable to a human serum preparation containing 1.8 mu./ml and 1.7 mu./ml FSH (Fig. 22).

The immunoreactive FSH and LH concentrations in calf serum were comparable to the lowest levels of FSH and LH found at any stage of the human menstrual cycle. Progesterone and oestradiol were undetectable in the calf serum (< 50 and < 6 pg/ml respectively).

Table 13. The mitotic activity of human granulosa cells in vitro when harvested from follicles containing different combinations of steroid and pituitary hormones.

	Hormones in follicular fluid					
	-FSH	+FSH	+FSH	+FSH	-FSH	-FSH
	-LH	-LH	-LH	+LH	+LH	+LH
	-O ₂	-O ₂	+O ₂	+O ₂	-O ₂	-O ₂
Mitotic index	0.66	1.29	3.67	0.98	0.50	0.25
± S.E.M.	±0.66	±0.10	±0.56	±0.20	±0.05	±0.11
(No. of obs.)	(186)	(43)	(53)	(72)	(9)	(11)

The mitotic index refers to the average number of cells remaining at the end of 10 days in culture divided by the number of live cells at the commencement of culture. The symbols + or - LH or FSH or oestradiol (O₂) indicate the presence or absence of these hormones in the follicular fluid from which the cells were harvested (see Table 14).

Table 14. Concentrations of hormones in follicular fluid.

+LH	>	2.8 mu./ml	» LH
+FSH	>	1.3 mu./ml	» FSH
+oestradiol	>	250 ng/ml	» oestradiol

The minimum detectable levels for LH and FSH in the smallest follicles (4 mm diameter) examined were 2.8 mu./ml and 1.3 mu./ml respectively, consequently these were chosen as the detection limits for all follicles for the purpose of group means. The detection limit for the measurement of follicular oestradiol was 8 ng/ml.

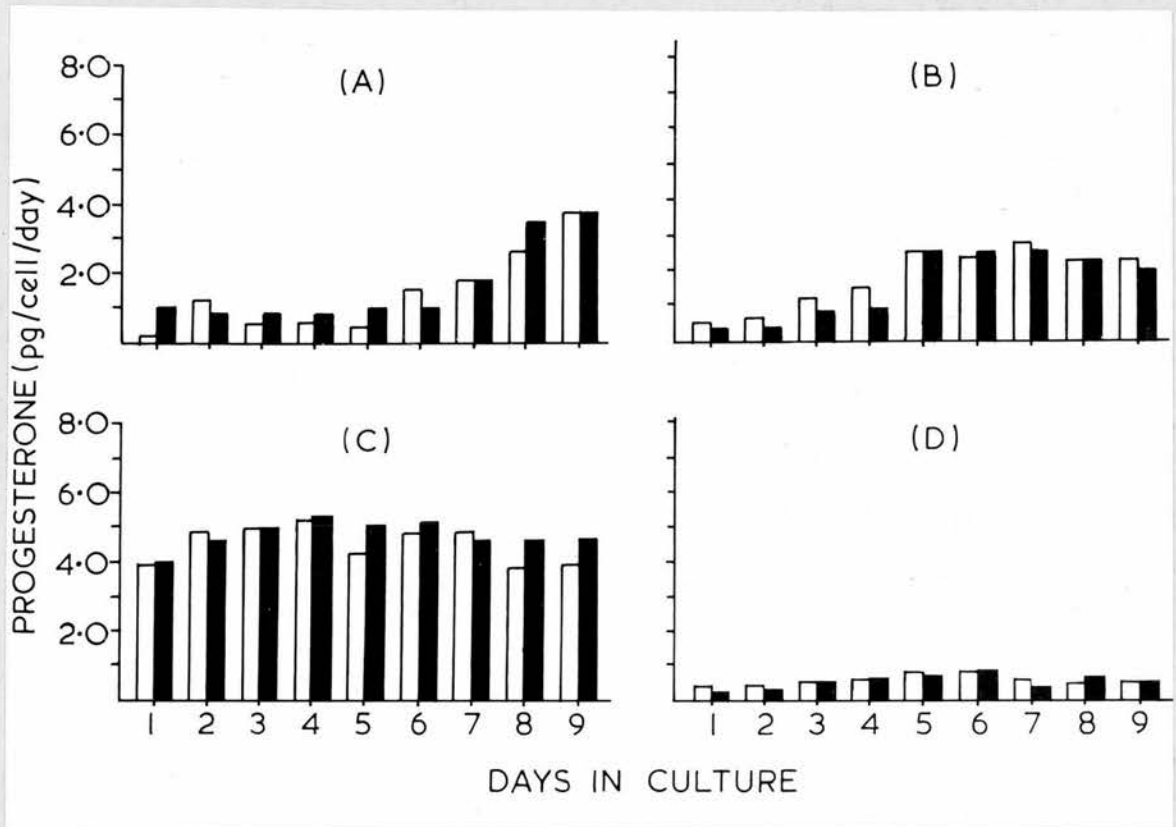


Fig. 22. Production of progesterone by granulosa cells grown in a medium of either calf serum or human serum with a known concentration of gonadotrophins. Cellular production of progesterone in calf (white bars) or human (black bars) serum culture medium for 9 days after the cells were harvested from a single 5 mm early follicular phase (a), 12 mm mid-follicular phase (b), 10 mm late follicular phase (c), or 6 mm late luteal phase follicle (d). The concentration of LH and FSH in the culture medium with 20% calf serum was unknown whereas in the 20% human serum preparation it was 1.8 and 1.7 mu./ml respectively. Each histogram is the mean of triplicate cultures.

The production of progesterone by granulosa cells in response to the addition of LH, FSH, or LH + FSH was variable. The doses of gonadotrophins which would induce the maximum steroidogenic response in all follicles was tested in granulosa cells harvested from the smallest follicles (4 mm diameter), and the results are shown in Figure 23. There was no significant change in production of progesterone when the concentration of LH was increased from 1.8 mu./ml to 30 mu./ml provided that the dose of FSH was held constant. Similarly there was no increase in the production of progesterone when FSH was increased to 30 mu./ml when LH was held constant. However, with increased concentrations of FSH the steroidogenic response to LH was enhanced (Figure 23). There was no significant increase in the production of progesterone when the concentration of both LH and FSH were increased from 30 mu./ml to 90 mu./ml ($P > 0.5$) (paired t test).

In all subsequent gonadotrophin stimulation experiments, the concentrations of LH and FSH were 30 mu./ml; there were no significant differences in response between FSH (CPDS/6) and FSH (73/519). The concentrations of LH and FSH which were added to the culture were within the normal physiological ranges for these hormones at the time of the mid cycle 'surge'.(e.g. 25-60 mu./ml) but about three times higher than the mean levels in follicular fluid (see Fig. 13).

The effect of differing hormone concentrations in follicular fluid on the production of progesterone by granulosa cells in vitro

Since there were no differences in the way granulosa cells grew or secreted progesterone when harvested from patients with stage 0 carcinoma of the cervix or menorrhagia, the results from all follicles were pooled. The variation in the daily production of progesterone

Fig. 23. Production of progesterone by granulosa cells in vitro in response to varying amounts of LH and FSH.

Production of progesterone per cell per day when the following concentrations of gonadotrophin were present (or added) in culture medium containing 20% serum from hypophysectomized patients: (x) 0.6 and 0.4 mu. LH and FSH/ml; (▲) 1.8 and 1.7, 4.4 and 1.7, 16.4 and 1.7 or 30.0 and 1.7 mu. LH and FSH/ml; (●) 4.0 and 30.0 mu. LH and FSH/ml; (Δ) 30.0 and 30.0 LH and FSH/ml, and (o) 90.0 and 90.0 mu. LH and FSH/ml. The cells were harvested from 5 early and mid-follicular phase follicles (all 4 mm in diameter) and each experiment is the mean result of duplicate cultures.

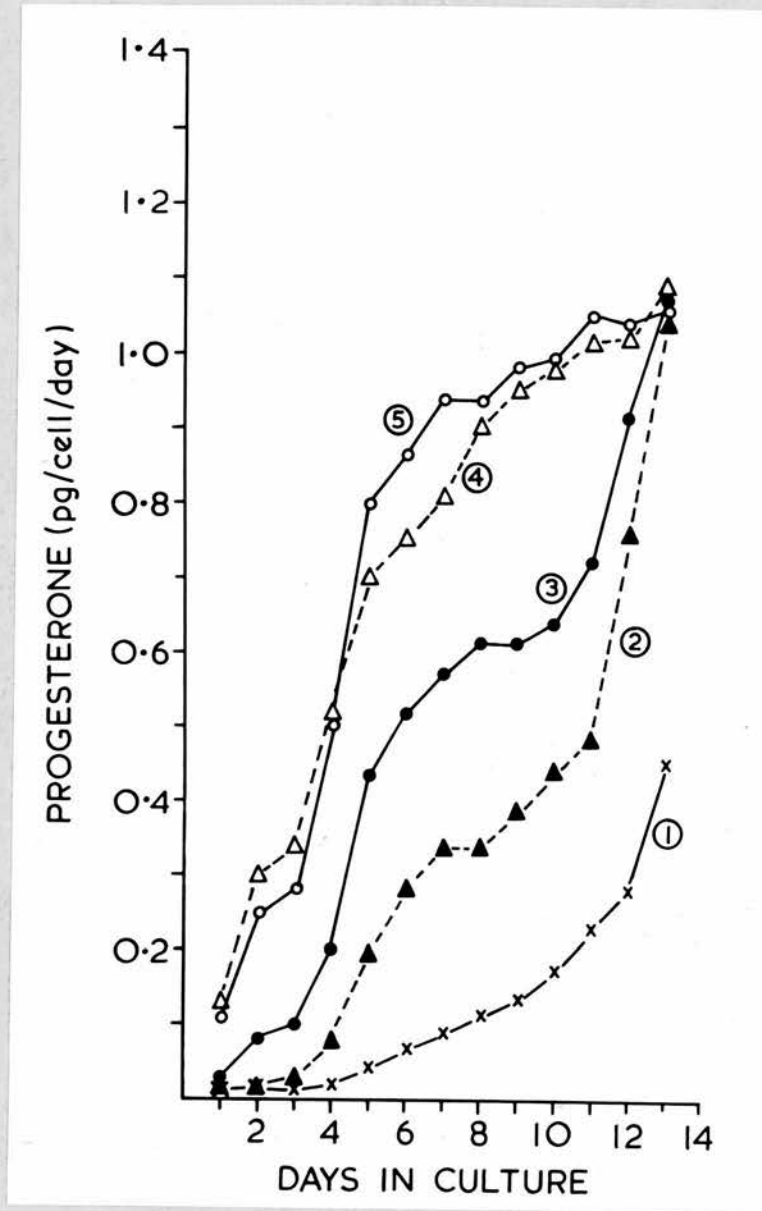


Fig. 23. (legend on p. 151)

between replicate cultures for all treatments was 4.9% (range 0.0-19.6%, n = 315).

The daily production of progesterone during 10 days in culture after various treatments in relation to the hormone concentrations in follicular fluid at the time of harvesting is shown in Figures 24-29.

Cells harvested from follicles containing LH, FSH and high concentrations of oestradiol secreted maximum amounts of progesterone into the culture medium, and were not influenced by the addition of LH, FSH, LH + FSH, LH + FSH + oestradiol or dibutryl cyclic AMP (DBC) (Fig. 27). By contrast cells from follicles lacking FSH, LH or oestradiol secreted less progesterone ($P < 0.001$), but were stimulated by the daily addition of LH + FSH, DBC or LH + FSH + oestradiol ($P < 0.01$, paired t test of the means) (see Figs. 24-29). The response to DBC was similar to LH + FSH and was significantly less effective than LH + FSH + oestradiol but more effective than LH, FSH, FSH + oestradiol or oestradiol alone with one exception (see Fig. 29) ($P < 0.001$ and $P < 0.01$ respectively). The steroidogenic response by granulosa cells in culture to the daily addition of LH + FSH or DBC was related to the hormone environment from which the cells were harvested (FSH + oestradiol $*$) > FSH + LH or FSH alone $> **$ LH or no hormone; $*P < 0.01$, $**P < 0.01$).

The maximum production of progesterone by a granulosa cell was about 5 pg/day and this was achieved within 1-2 days in culture if cells had been harvested from follicles containing LH, FSH and oestradiol. By contrast, cells harvested from follicles devoid of any hormone never achieved the maximum daily production during 10 days in culture, even after the addition of LH + FSH + oestradiol (Fig. 24).

Fig's 24-29. Average daily production of progesterone by human granulosa cells during 10 days of culture after various treatments in relation to the hormone concentrations in follicular fluid are shown in the next six figures. The average results (+ 1 S.E.M.) for the individual treatments are represented by the lines indicated. The average results of a number of different treatments were not significantly different from each other ($P > 0.05$, paired t test), consequently they are represented by the same line. The symbols + or - indicate the levels of hormones in follicular fluid: + LH > 2.8 mu./ml, - LH ≤ 2.8 mu./ml; + FSH > 1.3 mu./ml and - FSH ≤ 1.3 mu./ml; + oestradiol > 250 ng/ml and - oestradiol (O_2) ≤ 250 ng/ml. The follicular fluid contents are indicated in the boxes. The hormone treatments given in the culture media are shown on the figures. The number of experiments is shown in parentheses. The symbol DBC refers to dibutyl cyclic AMP.

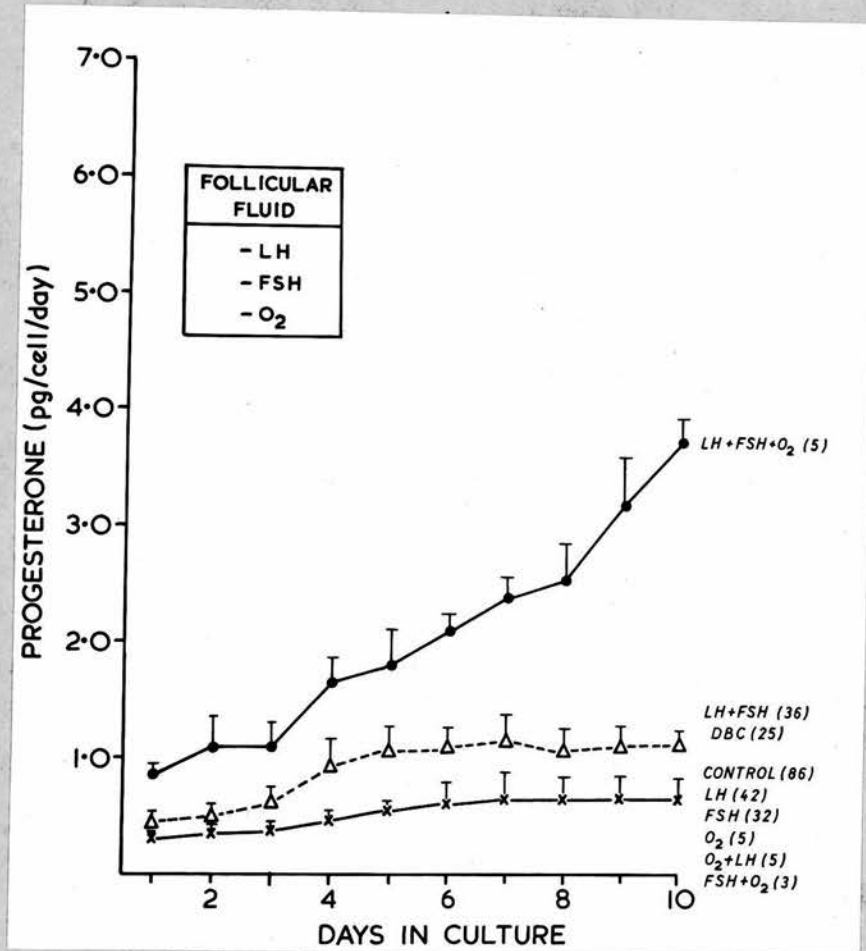


Fig. 24. (legend on p. 154)

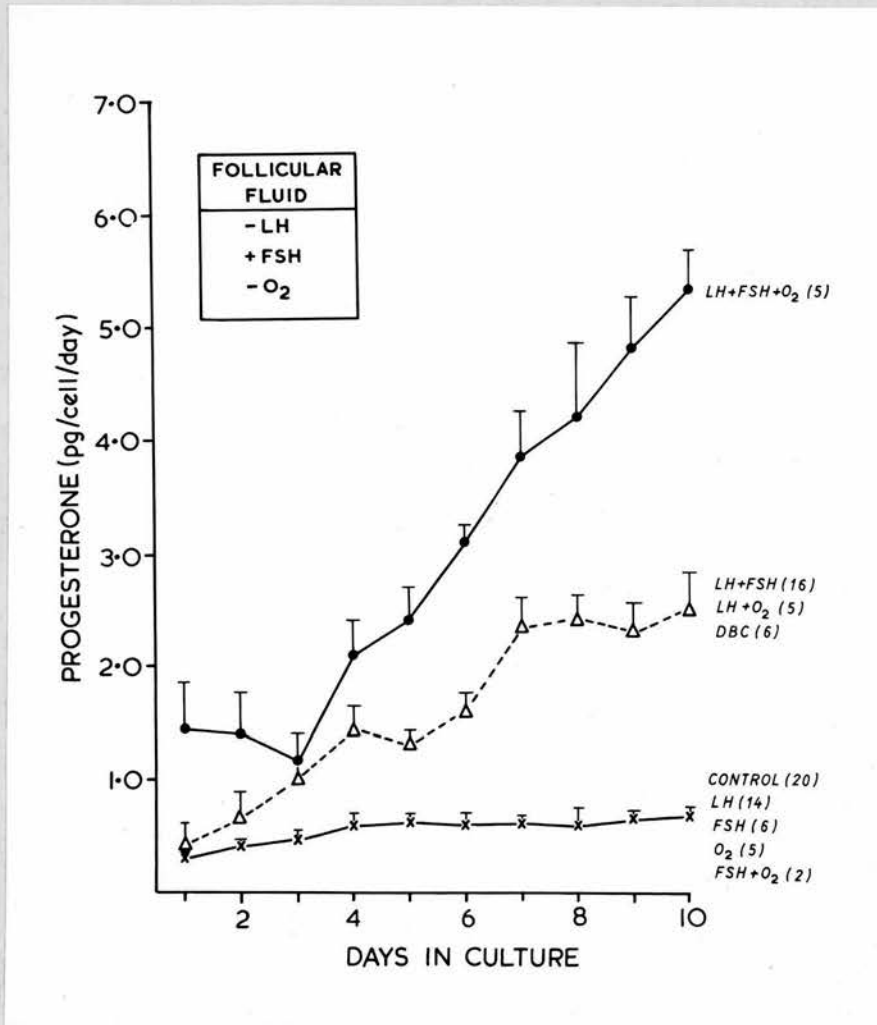


Fig. 25. (legend on p. 154)

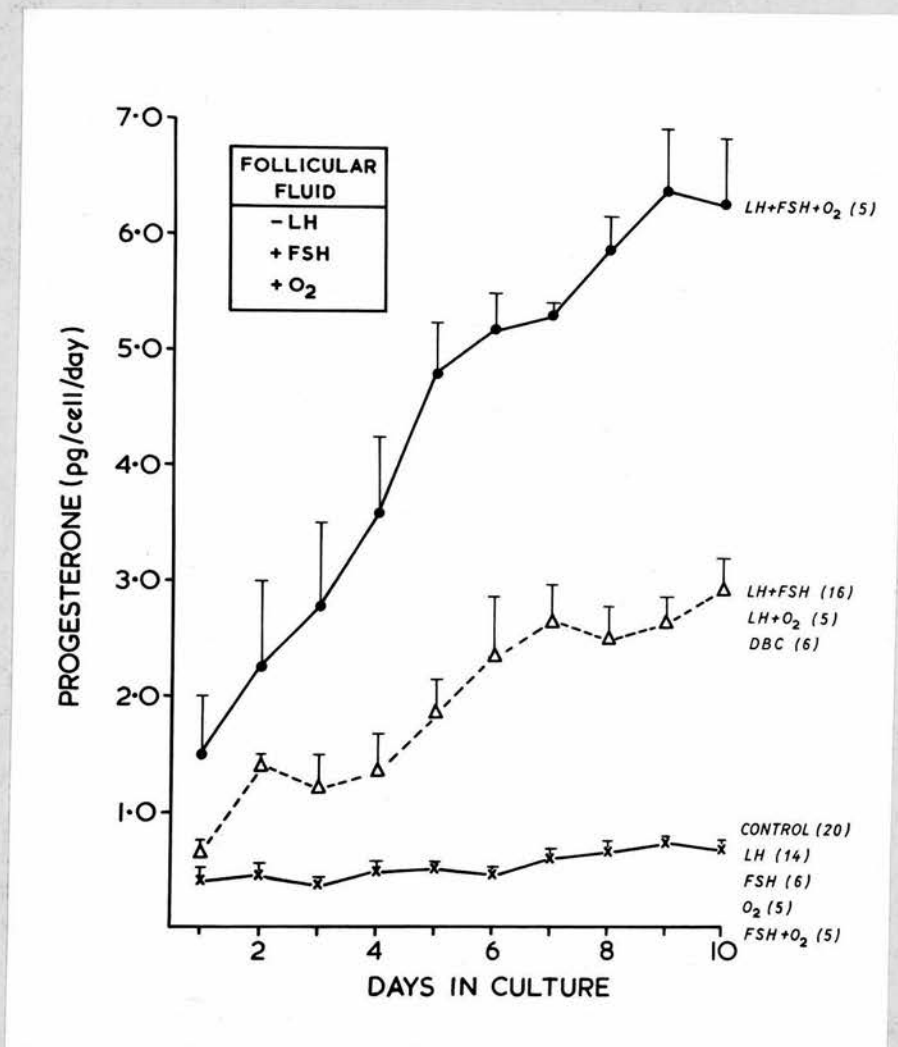


Fig. 26. (legend on p. 154)

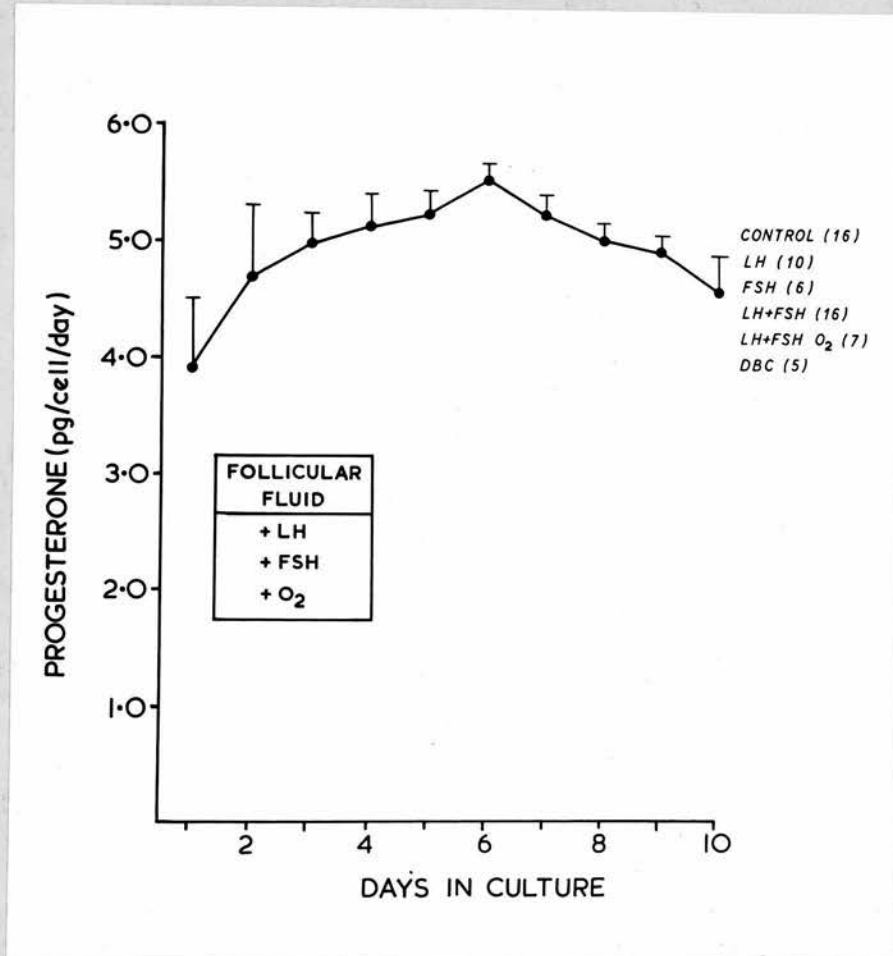


Fig. 27. (legend on p. 154)

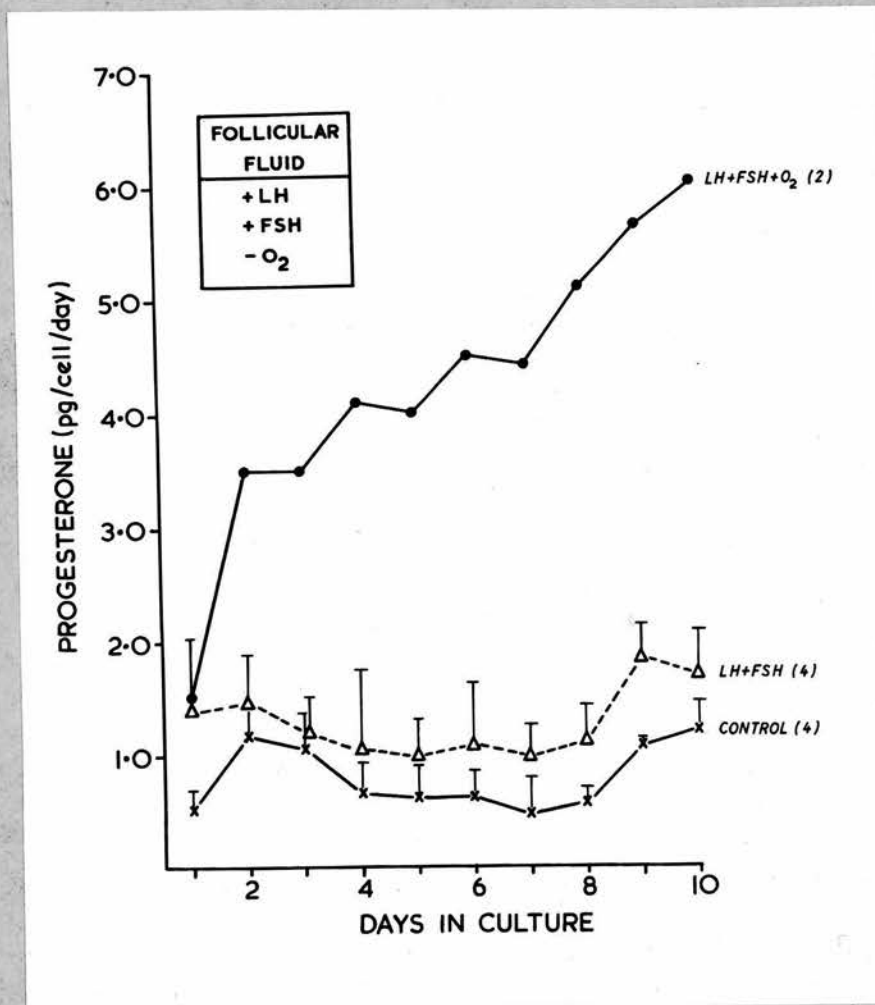


Fig. 28. (legend on p. 154)

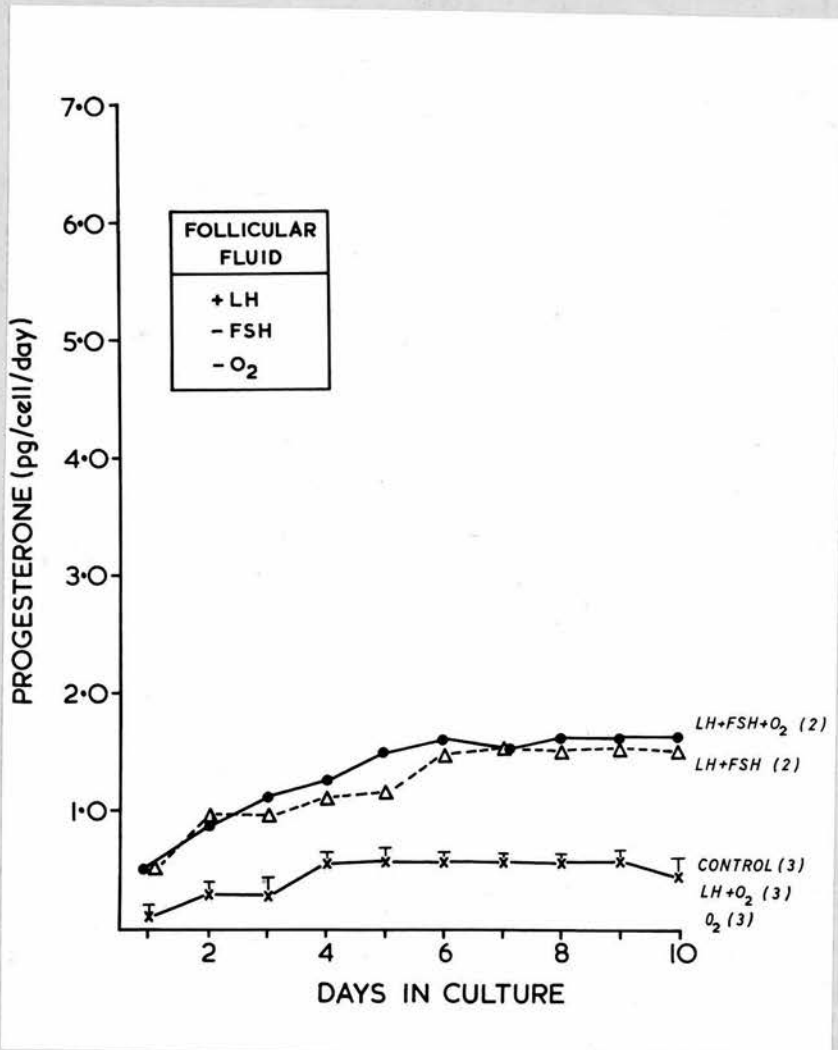


Fig. 29. (legend on p. 154)

DISCUSSION

The most interesting aspect of this study is the clear demonstration that the ability of human granulosa cells in culture to undergo mitosis or secrete progesterone is predetermined by the hormonal environment of the follicle from which they have been harvested. This therefore gives added point to the previous study (see discussion, Chapter 3) which documented a critical sequence of hormonal changes that take place within the follicular fluid prior to ovulation.

It seems that the mitotic activity of the granulosa cells in the intact follicle is stimulated by the presence of FSH and high concentrations of oestradiol in follicular fluid, an ability that is retained in culture. However, the presence of LH in the follicular fluid, or the addition of LH to culture medium impairs this mitotic activity (Delforge et al., 1972; see discussion, Chapter 3).

The biosynthetic activity of the granulosa cells in culture is also influenced by their prior hormonal environment. Progesterone secretion was greatest from cells harvested from follicles that contained high concentrations of FSH, LH and oestradiol. Similarly, the addition of these three hormones to the culture was able to stimulate progesterone secretion if the cells had been obtained from hormone deprived follicles. These findings are consistent with those reported by Goldenberg et al., 1972b) for porcine granulosa cells.

It is of interest to relate these findings to the sequence of hormonal changes taking place in the Graafian follicle during the course of follicular maturation (see Fig. 21). During the early follicular phase FSH and low concentrations of oestradiol were present in a large proportion of small antral follicles. By the mid-

follicular phase there were few small follicles with FSH, but some of the larger ones now contained high concentrations of both FSH and oestradiol. Just prior to ovulation, the largest follicle contained high concentrations of LH in addition to FSH and oestradiol. It is tempting to conclude that the initial appearance of FSH may be essential for follicular growth (Baird et al., 1975); subsequent exposure to FSH and oestradiol stimulates both the mitotic activity of the granulosa cells and an increase in the number of receptors for LH (Zelevnick et al., 1974; Channing, 1975) which prepares them for eventual steroid secretion. The final entry of LH into the follicle inhibits further mitosis and together with prolactin (see results in Chapters 6 and 8) initiates progesterone secretion.

There seems to be good agreement between the time course of these events in vivo and in vitro. For example, FSH treatment in vivo takes 6-10 days to mature a follicle (Brown et al., 1969; Gemzell & Johansson, 1971; Bertrand et al., 1972) and granulosa cells harvested from small early follicular phase follicles (containing FSH) achieve their maximum biosynthetic capacity in vitro after 8-10 days if LH, FSH and oestradiol is added to the cultures each day. Although it is necessary to add LH to assess the steroidogenic potential of granulosa cells in vitro, LH does not enter the follicle in appreciable amounts until the mid-cycle peak in plasma (see Fig. 13). These studies suggest therefore that a relatively long period, 8-10 days, of continual exposure to FSH and oestradiol is required before the maximum biosynthetic capacity of the granulosa cells is achieved; this synthetic potential is then only realised

and sustained in the presence of low physiological amounts of gonadotrophin and prolactin (see results section, Chapter 6).

During the luteal phase, Graafian follicles secrete little or no oestradiol (Baird & Fraser, 1974a), presumably because of the insufficient gonadotrophic support (Baird et al., 1975). These follicles have low concentrations of oestradiol in their follicular fluid (Baird & Fraser, 1974b; de Jong et al., 1974; Sanyal et al., 1974; Fig. 15),,but they may contain LH or LH + FSH. Granulosa cells harvested from these follicles do not undergo mitosis in culture and furthermore they secrete only low amounts of progesterone. Thus it appears that granulosa cells within follicles in the luteal phase and lacking oestradiol are unable to proliferate or achieve their maximum biosynthetic capacity. These findings also suggest that the premature appearance of LH in the follicular fluid of a Graafian follicle could arrest mitosis and interfere with the ability of granulosa cells to achieve their maximum biosynthetic potential. It appears that only the actively maturing follicle receives the critical sequence of hormones that will allow its granulosa cells to proliferate and achieve their maximum biosynthetic potential following ovulation.

These studies have shown that it is only granulosa cells from the actively maturing follicles whose steroidogenic potential is related to the stage of the ovarian cycle; Channing (1969c, 1970a, 1970b, 1970c) has made similar observations for horse, pig and rhesus monkey. However, most follicles recovered throughout the menstrual cycle were presumably not active because they were devoid of gonadotrophin(s) and/or oestradiol, and the steroidogenic potential of their granulosa cells were unrelated to the stage of the cycle.

These studies strongly suggest that the hormonal environment of

the Graafian follicle influences both the mitotic activity and biosynthetic potential of its granulosa cells, so that endocrine events occurring some considerable time before ovulation may dictate the subsequent activity of the corpus luteum.

SUMMARY

The steroidogenic potential of granulosa cells harvested from human Graafian follicles containing varying concentrations of pituitary and steroid hormones was examined. The mitotic activity and production of progesterone by granulosa cells in vitro was found to be correlated with their hormonal environment at the time of harvesting. Only cells from follicles containing some FSH and high concentrations of oestradiol underwent spontaneous mitosis in vitro. However, mitosis could be induced by adding FSH and high concentrations of oestradiol to the culture, provided that the concentration of LH was low. Cells harvested from follicles containing LH, FSH and high concentrations of oestradiol secreted significantly more progesterone than cells from follicles which did not contain all three hormones.

CHAPTER 6

**A POSSIBLE ROLE FOR PROLACTIN IN THE CONTROL OF STEROID
SECRETION BY THE HUMAN GRAAFIAN FOLLICLE**

INTRODUCTION

In an attempt to gain more insight into the possible role of prolactin in controlling ovarian activity in women, the effects of the addition or neutralisation of human prolactin in culture media on the production of progesterone by human granulosa cells growing in tissue culture were studied.

RESULTS

The effect of prolactin on the viability and mitotic activity of human granulosa cells in culture

When cells were exposed to prolactin at concentrations between 0.2 and 100 ng/ml with or without added LH + FSH there was no significant increase or decrease in cell numbers during the 10 days of culture when compared to untreated controls.

The production of progesterone by granulosa cells in culture medium in which the endogenous prolactin was neutralised with excess prolactin antiserum

Neutralisation of prolactin in the culture medium by the addition of rabbit anti-human prolactin serum (A4) (1/500, v/v final dilution) caused a significant decrease in the production of progesterone when compared to the controls (Fig. 30) ($p < 0.001$, Students t test). The controls were either untreated culture medium (5 duplicate experiments) or culture medium treated with normal rabbit serum (3), rabbit anti-bovine serum albumin (3) or rabbit anti-human growth hormone (5) all of which were present at a final dilution of 1/500 (v/v). There were no significant differences in the production of progesterone between these control cultures ($p > 0.05$) which were found to be within the precision achieved in replicate cultures. To confirm that free prolactin in the culture medium had been completely neutralised by the antiserum, the anti-prolactin-prolactin complex was precipitated out using a solid phase second antibody; the concentration of LH and

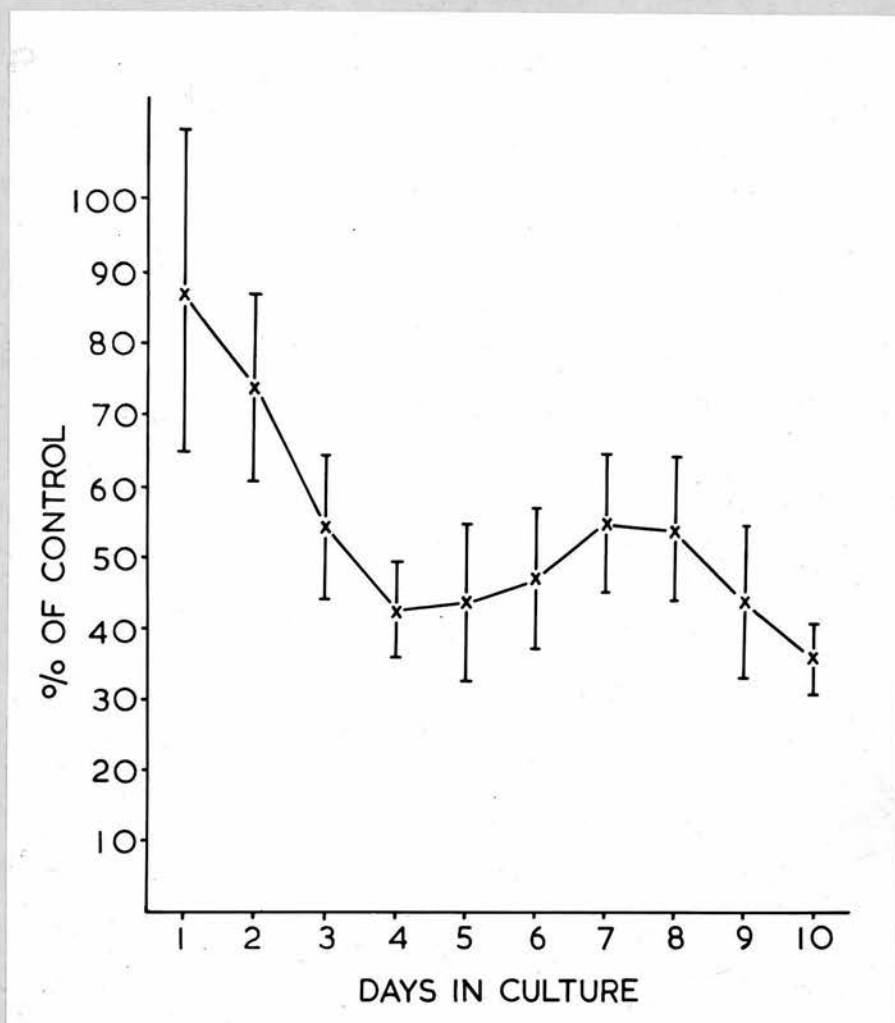


Fig. 30. The daily production of progesterone by human granulosa cells in a culture medium in which the endogenous prolactin activity was neutralised with excess rabbit anti-human prolactin serum. Results are expressed as a percentage of the controls and represent the mean of 5 replicate cultures. Vertical bars = ± 1 S.E.M.

FSH in the medium were unaltered whereas prolactin was undetectable (<0.2 ng/ml).

The daily production of progesterone by granulosa cells in a culture medium containing high concentrations of prolactin

The effect of adding prolactin to culture medium on the daily production of progesterone is shown in Fig. 31. There was no effect on progesterone secretion if the concentration of prolactin was between 5 and 20 ng/ml; the mean concentration of prolactin in peripheral blood of women during the menstrual cycle is 15 ± 1 (\pm S.E.M.) (McNeilly, Evans & Chard, 1973). When the concentrations in the culture medium were increased from 25 to 100 ng/ml, there was a progressive decrease in the daily production of progesterone. When the concentration of prolactin in the culture medium was 100 ng/ml, the total progesterone output after 10 days culture was only 10% of that achieved in the control (Fig. 31). This inhibitory effect persisted even when the concentrations of LH (1.8 mu./ml) and FSH (1.7 mu./ml) were both increased to 30 mu./ml (Fig. 32) and it could be obtained with granulosa cells collected from follicles at any stage of the menstrual cycle.

When the concentrations of prolactin were increased on the 5th and 6th day of culture from 10 to 100 ng/ml there was an immediate fall in the daily secretion of progesterone (Fig. 33) which returned to control values when the prolactin concentrations were lowered once more.

DISCUSSION

These studies clearly demonstrate that granulosa cells are dependent on low physiological levels of prolactin for the secretion of progesterone. Furthermore the cells appear to secrete at maximum capacity when the concentration of prolactin is within the

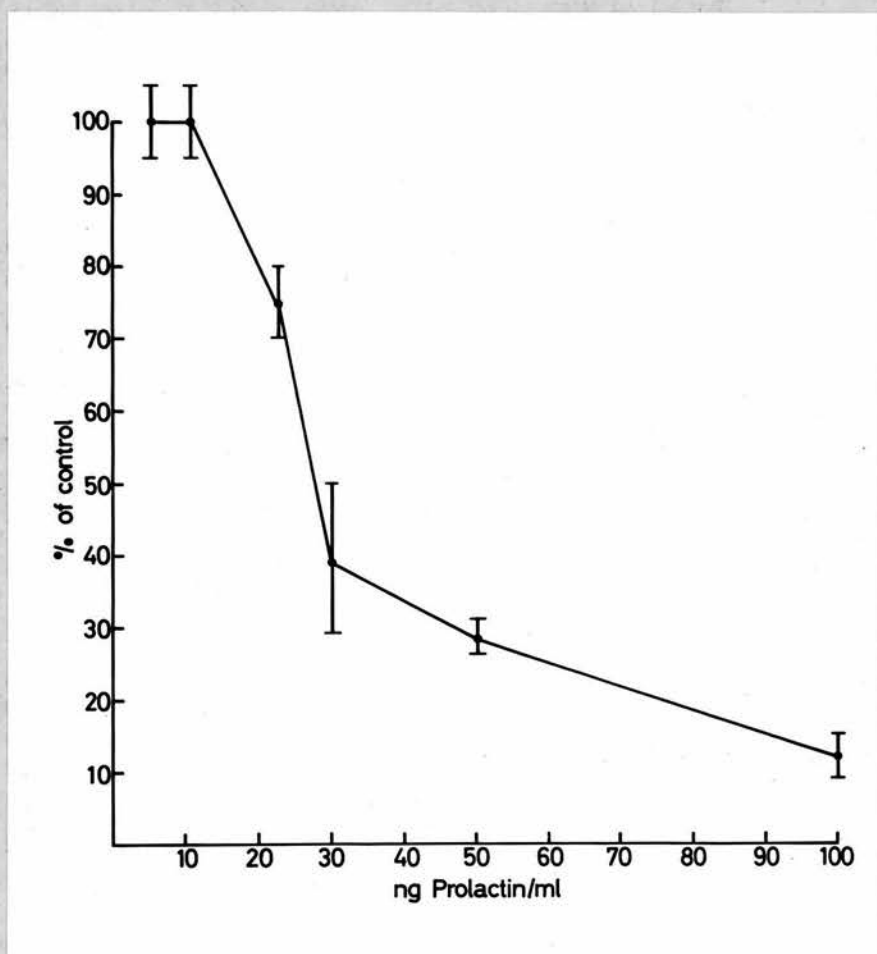


Fig. 31. Effect of varying concentrations of prolactin on the total production of progesterone by human granulosa cells during 10 days culture when the cells were exposed to low levels of LH and FSH (each < 2 mu./ml). Each point (x) is the mean result of 5 duplicate experiments. The vertical bars represent ± 1 S.E.M. The results are expressed as a percentage of the controls.

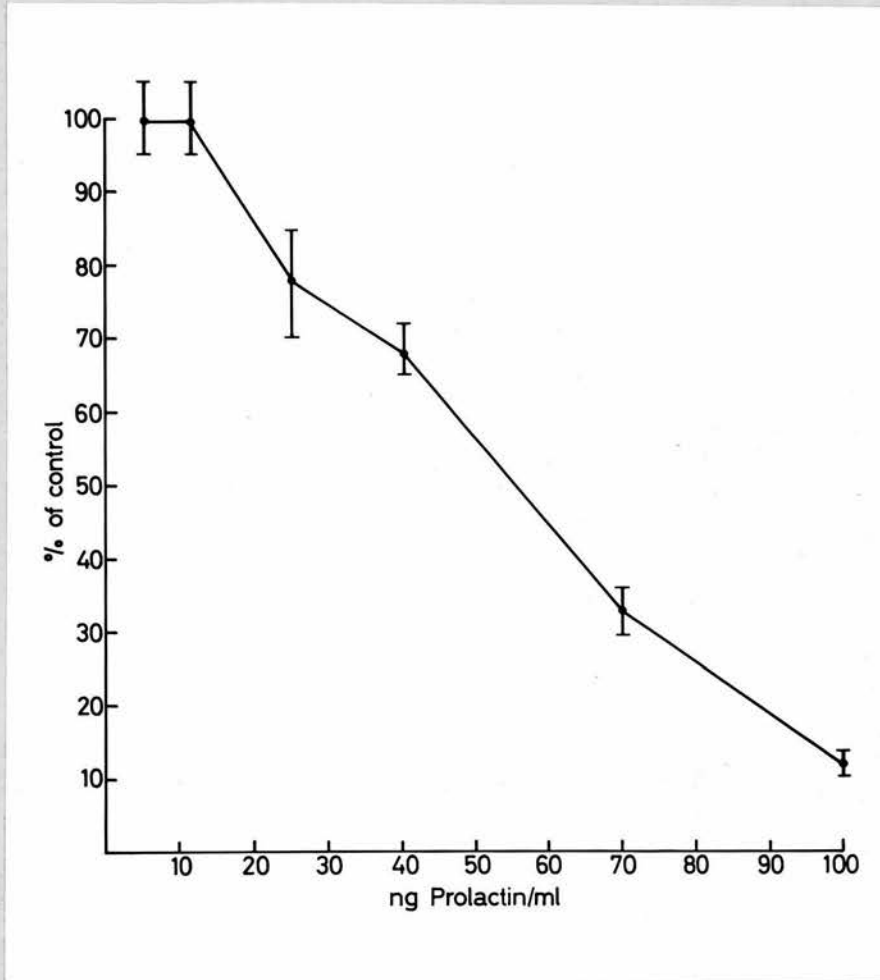


Fig. 32. Effect of varying concentrations of prolactin on the total production of progesterone by human granulosa cells during 10 days of culture when the cells were exposed to high concentrations of LH and FSH (each 30 mu./ml). Each point (x) is the mean result of 5 replicate experiments. The vertical bars represent \pm S.E.M. The results are expressed as a percentage of the controls.

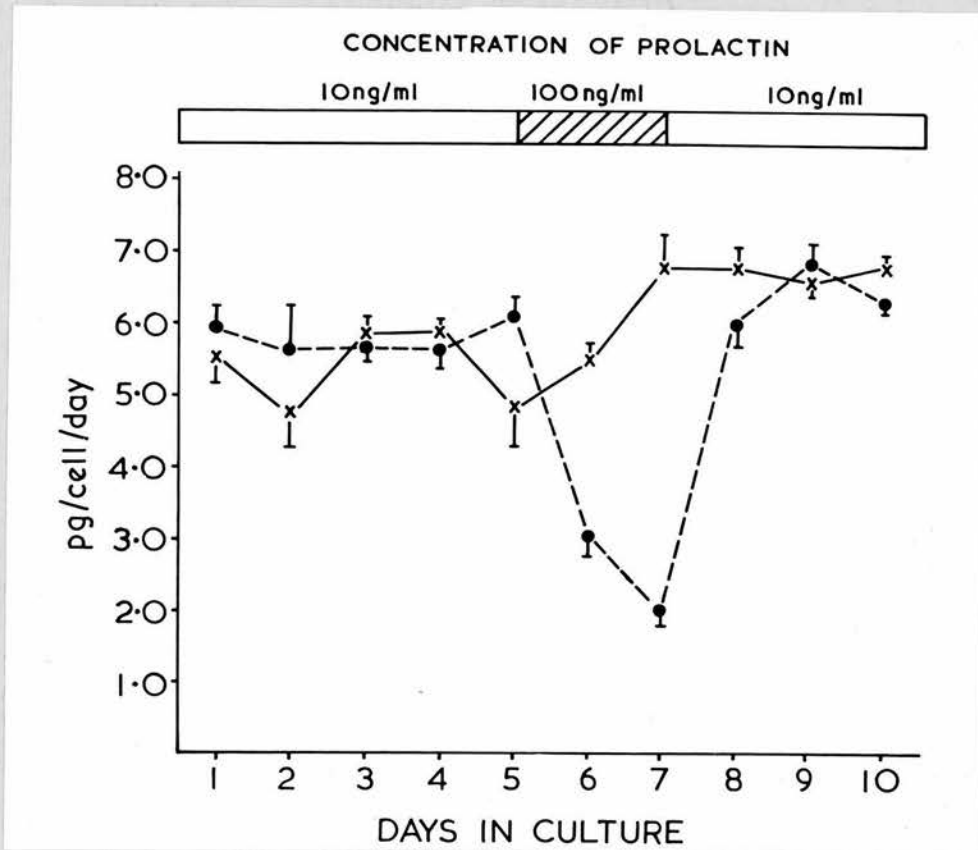


Fig. 33. Effect of increasing the concentration of prolactin in culture medium on Days 5 and 6 on the daily production of progesterone by human granulosa cells. ●—● represents the mean daily production by cells exposed to 100 ng prolactin/ml on Days 5 and 6 (6 duplicate experiments); X—X represents the mean daily production by cells exposed to 10 ng prolactin/ml each day (6 duplicate experiments). Vertical bars represent ± 1 S.E.M.

normal physiological range (i.e. 5-20 ng/ml) (McNeilly et al., 1973). If the concentration exceeds 20 ng/ml however, there is a marked inhibition of progesterone secretion which is not overcome by increasing the levels of LH and FSH although secretion returns to normal immediately after the levels of prolactin are lowered. Since neither the viability nor the mitotic activity of the cells are affected when they are exposed to high concentrations of prolactin, the inhibitory effect is probably a consequence of changes in the biosynthetic potential of the cell. Similar results have been obtained with intact mouse follicles maintained in organ culture using human, rat or sheep prolactin (McNatty, Neal & Baker, unpublished observations). These findings suggest that prolactin may have an important role in controlling steroidogenesis by the Graafian follicle. Prolactin is probably essential for steroidogenesis since it influences the amount of cholesterol available for metabolism through to progesterone (Armstrong, Knudsen & Miller, 1969; Behrman et al., 1970; Behrman, MacDonald & Greep, 1971b). However the way in which high levels of prolactin alter the biosynthetic potential of the cells remains unknown.

These in vitro experiments suggest that high concentrations within the follicular fluid may actually depress progesterone secretion by the granulosa cells. This might provide an explanation for the fact that galactorrhoea with high prolactin levels is commonly associated with amenorrhoea (Perez, Vela, Masnick & Potter, 1972), and that if the prolactin levels are lowered by treatment with 2-Br- α -ergocryptine-methanesulphonate (CB-154), ovulation and menstruation recur (Besser, Parke, Edwards, Forsyth & McNeilly, 1972; del Pozo, Brun del Re, Varga & Friesen, 1972). It is well recognised that post-partum

lactation can inhibit ovulation in women and other animals (Perez et al., 1972; Bonte & van Balen, 1969) although the mechanism has never been explained.

Although there are now numerous descriptive accounts of the changing hormone levels in blood and urine throughout the menstrual cycle, we do not really understand what factors are responsible for the formation, maintenance and regression of the human corpus luteum. This is an area of great potential interest for the development of new forms of contraception. The in vitro results presented here cast doubt on the simplistic view that LH is the only gonadotrophin necessary for luteal maintenance (Vande Wiele et al., 1970) and suggest that prolactin is also involved. Furthermore, they add further support to the hypothesis that the gonadotrophin content of the follicular fluid has important consequences for the secretory activity of the granulosa cells both before and after ovulation.

SUMMARY

Granulosa cells were cultured in a medium in which the prolactin activity was neutralised with excess antiserum to prolactin or in a medium in which the concentration of prolactin was increased with or without a concomitant increase in LH and FSH.

The production of progesterone by granulosa cells was inhibited either after complete neutralisation of free prolactin in the culture medium or when the concentration of prolactin was > 25 ng/ml. The inhibitory effect with high concentrations of prolactin with or without LH + FSH was not due to changes in cell viability or mitotic activity. Furthermore the inhibitory effect with high concentrations of prolactin was reversible.

CHAPTER 7

**IMMUNOREACTIVE SUBUNITS OF LUTEINIZING HORMONE IN PERIPHERAL
PLASMA AND FOLLICULAR FLUID THROUGHOUT THE MENSTRUAL CYCLE AND
THEIR EFFECT ON THE SECRETION RATE OF PROGESTERONE BY
GRANULOSA CELLS IN CULTURE**

INTRODUCTION

The pituitary glycoproteins, LH, FSH and thyroid-stimulating hormone (TSH) consist of two non-identical, non-covalently linked polypeptide chains or subunits termed α and β (Ryan, 1968; Papkoff & Ekblad, 1970; Papkoff, Sairam & Li, 1971; Pierce, Liao, Howard & Shome, 1971; Saxena & Rathnam, 1971; Stockell-Hartree, Thomas, Braikevitch, Bell, Christie, Spaul, Taylor & Pierce, 1971). Similarly the subunit nature of HCG has been established (Swaminathan & Bahl, 1970; Canfield, Morgan, Kammerman, Bell & Agosto, 1971; Morgan & Canfield, 1971). The α -subunits of all four hormones TSH, LH, FSH and HCG are chemically and immunologically similar whereas the β -subunits are all different and responsible for the biological and immunological specificity of the hormones (Vaitukaitis & Ross, 1972). Thus the α -subunit of one of the four glycoproteins can be combined with the β -subunit of any of the other three and the biological activity is characteristic of the hormone from which the β -subunit is derived (Greep, 1974).

Most studies have generally shown that the α - and β -subunits are both very low in biological activity (i.e. $\sim 33\%$ or less of the activity of the native molecule (Papkoff & Li, 1970; Pierce et al., 1971; Channing & Kammerman, 1973b; Stockell-Hartree, personal communication) whilst the biological activity of the recombined subunits is largely or completely restored. However, a few studies have suggested that the subunits may have considerable biological activity. For example the β -subunit of ovine FSH, when tested in lizards, has all the activity of the native or reconstituted ($\alpha + \beta$) hormone, whereas the α -subunit is totally inactive (Licht & Papkoff, 1971). Similarly the β -subunit of ovine LH when tested in rats for lipotropic activity is as active as the complete molecule (Gospodarowicz, 1971)

More recently the β -subunit of ovine LH has been shown to be effective in the induction of ovulation in intact and hypophysectomized hamsters, whilst the α -subunit is totally inactive (Yang, Sairam, Papkoff & Li, 1972).

The subunits of human LH probably circulate in plasma independently of the intact hormone (Laburthe, Dolais & Rosselin, 1973; Hagen & McNeilly, 1975a; Hagen, McNatty & McNeilly, unpublished data), with peaks occurring during the late follicular phase of the menstrual cycle. It is not known whether the subunits are released independently by the pituitary or whether their presence in plasma is a consequence of intact hormone dissociating (Stockell-Hartree, personal communication).

Proteins can cross the blood-follicle barrier and the distribution of serum proteins in follicular fluid is approximately proportional to their molecular weights (Johnson, 1973). It is reasonable to assume therefore that the subunits of LH are present in follicular fluid in greater amounts than the intact hormone.

The possibility of raising antibodies against the β -subunit of a protein hormone may provide a method of neutralising the biological activity of the intact hormone from which the β -subunit was derived without interfering with the biological activities of other closely related protein hormones (Hearn, Short & Lunn, 1975; Stevens, 1975). This technique provides an immunological method for the control of fertility. Since there are probably circulating levels of subunits in plasma and follicular fluid it seems necessary to examine the effects of the subunits on follicular development and/or ovarian steroid secretion. Furthermore it seems necessary to use an in vivo or in vitro biological test system from the same species as that from which the pituitary or placental subunit is derived in view of the

potent activities they can have in an unrelated species (see Licht & Papkoff, 1971)

The concentrations of α - and β -subunits of luteinizing hormone (LH α and LH β respectively) were measured in samples of peripheral plasma and follicular fluid collected from women at varying stages of the menstrual cycle. The aim of this study was to investigate the relationship between the concentrations of LH α and LH β in follicular fluid and to relate them to concentrations in plasma, follicle size and the stage of the menstrual cycle. In addition the rate of progesterone secretion by human granulosa cells in tissue culture after exposure to the α - and β -subunits of LH was compared to that achieved after the cells were exposed to intact LH.

RESULTS

Concentration of LH α and LH β in plasma

Figure 34 shows the concentration of LH α and LH β in plasma in relation to the stage of the menstrual cycle. The samples were obtained from patients during surgery and immediately prior to oophorectomy.

Concentration of LH α in follicular fluid

The mean concentration of LH α in all antral fluids in relation to follicular size and the stage of the menstrual cycle is shown in Fig. 35. The minimum detectable level of LH α in the smallest follicles (4 mm) examined was 0.6 ng/ml which was therefore chosen as the limit of detection for all follicles. The percentage of follicles with detectable LH α at each phase of the cycle is shown in Table 15. The greatest proportion of follicles with α -subunit are found when there are peak levels in peripheral plasma (Fig. 35). The α -subunit was detectable in a proportion of large follicles

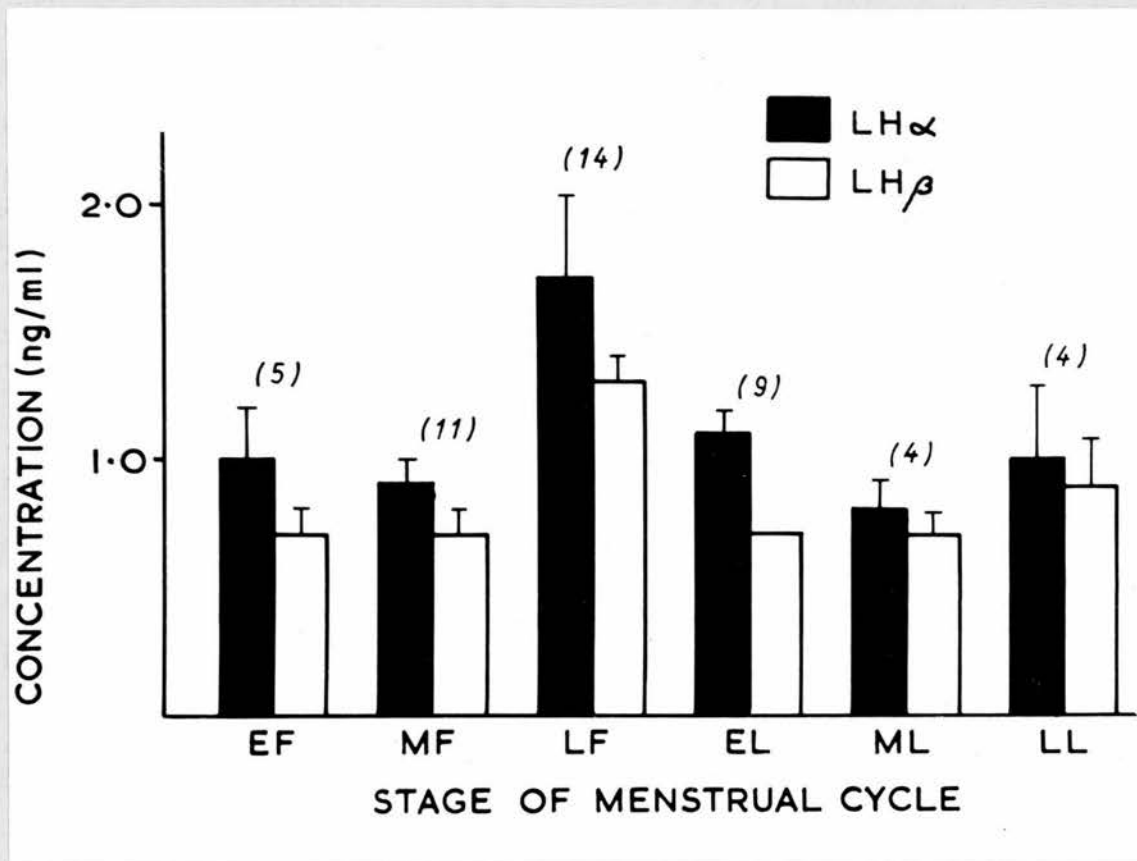


Fig. 34. Concentration of α and β subunits of LH in peripheral plasma in relation to the stage of the menstrual cycle. Results shown represent means + 1 S.E.M. For abbreviations see Fig. 10. Numbers in parentheses represent number of observations.

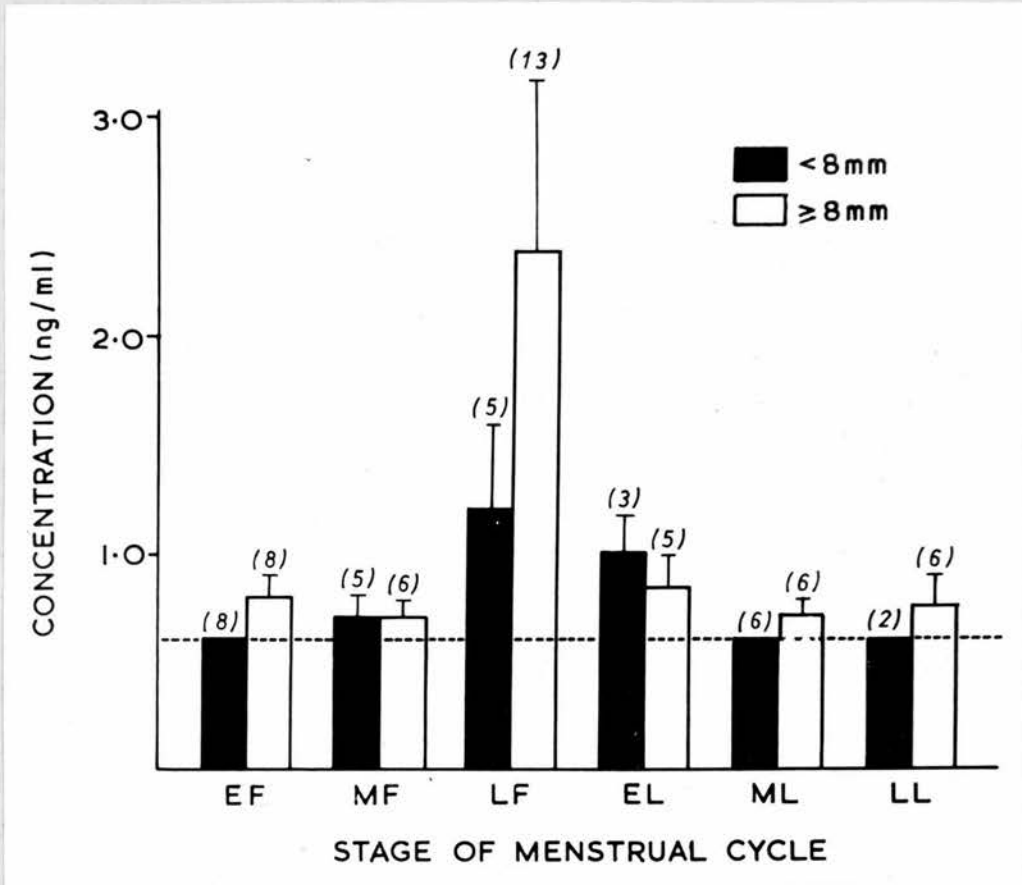


Fig. 35. Concentration of LH α in follicular fluid in relation to follicle size and stage of the cycle. Results shown represent means + 1 S.E.M. For abbreviations see Fig. 10. Numbers in parentheses represent number of observations. The dotted line represents the detection limit of the assay (0.6 ng/ml).

Table 15. Percentage of follicles with detectable levels of LH α and LH β in relation to size at each phase of the human menstrual cycle.

Size of follicle (diam.)	LH Subunit	Stage of Menstrual Cycle					
		M-EF	MF	LF	EL	ML	LL
< 8 mm	α	0.0	25.0	45.6	25.0	0.0	0.0
	β	0.0	0.0	30.0	25.0	0.0	0.0
\geq 8 mm	α	33.0	25.0	50.0	25.0	16.0	16.0
	β	0.0	0.0	45.6	25.0	0.0	0.0

EF, MF and LF refer to early, patient still menstruating, mid- and late follicular phase respectively, while EL, ML and LL refer to early, mid- and late luteal phase respectively.

(≥ 8 mm) at every phase of the menstrual cycle at concentrations similar to those found in plasma. By contrast α -subunit was only detectable in small follicles (< 8 mm) during the mid- to late proliferative phase and the early secretory phase of the cycle.

Concentration of LH β in follicular fluid

The mean concentration of LH β in all antral fluids in relation to follicular size and the stage of the menstrual cycle is shown in Fig. 36. The percentage of follicles with detectable LH β at each phase of the cycle is shown in Table 15. The minimum detectable level of LH β in the smallest follicle examined was 0.6 ng/ml which was therefore chosen as the limit of detection for all follicles. The β -subunit was only detectable in a similar proportion of small or large follicles either during or just after peak concentrations in peripheral plasma. The concentration of β -subunit in follicular fluid at mid-cycle was similar to that in plasma.

The effect of subunits of LH on the viability and mitotic activity of granulosa cells in vitro

When cells were exposed to LH α and LH β alone or in combination, there was no significant increase or decrease in cell numbers during the 10 days of culture, when compared to untreated controls.

The effect of differing doses of LH α or LH β subunit on the daily production of progesterone by granulosa cells in culture.

Figures 37 and 38 show the daily production of progesterone by granulosa cells after daily exposure to LH α and LH β at concentrations between 1 and 100 ng/ml. The daily production was expressed as a fraction of that achieved by the untreated control; if the subunit had no effect on steroidogenesis then the production rate/day was 1, but if stimulation occurred then the production rate was > 1 . There

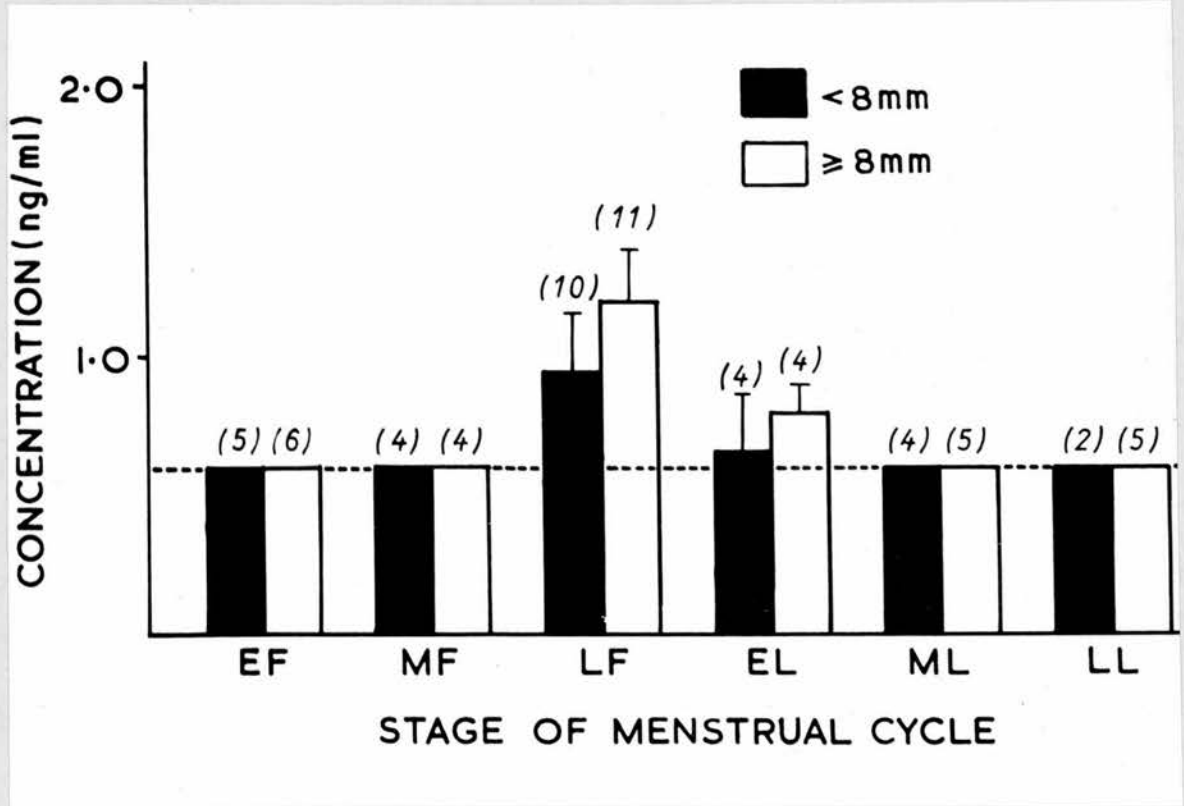


Fig. 36. Concentration of LH β in follicular fluid in relation to follicle size and stage of the cycle. Results shown represent means + 1 S.E.M. For abbreviations see Fig. 10. Numbers in parentheses represent number of observations. Dotted line represents the detection limit of the assay (0.6 ng/ml).

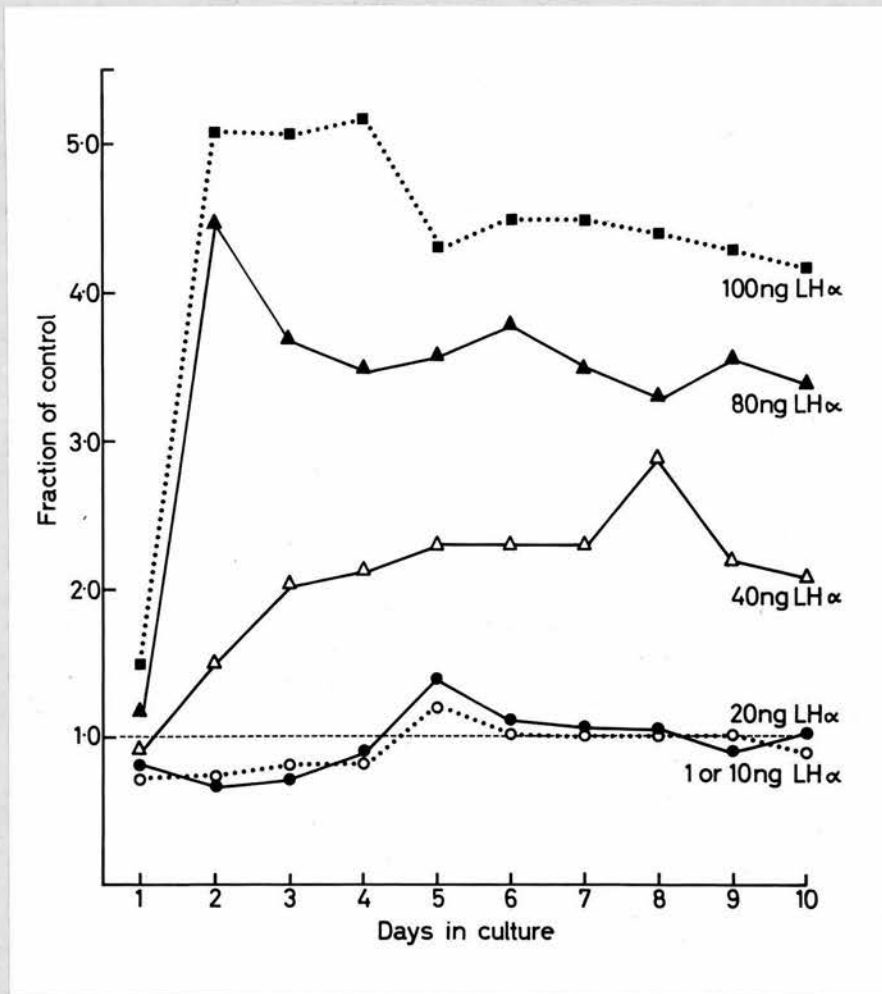


Fig. 37. Effect of differing doses of LH α on the daily production of progesterone by granulosa cells. The results are expressed as the ratio of LH α stimulated cells divided by the controls. Results of each experiment are the mean of 4 duplicate cultures. Every culture contained 30 mu. FSH/ml of the medium each day.

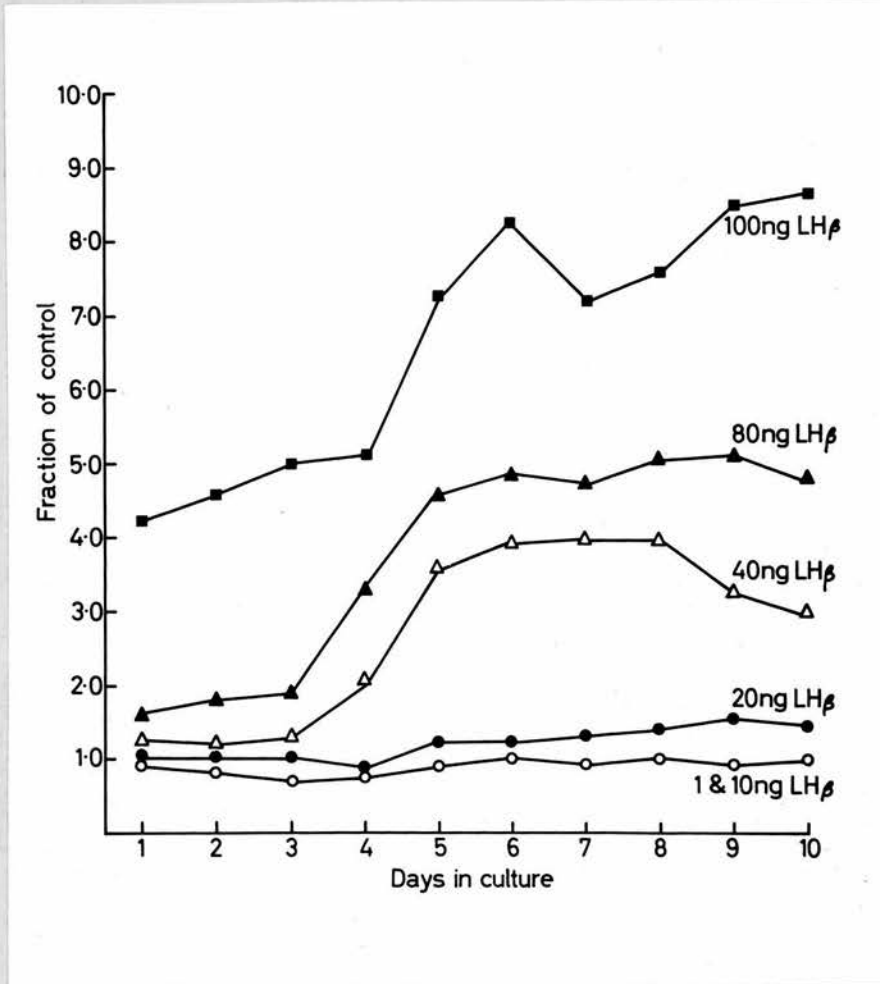


Fig. 38. Effect of differing doses of LH β on the daily production of progesterone by granulosa cells in culture. The results are expressed as the ratio of the LH β stimulated cells divided by the controls. Results of each experiment are the mean of 4 duplicate cultures. Every culture contained 30 mu. FSH/ml of medium each day.

was no increase in the daily production of progesterone when the concentration of α - or β -subunit was between 1 and 20 ng/ml; however there was a progressive increase in production if the concentration of either subunit was increased from 40 to 100 ng/ml.

The effect of adding both LH α and LH β subunits on the daily production rate of progesterone by granulosa cells in culture.

The daily addition of 10 or 40 ng LH α and LH β simultaneously is shown in Fig. 39. By the 10th day of culture, 10 ng LH α + 10 ng LH β increased the daily production rate 7-fold while 40 ng LH α + 40 ng LH β induced a 15 fold increase. By contrast 30 mu. LH induced a 10 fold increase in the production rate on the 10th day of culture. Since 7 mu. of LH is approximately equivalent to 1 ng LH (W. M. Hunter, personal communication) then equivalent amounts of α + β subunits only produced ~30% of the activity of intact LH (m/m).

DISCUSSION

During the normal menstrual cycle there are peak concentrations of LH and FSH at mid cycle (Faiman & Ryan, 1967; Burger, Catt & Brown, 1968; Midgley & Jaffe, 1968; Franchimont, 1969; Zbraham, Odell, Swerdloff & Hopper, 1972). It is also apparent that there are peak concentrations of both subunits coincident with both LH and FSH.

The pattern of LH α and LH β in follicular fluid is reminiscent to that for FSH and LH (see discussion, Chapter 3). Follicle-stimulating hormone was present in a greater number of large follicles (≥ 8 mm) both at the early and late follicular phases. Similar results were found for LH α . Unlike FSH however, the concentration of the α -subunit in fluid was similar to that found in plasma. This difference may either be due to lack of uptake or utilization of the

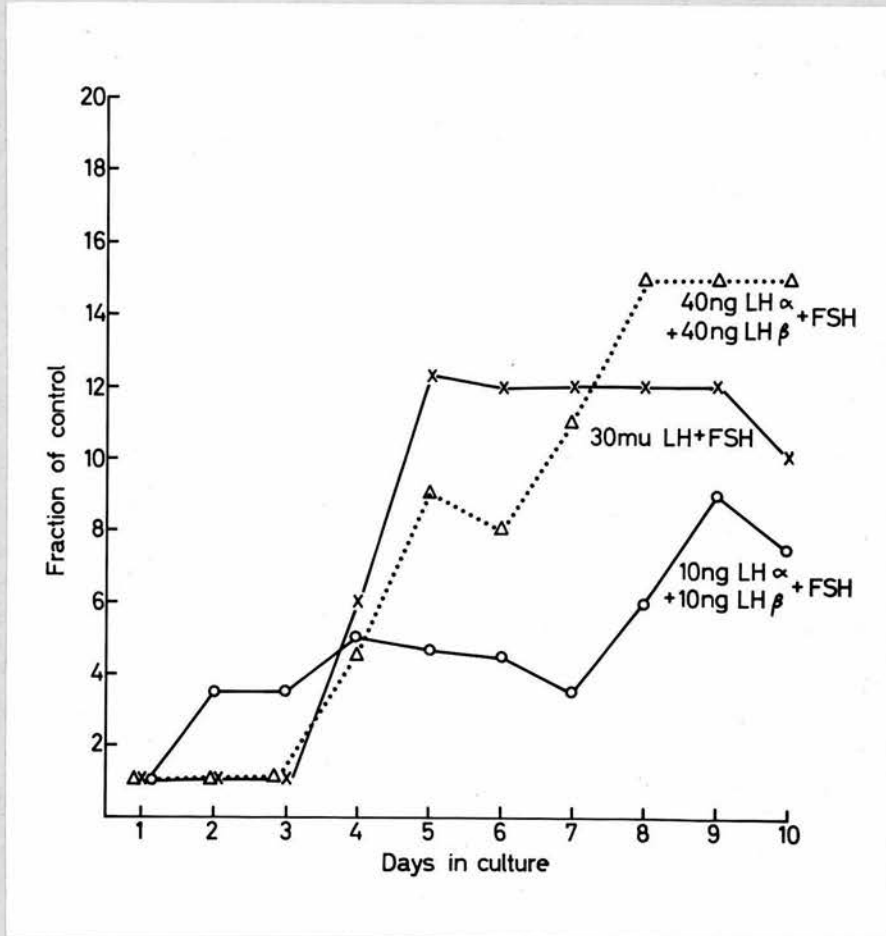


Fig. 39. Effect of LH α + LH β on the production of progesterone by granulosa cells in culture. The results are expressed as the ratio of the LH α + LH β stimulated cells divided by the controls. Results for each experiment are the mean of 3 duplicate cultures. Every culture contained 30 mu./FSH/ml of medium each day.

subunit or merely due to differences in the rate of transfer across the blood-follicle barrier. Intact LH was found only in follicles either at the late-follicular or early luteal phase of the cycle. Similar results were found for LH β . Although LH β was not detectable in any follicle other than at mid-cycle, this may be because the minimum detectable level in fluid was close to the actual levels in plasma. Nevertheless the results clearly show that the concentrations of LH β in follicular fluid at mid-cycle were similar to those in plasma.

It seems unlikely in view of the in vitro findings that the subunits either alone or in combination influence the rate of cell mitosis or their viability unless both subunits are present in follicular fluid in high concentrations prior to the mid-cycle gonadotrophin peak (Table 13). Furthermore when LH α is present in follicular fluid even with FSH it is unlikely to alter or disrupt the developing steroidogenic potential of the granulosa cells. However at mid-cycle when both α - and β -subunit are present in the fluid of a large preovulatory Graafian follicle it is not unreasonable to expect recombination of the subunits to occur which would enhance steroidogenesis by the granulosa cells undergoing luteinization at this time (see Fig. 39).

The biological activity of the glycoproteins and their subunits have been studied in vivo and in vitro and most studies have shown that the subunits themselves have little or no intrinsic biological activity (Reichert, 1972; Catt, Dufau & Tsuruhara, 1973; Cole, Davis, Huseby & Rice, 1973). The present studies support these conclusions and suggest that the role of the subunits of LH in follicular development is at best only of minor significance.

SUMMARY

The concentration of the α - and β -subunits of LH in peripheral plasma and follicular fluid was measured throughout the menstrual cycle, and the effects of LH α and LH β either alone or in combination on the production of progesterone by human granulosa cells in tissue culture was investigated.

Changes in the plasma concentrations of LH α and LH β throughout the menstrual cycle were similar to those of LH and FSH. In follicular fluid, LH α was detectable in large follicles (≥ 8 mm) throughout the menstrual cycle at concentrations similar to those found in plasma. By contrast LH α was only detectable in small follicles (< 8 mm) and LH β in all follicles during or just after peak concentrations in peripheral plasma. The LH subunits, even at levels 5 times higher than those in plasma did not increase the rate of progesterone secretion by human granulosa cells when each were added alone. However when both subunits were added simultaneously the increased rate in progesterone secretion was about 30% of that achieved with intact LH.

CHAPTER 8

**THE EFFECTS OF ANTIBODIES TO HUMAN GONADOTROPHINS ON THE
VIABILITY OF, AND RATE OF PROGESTERONE SECRETION BY
HUMAN GRANULOSA CELLS IN CULTURE**

INTRODUCTION

Human serum was treated with antibodies to gonadotrophins to remove selectively either FSH, prolactin and/or LH before adding the serum to preovulatory granulosa cells in tissue culture. In this way the role of each pituitary hormone in the initiation and maintenance of steroidogenesis by luteinizing granulosa cells in tissue culture was investigated.

RESULTS

Concentrations of gonadotrophins in human serum after antigonadotrophin treatment

The concentrations of LH, FSH and prolactin in human serum before and after treatment with antigonadotrophin sera are shown in Table 16. The concentrations of LH and FSH were generally unaffected after treatment with normal rabbit or guinea pig serum, or rabbit anti-bovine serum albumin. Rabbit anti-growth hormone treatment removed about 50% of the endogenous prolactin. The rabbit anti-human prolactin A₄ removed selectively >95% of the endogenous prolactin while the anti prolactin A₃ removed >95% of both LH and prolactin. Guinea-pig anti-human LH treatment removed ~95% of the FSH and about 80% of the LH while rabbit anti-human LH removed ~95% of the LH and ~50% of the FSH; in both cases the concentration of prolactin did not change.

Viability of HeLa cells cultured in antigonadotrophin treated serum

There was no morphological evidence to suggest that the antigonadotrophin treated culture media or untreated control media contained cytotoxic agents or lacked serum components essential for the maintenance of cells in culture. In all cultures mitosis had occurred, and the coverslips on which ~90% of the surface area was covered in cells at the start of culture were nearly all completely covered (100%) after six days of culture.

Table 16. Effect of adding Gonadotrophin antisera on gonadotrophin concentrations in human sera.

Gonadotrophin concentrations in human serum before treatment			Antiserum added	Final dilution of antiserum	Gonadotrophin concentrations in human serum after addition of antisera		
LH mu./ml	FSH mu./ml	Prolactin ng/ml			LH mu./ml	FSH mu./ml	Prolactin ng/ml
15.4	14.3	12.8	Normal rabbit serum	1:200	16.8	12.0	11.7
"	"	"	Rabbit anti BSA	1:200	17.1	11.1	10.4
"	"	"	Rabbit anti HGH (M105)	1:200	16.0	15.0	6.5
"	"	"	Rabbit anti human prolactin (A ₄)	1:400	12.8	11.8	< 0.2
"	"	"	Rabbit anti human prolactin (A ₃)	1:200	< 0.2	2.2	< 0.2
"	"	"	Rabbit anti human LH (R6)	1:200	0.8	7.1	9.9
"	"	"	Normal guinea pig serum	1:200	16.1	13.7	13.6
"	"	"	Guinea pig anti human LH (7252)	1:200	2.9	< 0.3	12.6

Concentrations of gonadotrophins and oestradiol in the follicular fluid of the Graafian follicles

All 30 samples of follicular fluid contained detectable amounts of LH (range 0.8-1.8 mu./ml), FSH (mean 2.0, range 1.3-3.9 mu./ml), prolactin (mean 9.6 range 1.5-18.1 ng/ml), and high concentrations of oestradiol (mean 704, range 239-3600 ng/ml). Thus all the granulosa cells were harvested from actively developing follicles (see Chapter 3 and 5).

Progesterone secretion by granulosa cells grown in a culture medium devoid of gonadotrophin(s)

The secretion of progesterone by granulosa cells in normal human serum (36 experiments) was not significantly different from that achieved by cells cultured in human serum containing antibodies to steroid producing tissues (42) ($P > 0.2$, paired t test). Similarly there were no significant differences in the secretion of progesterone between cells cultured in normal human serum (36 experiments), human serum treated with normal rabbit serum (20), normal guinea pig serum (10), rabbit anti-BSA serum (8) or rabbit anti-HGH serum (12) ($P > 0.05$, paired t test). Consequently the secretion of progesterone from the granulosa cells cultured in gonadotrophin deprived culture medium was compared to the average secretion rate from all control cultures in the same experiment. The variation in the secretion of progesterone between duplicate cultures was $7.3 \pm 1.9\%$, mean \pm S.E.M.; ($n = 174$).

Granulosa cells in the control cultures achieved their maximum rate of secretion (about 5 pg/cell/day) after 2 to 5 days in culture and maintained this secretion rate until the cultures were stopped on Day 10 (see Fig. 26 and 27).

The daily progesterone secretion by granulosa cells cultured in

a medium devoid of prolactin alone or prolactin + LH is shown in Fig. 40. After 24 hours in either medium, the secretion of progesterone was only 60% of the controls, and from Day 6 to 10 of culture the secretion rates were only 30 and 20% of the controls respectively although the number of cells was similar in treatment and control groups. In all experiments, between 80 and 125% of the cells remained after 10 days culture when compared to the number of live cells on the first day of culture. The daily secretion of progesterone by granulosa cells grown in a culture medium with a concentration of LH which was about 20 times lower than the controls (0.17 mu./ml compared to 3.1 mu./ml LH) is shown in Fig. 41. The number of cells remaining in the treatment and control groups was similar and was between 91 and 140% of the number at the start of culture. Although the cells remained viable when exposed to extremely low levels of LH their daily secretion of progesterone in vitro declined in a manner similar to those cultured in a medium devoid of prolactin.

The number of cells remaining on the coverslips after 10 days culture in serum devoid of FSH (<0.06 mu./ml) was only $7.8 \pm 1.4\%$ (n = 5) of those remaining in the controls. The addition of 30 mu. FSH (MRC Std. 73/519) each day in 50 μ l of phosphate buffered saline to the culture medium devoid of FSH maintained the cells at similar numbers to those in the controls (Table 17).

DISCUSSION

This study has clearly demonstrated the involvement of all three human pituitary gonadotrophins (LH, FSH and prolactin) in the initiation and maintenance of progesterone secretion by human granulosa cells in vitro. The granulosa cells were obtained from large active follicles just prior to ovulation, and they secreted maximum amounts

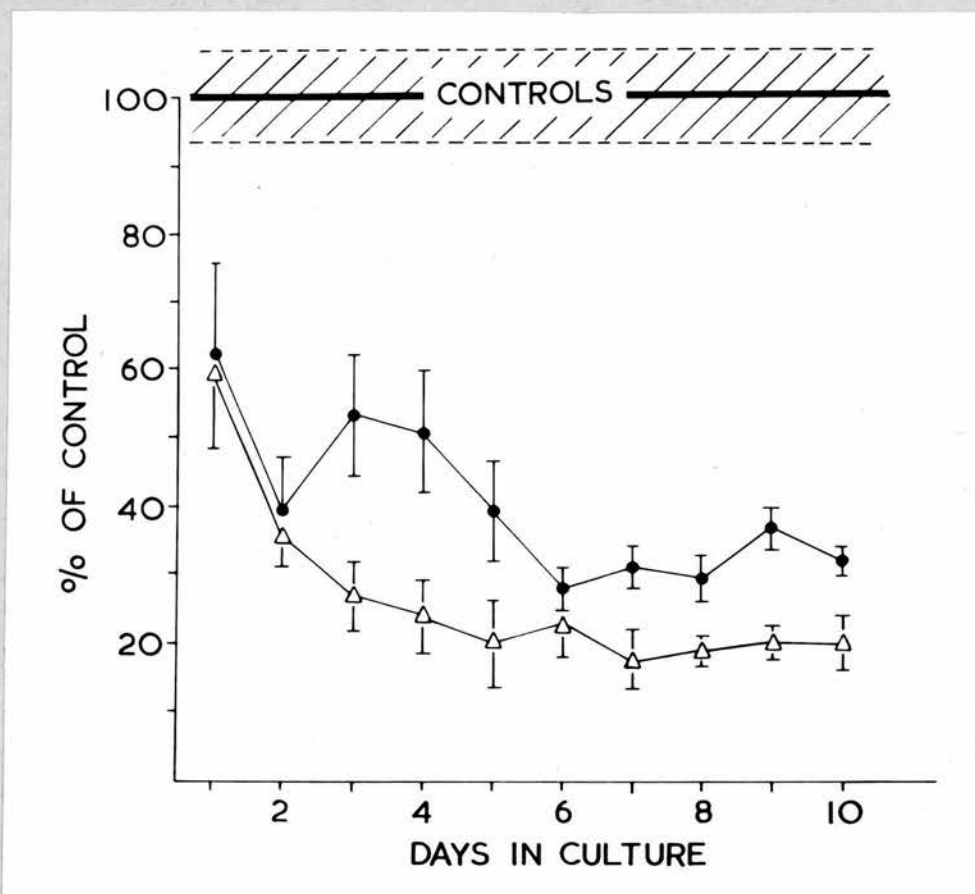


Fig. 40. The daily production of progesterone by human granulosa cells in culture medium devoid of prolactin (●—●) (5 duplicate experiments) or prolactin plus LH (Δ—Δ) (3 duplicate experiments) expressed as a percentage of the controls. The vertical lines represent ± 1 S.E.M. The controls are represented by the hatched lines which illustrate the mean and 1 S.E.M.

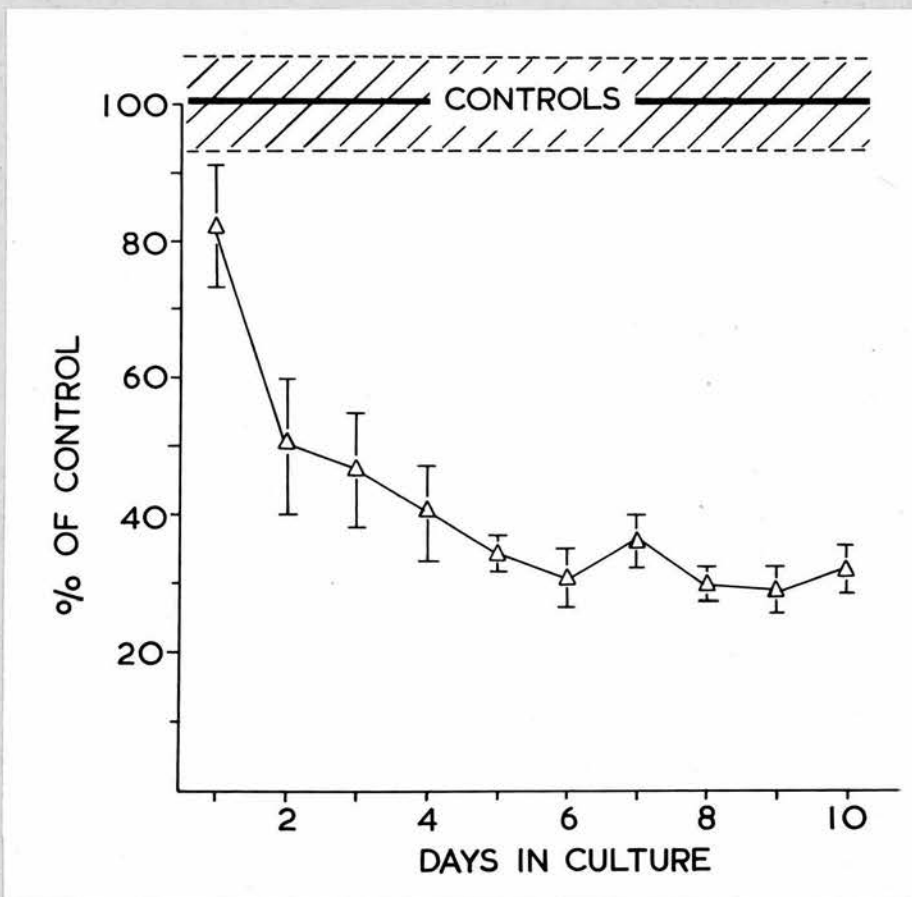


Fig. 41. The daily production of progesterone by human granulosa cells in a culture medium containing 20 times less LH than the controls (Δ — Δ) (7 duplicate experiments), expressed as a percentage of the controls. The vertical lines represent ± 1 S.E.M. The controls are represented by the hatched lines which illustrate the mean and ± 1 S.E.M.

Table 17. The effect of FSH on the viability of granulosa cells in culture

	No. of cells alive at time of inoculation ($\times 10^3$)	No. of cells alive after 10 days culture ($\times 10^3$)		
		Control	-FSH	-FSH + FSH
1	342	351	35	363
2	50	52	6	49
3	264	187	15	191
4	236	249	15	250
5	306	317	15	308

-FSH refers to culture medium devoid of FSH

-FSH + FSH refers to culture medium devoid of FSH with 30 mu.

FSH/ml added each day

Control refers to culture medium with untreated human serum with 2.5 mu./FSH/ml.

of progesterone after 2 to 5 days in culture, when exposed continuously to low physiological levels of gonadotrophins (see Fig. 27). During the first few days of culture, when the cells in the controls secreted progressively more progesterone, those in culture medium devoid of LH and/or prolactin were unable to increase their secretion rate in a similar manner. This suggests that granulosa cells undergoing luteinization require both LH and prolactin to express their full biosynthetic potential. The declining concentration of prolactin in the follicular fluid as the follicle approaches ovulation could be a result of prolactin utilisation in the early stages of luteinization (see Figs. 14 & 16). When granulosa cells finally achieved their maximum secretion rate in culture (i.e. from Days 6 to 10) they required continuous exposure to both LH and prolactin; these findings give added support to the earlier hypothesis which suggested that both pituitary hormones were essential for maintaining the secretory activity of the human corpus luteum (see discussion for Chapter 6).

Granulosa cells also seem to require FSH in order to prepare for their subsequent secretory activity (Zelevnik et al., 1974; Channing, 1975; see results for Chapter 5). However, the role of FSH in either initiating or maintaining steroid secretion by these cells has not been defined. Although the granulosa cells in this study were harvested from follicles containing FSH, the decline in cell numbers during 10 days culture in a serum devoid of FSH suggests that the continued presence of this gonadotrophin is essential. This is in agreement with the in vivo findings of Peters et al. (1975) for the mouse; they have suggested that the role of FSH may be to prevent the granulosa cells undergoing premature atresia.

Human granulosa cells in vivo show no histological signs of

secretory activity until immediately prior to ovulation, when changes consistent with early luteinization first appear. The cytoplasm of the granulosa cells enlarges and becomes granular in appearance (Hertig, 1967; Baird et al., 1975; see results Chapter 4) and there appears to be an increase in the amount of smooth endoplasmic reticulum and mitochondria (Crisp et al., 1970; Delforge et al., 1972). Histochemically, 3β -ol steroid dehydrogenase activity can be demonstrated in the granulosa cells of preovulatory follicles (Friedrich et al., 1974). At this time and coincidental with the LH peak, the concentration of progesterone rises in peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970) in ovarian venous plasma (Mikhail, 1970; Lloyd et al., 1971; de Jong et al., 1974) and particularly in follicular fluid (Sanyal et al., 1974; Fig. 16). These findings taken together suggest that the preovulatory source of progesterone originates from the luteinizing granulosa cells.

It is suggested therefore that LH, prolactin and FSH may constitute the lutetrophic complex necessary for the initiation and maintenance of normal secretory activity by luteinized human granulosa cells.

SUMMARY

The role of FSH and prolactin in the initiation and maintenance of steroidogenesis by luteinizing human granulosa cells in vitro was investigated. Human serum was treated with antibodies to human gonadotrophins to remove selectively either FSH, prolactin and/or LH before adding the serum to preovulatory granulosa cells in tissue culture.

Granulosa cells remained viable when cultured for 10 days in a serum devoid of LH and/or prolactin. After 6 days of culture the cells were secreting approximately 70% less progesterone each day than in the controls. Only 8% of the granulosa cells remained viable after 10 days culture in a serum devoid of FSH. The addition of FSH to granulosa cells in culture media devoid of FSH maintained the cell numbers at a similar level to those found in the controls.

CHAPTER 9

**THE CYTOTOXIC EFFECT OF SERUM FROM PATIENTS WITH
AUTOIMMUNE OVARIAN FAILURE ON HUMAN GRANULOSA CELLS
IN CULTURE**

INTRODUCTION

The sera of a proportion of patients with idiopathic Addison's disease are known to contain antibodies that cross-react in vitro with other steroid producing tissues, i.e. ovary, trophoblast and Leydig cells, as well as to adrenal cortical tissue (Anderson, Goudie, Gray & Stuart-Smith, 1968; Irvine, Chan, Scarth, Kolb, Hartog, Bayliss & Drury, 1968; Irvine et al., 1969; Irvine & Barnes, 1974). Female patients in this group have a high incidence of premature ovarian failure, with either primary or secondary amenorrhoea, and the ovaries are characterised by total fibrous replacement or lymphocytic infiltration around developing Graafian follicles (Irvine et al., 1968, 1969 & 1974). The ovaries of women with premature ovarian failure often contain numerous primary follicles but are completely devoid of large Graafian follicles. It seems that as the follicles attempt to develop they fail to do so and are apparently destroyed (Irvine & Barnes, 1974; E. Barnes, personal communication).

Slices of human luteal tissue previously incubated with Addisonian sera from patients with ovarian failure gave rise to three distinctive patterns of immunofluorescence after further incubation in the presence of horse IgG-fluorescein isothiocyanate (IgG - FITC). The staining patterns are observed most frequently in the cytoplasm of the granulosa-derived luteal cells and are described as "clumpy", "patchy" or "confluent" (Irvine, 1969). These findings raise the possibility that in patients with antibodies to luteal tissue, the lack of follicular development may partly be due to destruction of the granulosa cells after the antibodies have crossed the blood-follicle barrier. Irvine (1960)

and Pulvertaft, Doniach, Roitt & Hudson (1961) have demonstrated a cytotoxic effect on thyroid and adrenal cells in tissue culture only if complement was present. Thus the role of complement-fixing antibodies in the destruction of human Graafian follicles must also be considered. Serum contains complement which consists of nine distinct components (C_1 to C_9); these can be inactivated when serum is heated at 56°C for 30 minutes. Presumably complement is present in follicular fluid since the immunoelectrophoretic or disc-gel electrophoretic patterns of follicular fluid are similar to those of serum (Edwards, 1974). The action of complement in cell destruction remains obscure. The antibody binds to the antigenic site on the cell membrane, then complement binds to the antibody thus forming a complex; in the absence of this complex formation it seems that cell destruction does not normally occur (Irvine, 1974; Roitt, 1974).

Granulosa cells can readily be obtained from human Graafian follicles as a dispersed cell suspension without chemical or enzymatic pretreatment. In culture, granulosa cells grow as a monolayer, undergoing hypertrophy and secreting progesterone into the medium (Channing, 1969a) see also Chapter 5).

Serum containing antibodies reactive with human ovarian tissue was obtained from patients with Addison's disease and premature ovarian failure. The effects of these sera on both the morphology and steroid producing ability of granulosa cells in tissue culture was investigated.

RESULTS

A cytotoxic effect was only observed when some 'Addisonian' sera were added to granulosa cells in culture in the presence of

complement (Plate 16b); no cytotoxic effect was observed in the absence of complement (Plate 16a). Control sera from post menopausal or normally menstruating women, or sera containing other antibodies (i.e. to antinuclear factors or to gastric parietal cells, etc.), were not cytotoxic to granulosa cells in culture in the presence of complement. Neither the test nor the control sera which failed to produce a cytotoxic effect reduced cell viability.

The cytotoxic effect was related to the immunofluorescent staining pattern of the serum, since none of the 9 sera giving a "clumpy" immunofluorescent staining pattern produced a cytotoxic effect (Group 4, Table 18) and three of the eight "patchy" staining sera were also non-cytotoxic (Group 3, Table 18). However, all four "confluent" staining sera produced a marked cytotoxic effect, as did five of the "patchy" staining sera (Groups 1 and 2, Table 18). The degree of cellular damage in culture was also related to the immunofluorescent titre of the antibody (Table 19). There was evidence that the affinity of the granulosa cells for antibody declined markedly with time in culture since horse anti-human IgG - FITC conjugate showed the most intense staining after only 24 hours (++) with a marked decline in intensity after 48 hours (+ and ⁺) and was undetectable after 72 hours (negative). This would explain why no cytotoxic effect was observed if the addition of a cytotoxic 'Addisonian' serum plus complement to granulosa cell cultures was delayed for 24 hours.

The inhibitory effect of 'Addisonian' sera plus complement on the production of progesterone by granulosa cells in culture is shown in Fig. 42. A progressive fall in progesterone output occurred in those cultures in which a cytotoxic effect was observed, and after 3 days the production rate was 30% of that



Plate 16a. Morphological appearance of granulosa cells after culture in serum giving a "confluent" staining pattern in the indirect immunofluorescence test with sections of human corpus luteum. No complement was present. Mag. X 640.

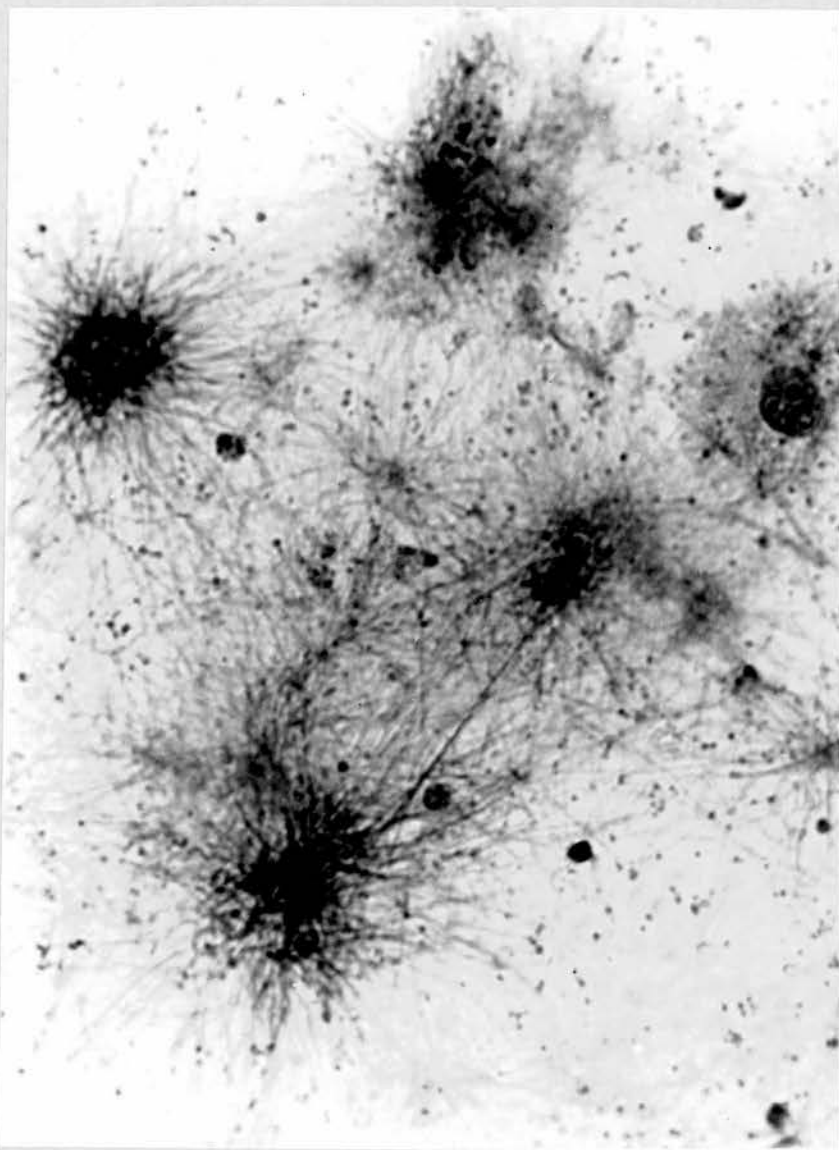


Plate 16b. Morphological appearance of granulosa cells after the cells were cultured in the same serum as described in Plate 16a together with complement. Mag. X 640.

Table 18. Clinical features of patients with Addison's disease

Sex	Age at Study	Reproductive History	Associated Diseases	Staining pattern		Titre	Effect on cultured granulosa cells
				On Corpus luteum			
1. Female							
Female	22	Ovarian failure	Diabetes Mellitus			32	
Female	22	Ovarian failure	-			32	
Female	24 ^{3/4}	Ovarian failure	-		Confluent	8	
Female	42	Ovarian failure	-			Neat	
2. Female							
Male	18	Primary amenorrhoea	Hypoparathyroid			8	Cytotoxic
Female	16	Atrophic testis	-			16	
Female	23	Ovarian failure	-		Patchy	8	
Female	26	Ovarian failure	-			64	
Female	35	Ovarian failure	-			4	
3. Female							
Female	9	Prepubertal	Arthralgia			4	Non
Female	36	Menorrhagia	Thyrototoxicosis			16	
Female	40	Ovarian failure	Perinicious anaemia, hypothyroid	Patchy		64	Cytotoxic

Cont

Table 18.

CLINICAL FEATURES

IMMUNOFLUORESCENCE

<u>Sex</u>	<u>Age at Study</u>	<u>Reproductive History</u>	<u>Associated Diseases</u>	<u>Staining Pattern on corpus luteum</u>	<u>Titre</u>	<u>Effect on cultured granulosa cells</u>
4. Female	14	Menstruating				
Male	14	Undergoing normal puberty	Hypoparathyroid Hypoparathyroid		128 4	
Female	15	Primary amenorrhoea	Keratitis & chronic monilia, hypoparathyroid		16	
Female	26	Ovarian failure	Hashimoto thyroiditis	Clumpy	32	Non Cytotoxic
Female	29	Oligomenorrhoea	-		128	
Female	30	Ovarian failure	-		128	
Female	36	Ovarian failure	-		2	
Female	40	Ovarian failure	Thyrototoxicosis		4	
Female	46	Ovarian failure	-		64	
5. Female	51	Menorrhagia	Pernicious anaemia		0	
Female	55	Normal menopause	-	Negative	0	

The clinical features of 23 patients with idiopathic Addison's disease together with the type of immunofluorescent staining pattern the individual sera gave on unfixed, air-dried, cryostat sections of human corpus luteum and the reciprocal titre of the antibody in the sera. The sera which were cytotoxic to human granulosa cells after three days in culture are indicated.

Table 19.

Immunofluorescent titre of antibody to luteal tissue	Pattern of immunofluorescent staining using corpus luteum as antigen	Cytotoxic effects of differing volumes of serum added to granulosa cell cultures (ml serum added)						
		(0.5)	(0.2)	(0.1)	(0.05)	(0.025)	(0.01)	
32	confluent	++	++	++	++	++	++	+
undiluted	confluent	+	-	-	-	-	-	-
64	patchy	++	++	++	++	++	++	+
4	patchy	+	-	-	-	-	-	-
128	clumpy	-	-	-	-	-	-	-
128	clumpy	-	-	-	-	-	-	-
4	clumpy	-	-	-	-	-	-	-
4	clumpy	-	-	-	-	-	-	-

- Indicates that less than 15% of the cells were damaged. + Indicates that 15-80% of the cells were damaged.
 ++ Indicates that 80% were damaged after three days of culture.

The relationship in 8 patients between the titre of antibody in the immunofluorescence test using sections of human corpus luteum and the volume of serum required to produce a cytotoxic effect in 3-day cultures of human Granulosa cells.

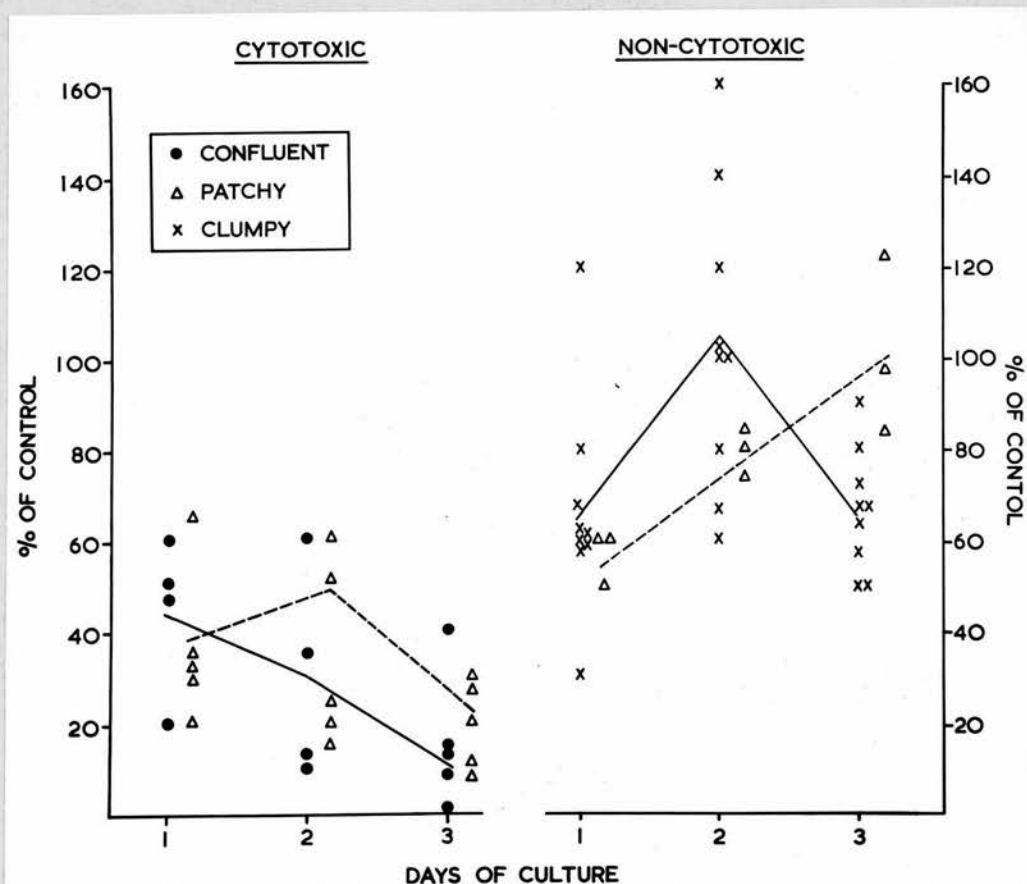


Fig. 42. The production of progesterone by human granulosa cells cultured in 'Addisonian' serum expressed as a percentage of progesterone produced by cells in control serum. Individual values are shown by the points and the means by the lines:

(a) cytotoxic sera; (b) non-cytotoxic sera.

● = "confluent") Immunofluorescent staining pattern of the
)
 Δ = "patchy") serum using unfixed, air dried, cryostat sections
)
 x = "clumpy") of human corpus luteum as antigen.

achieved by the controls (Fig. 42). Both the "patchy" and "confluent" staining cytotoxic sera produced a similar reduction in progesterone. In contrast, those "clumpy" and "patchy" staining sera which failed to produce a cytotoxic effect gave significantly higher progesterone concentrations ($P < 0.01$), which were comparable to the values obtained with control sera (Fig. 42).

DISCUSSION

Premature ovarian failure is the most recently recognised condition in a group of interrelated disorders which are characterised by organ-specific autoimmunity (Irvine & Barnes, 1974). The demonstration in the present paper of a direct cytotoxic effect of sera from 'Addisonian' patients with ovarian failure on human granulosa cells in tissue culture clearly confirms the existence of cytotoxic "steroid cell antibodies". There can be little doubt that in vivo these antibodies can gain access to the granulosa cells within the Graafian follicle (von Kaula, Aidawa & Pettigrew, 1958; Heglar, 1962; Menge, 1970) and in man the concentration of IgG in follicular fluid is about 45% of that in serum (Johnson, 1973). The cytotoxic effect demonstrated was complement dependent and proportional to the titre of the antibody in the serum.

Human granulosa cells do not require prior treatment with chemical agents in order to obtain an isolated cell suspension for culture. It therefore seems likely that the cytotoxic effect observed with some 'Addisonian' sera in this study reflects an affinity of certain antibodies for the intact surface of granulosa cells. The absence of a cytotoxic effect with other 'Addisonian' sera may reflect either an intra cellular localisation of the antigens or a lower antigen affinity. Irvine (1960) and Pulvertaft et al., (1961)

demonstrated a similar cytotoxic effect on thyroid and adrenal cells in tissue culture by complement-fixing thyroid and adrenal antibodies, respectively. However, in these studies the cell suspensions were obtained after treatment with trypsin. It is likely that trypsin might expose receptors on the cell surfaces which then become available for reaction with the auto-antibody (Irvine, 1969). The present study is therefore the first demonstration of a direct cytotoxic effect of auto-antibody on intact cells.

The antigenic characteristics of the surface of the cultured granulosa cells alters with time, as demonstrated by the lack of effect when cytotoxic sera are added later than 24 hours after initial culture, and the rapid decline in immunofluorescent staining of granulosa cells with increasing time in culture. A similar effect has been noted with thyroid cells in culture (Irvine, 1960).

The visible cytotoxic effects were accompanied by a marked reduction in steroidogenesis. This, taken together with the close correlation between the presence of steroid cell antibodies in the serum and a history of amenorrhoea (Table 18), makes it attractive to speculate that cytotoxic auto-antibody may at least be partially responsible for the ovarian failure in patients with idiopathic Addison's disease and premature ovarian failure.

SUMMARY

Human granulosa cells growing in culture were used to study the cytotoxic effects of sera from 23 patients with idiopathic Addison's disease. There were two women with primary amenorrhoea, thirteen with premature ovarian failure following a normal menarche, and one man with testicular atrophy in this group. The sera from all 23 patients cross-reacted with fresh sections of human corpus luteum using an in vitro immunofluorescent test.

Serum from 9 of the 23 patients contained antibodies which were cytotoxic to human granulosa cells in tissue culture, the cytotoxic effect was complement dependent, and related to the immunofluorescent staining properties of the serum and dilution of antibody present. No cytotoxic effect was observed when granulosa cells were cultured in serum from the remaining 14 'Addisonian' patients, or from normally menstruating women, post menopausal women or in serum containing organ-specific antibodies to other endocrine tissues.

The cytotoxic effect was paralleled by a fall in progesterone production by the granulosa cells. The production of progesterone by cells cultured in any of the non-cytotoxic sera was significantly greater than that produced by cells in the presence of cytotoxic antibody.

CHAPTER 10

**THE EFFECTS OF PROSTAGLANDIN F₂α AND E₂ ON THE
PRODUCTION OF PROGESTERONE BY HUMAN GRANULOSA CELLS
IN CULTURE**

INTRODUCTION

Human granulosa cells cultured in vitro provide a convenient biochemical model system for studying some of the activities of the human corpus luteum. In this study granulosa cells of differing steroidogenic potentials were used to investigate the possible effects of PGF₂α and PGE₂ on the human corpus luteum.

RESULTS

The effect of prostaglandins on the viability or mitotic activity of granulosa cells in vitro

When cells were exposed to PGF₂α and PGE₂ alone or in combination there was no significant increase or decrease in cell numbers during the 10 days of culture, when compared to untreated controls with or without gonadotrophins. The single exception to this was when PGE₂ was added to culture medium containing cells harvested from inactive follicles; i.e. those containing undetectable levels of FSH (< 1.3 mu./ml) and low concentrations of oestradiol (< 250 ng/ml); in these experiments, a 2 fold increase in cell numbers occurred ($r = 1.91 \pm 0.22$, n = 4) when compared to the controls ($r = 1.08 \pm 0.11$, n = 4) (r = no. of cells after 10 days culture divided by the number of live cells at the start of culture).

The effect of differing doses of prostaglandin F₂α on the total production of progesterone by granulosa cells in culture

The total production of progesterone by granulosa cells during 10 days culture after daily exposure to PGF₂α at concentrations between 1 and 8000 ng/ml is shown in Table 20. At every dose level, PGF₂α lowered the total production of progesterone by 60% or more when added to cells harvested from 'active' follicles, i.e. those containing detectable levels of FSH, (> 1.3 mu./ml) and high

Table 20. Total production of progesterone by human granulosa cells after ten days culture with varying amounts of PGF2 α expressed as a percentage of the controls (mean \pm S.E.M.).

Source of Granulosa Cells	Dose of PGF2 α added (ng/ml of culture medium)						
	1	10	50	100	500	1000	8000
'Inactive' follicles	93.5	88.6	85.7	84.3	70.0	50.0	45.7
			± 6.2	± 5.7	± 6.6	± 4.7	
	(2)	(2)	(4)	(4)	(4)	(4)	(4)
'Active' follicles	39.3	33.3	32.1	22.0	16.0		
		± 4.4	± 1.1	± 2.9	± 3.3	-	-
	(2)	(4)	(4)	(5)	(4)		

'Inactive' follicles contained < 1.3 mu. FSH/ml and < 250 ng oestradiol-17 β /ml in follicular fluid. 'Active' follicles contained > 1.3 mu. FSH/ml and > 250 ng oestradiol-17 β /ml. The numbers in parentheses refer to the number of experiments on individual follicles.

concentrations of oestradiol (> 250 ng/ml). By contrast a much smaller reduction in total progesterone output was observed in cells harvested from 'inactive' follicles (see Table 30). In both cases the higher the concentration of PGF2 α the greater the inhibition in progesterone output.

The effect of prostaglandin F2 α 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 PGF2 α with or without LH + FSH is shown in Fig. 43. There was a marked inhibition (~60%) in the rate of progesterone secretion compared to the controls when 50 ng PGF2 α was added. Although LH + FSH induced a 2 fold increase in progesterone secretion, the addition of 50 ng PGF2 α markedly inhibited this stimulatory effect.

The daily production of progesterone by granulosa cells harvested from 'inactive' follicles and treated daily with PGF2 α with or without LH + FSH is shown in Fig. 44. A small decrease (~10%) in production occurred compared to the controls when 50 ng PGF2 α was added. Although LH + FSH induced a 3 to 6 fold increase in progesterone secretion, the addition of 50 ng PGF2 α markedly inhibited this stimulatory effect.

The effect of delaying the addition of PGF2 α on the production of progesterone by granulosa cells harvested from an 'active' follicle and exposed to LH + FSH daily is shown in Fig. 45. The addition of 50 ng of PGF2 α each day from Day 6 had no effect on the daily production of progesterone. However, when 1000 ng of PGF2 α was added daily from Day 6 there was a progressive decrease in progesterone output so that by Day 12 the output was only one third of that achieved by the control.

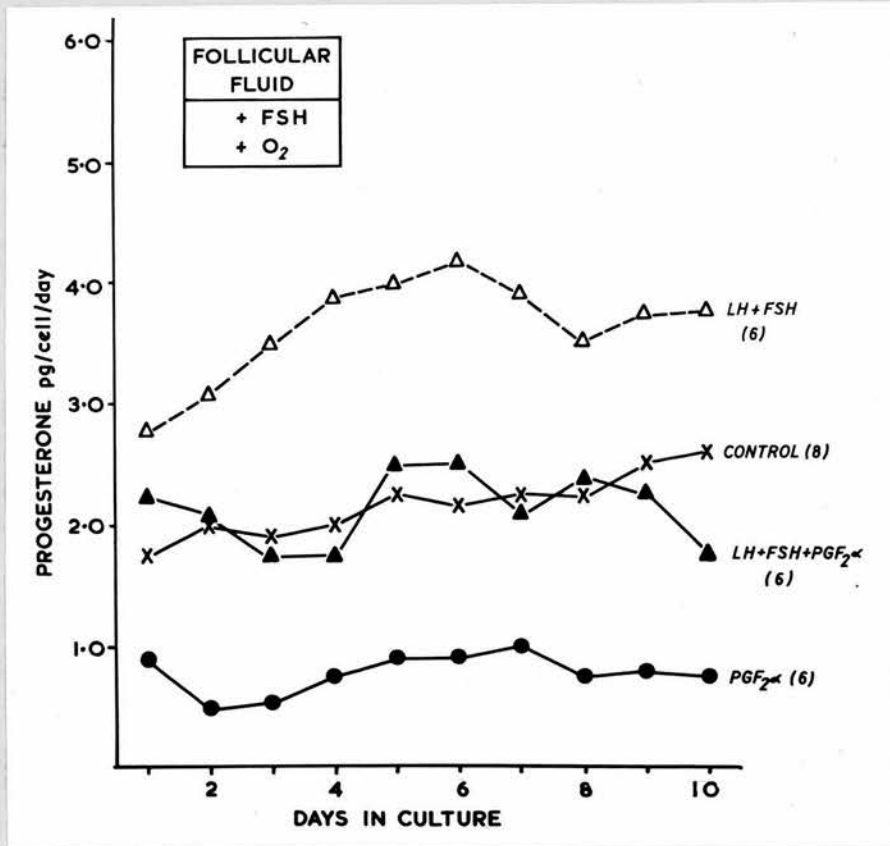


Fig. 43. The average daily production of progesterone by cells in vitro which were exposed to PGF₂α (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing FSH and O₂ (oestradiol-17β): + FSH > 1.3 mu./ml; + O₂ > 250 ng/ml. The numbers in parentheses refer to the number of experiments.

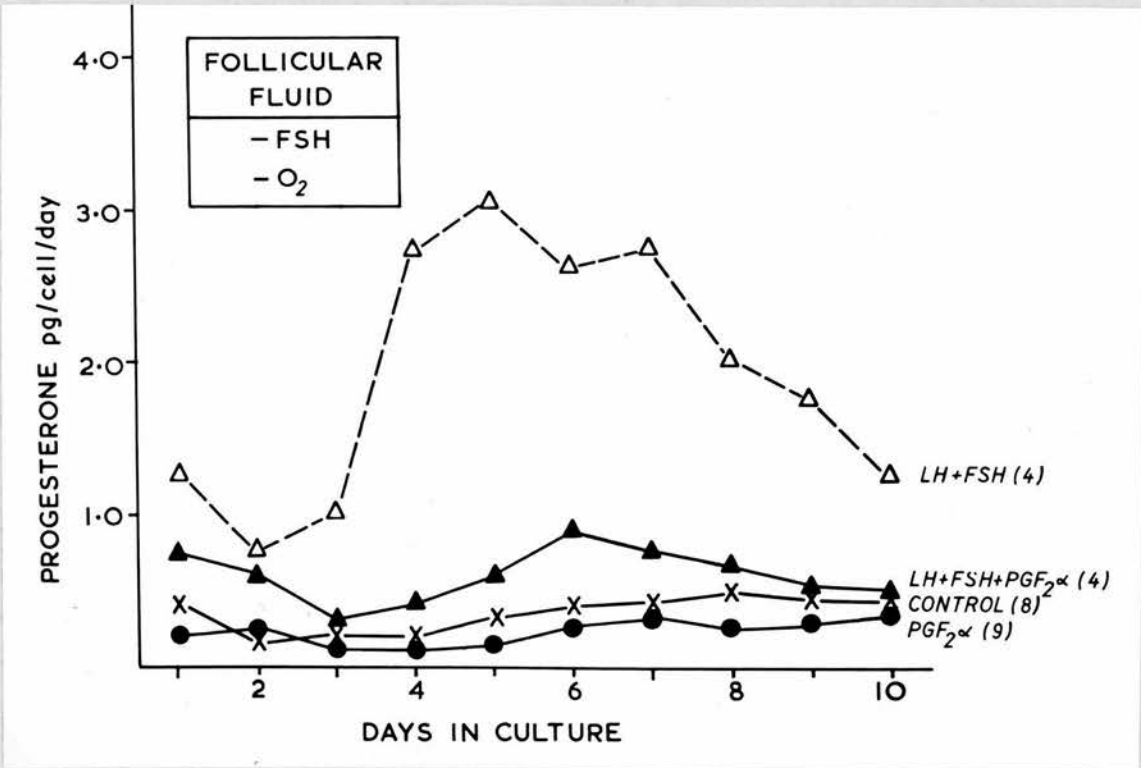


Fig. 44. The average daily production of progesterone by granulosa cells in vitro which were exposed to PGF₂α (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing low concentrations of FSH and O₂ (oestradiol-17β): FSH < 1.3 mu./ml; O₂ < 250 ng/ml. The numbers in parentheses refer to the number of experiments.

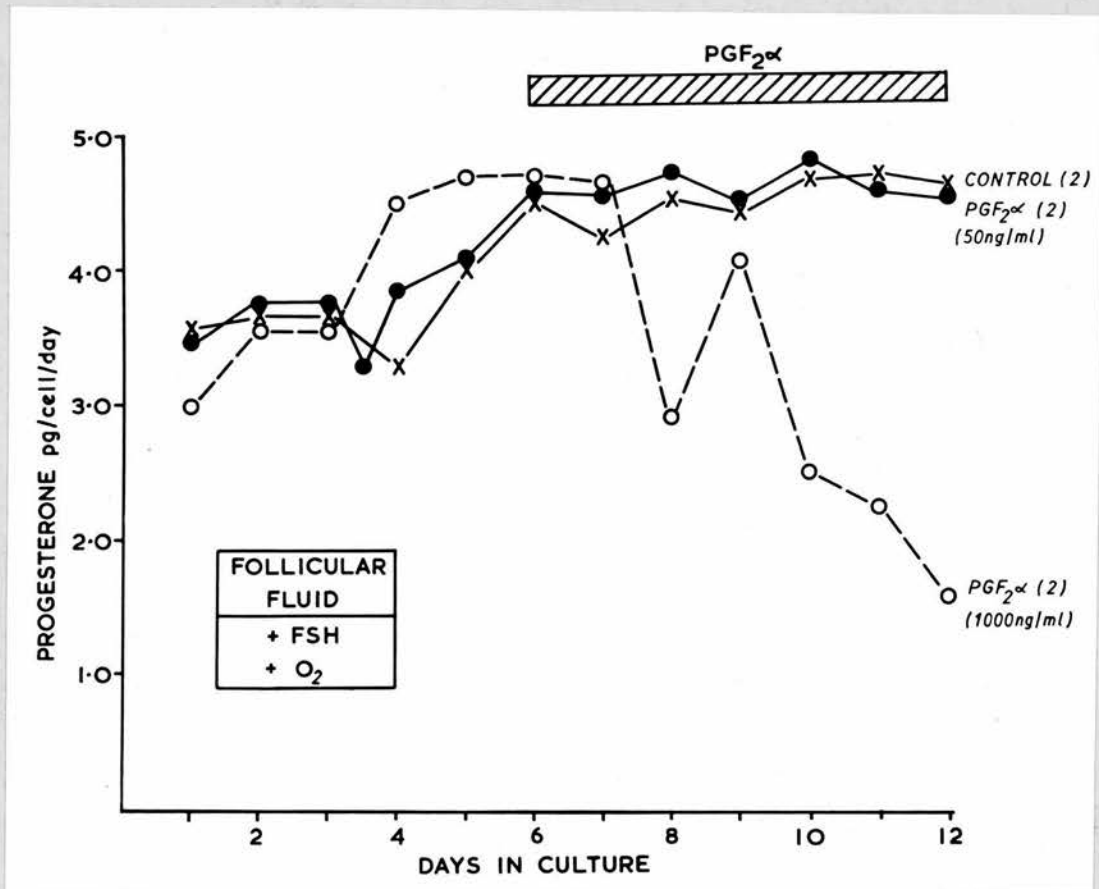


Fig. 45. The effect of delaying the addition of prostaglandin F₂α on the daily production of progesterone by granulosa cells in culture. The Fig. shows the average daily production of progesterone by granulosa cells which were exposed to either 50 or 1000 ng PGF₂α/ml daily from Day 6. The cells were harvested from a follicle containing FSH and O₂. See Fig. 43 for abbreviations. Numbers in parentheses refer to the number of experiments.

The effect of prostaglandin E2 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 PGE2 with or without LH + FSH is shown in Fig. 46. Luteinizing hormone + FSH only stimulated a two-fold increase in the production of progesterone, whilst the daily addition of 50 ng PGE2 stimulated a 3-4 fold increase so that a maximum secretion rate of about 5 pg/cell/day (see Fig. 27) was reached within 3-4 days in culture. The daily addition of LH + FSH together with 50 ng PGE2 did not increase the production rate of progesterone any further, and the cells could not be stimulated any more by further additions of PGE2 from 1 to 500 ng/ml.

The daily production of progesterone by granulosa cells harvested from 'inactive' follicles and exposed to LH + FSH + PGE2 or PGE2 when compared to the controls is shown in Fig. 47. In this case the addition of between 1-500 ng PGE2 to cells harvested from 'inactive' follicles failed to stimulate the production of progesterone more than by LH + FSH. Furthermore, the addition of PGE2 together with LH + FSH failed to produce any further stimulation. The effect of adding prostaglandins F2 α and E2 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. 46; the daily production of progesterone was similar to that achieved by adding PGE2 alone. The 10 day production of progesterone by granulosa cells harvested from preovulatory follicle when exposed daily to varying doses of PGF2 α , PGE2 or PGF2 α + PGE2 is shown in Table 21. Addition of PGF2 α at 10 or 500 ng lowered the total

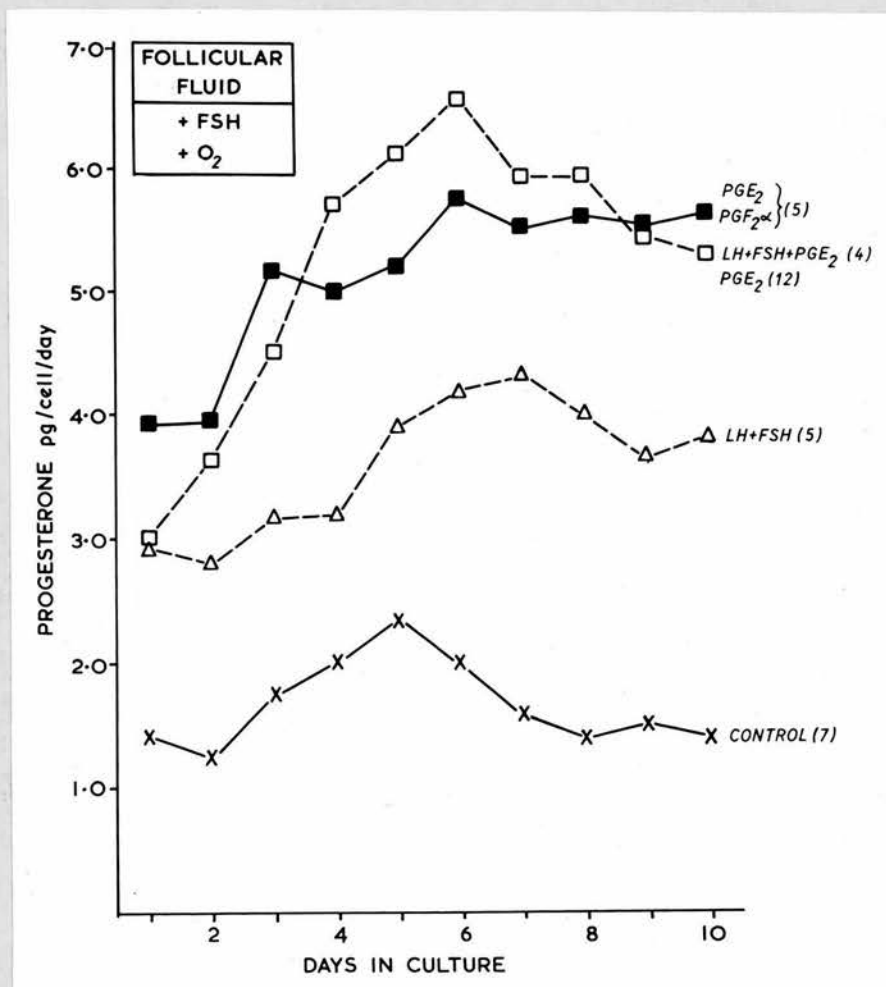


Fig. 46. The effect of prostaglandin E₂ on the daily production of progesterone by granulosa cells in culture. The Fig. shows the average daily production of progesterone by cells which were exposed to PGE₂ (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing FSH and O₂ (oestradiol-17 β). See Fig. 44 for abbreviations. The numbers in parentheses refer to the number of experiments.

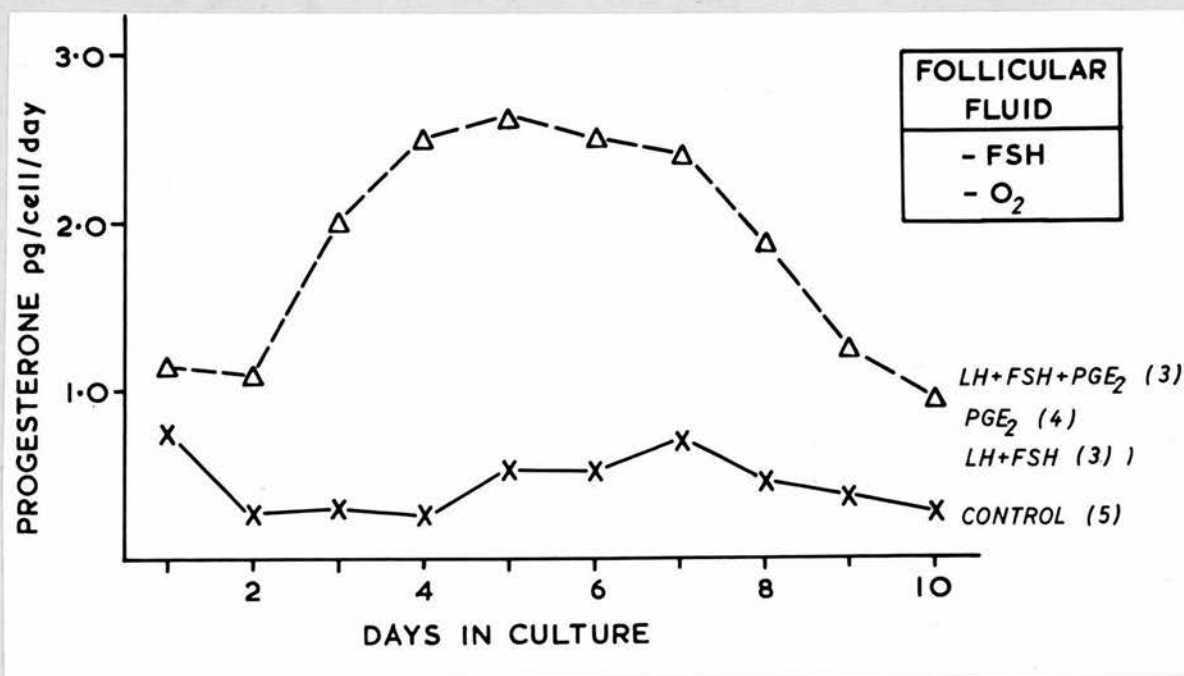


Fig. 47. The effect of prostaglandin E₂ on the daily production of progesterone by granulosa cells in culture. The Fig. shows the average daily production of progesterone by cells which were exposed to PGE₂ (50 ng/ml) with or without added LH and FSH (both 30 mu./ml). The cells were harvested from follicles containing low concentrations of FSH and O₂. See Fig. 44 for abbreviations. The numbers in parentheses refer to the numbers of experiments. The average results after treatment with either LH + FSH + PGE₂, PGE₂ or LH + FSH were identical and are indicated by the single line.

Table 21. The effect of adding prostaglandin F2 α and E2

on the production of progesterone by granulosa cells.

Prostaglandin	Concentration (ng/ml)	Total production of progesterone during 10 days of culture (μ g progesterone $\times 10^6$) cells mean \pm S.E.M.	Number of experiments
Control	0.04	51.8 \pm 4.6	5
PGE2	10	60.3 \pm 1.9	5
	500	61.9 \pm 2.3	5
PGF2 α	10	23.2 \pm 3.7	5
	500	17.5 \pm 2.1	5
PGE2	10	49.9 \pm 4.9	3
PGF2 α	10		
PGE2	50	48.8 \pm 5.1	3
PGF2 α	50		
PGE2	100	57.6 \pm 4.3	3
PGF2 α	100		
PGE2	500	57.2 \pm 6.6	3
PGF2 α	500		

Cells were harvested from a single 15 mm late follicular phase follicle (15 mm diameter) with detectable levels of LH (4.8 mu./ml) FSH (3.3 mu./ml) and oestradiol (2100 ng/ml) in the follicular fluid. Concentration refers to the final concentration of PGF2 α or PGE2 in the medium.

production of progesterone achieved by adding PGE2 alone.

DISCUSSION

These results show clearly that PGF2 α inhibits the production of progesterone by human granulosa cells in vitro without affecting cell viability. They also show that provided PGF2 α 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 (see Fig. 24-29). Furthermore, when PGF2 α 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 PGF2 α , the production of progesterone was not inhibited. Although a detailed dose-relationship was not established, a much higher concentration of PGF2 α (1000 ng/ml medium) was required to inhibit the production of progesterone. ?

The addition of PGE2 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 PGE2 was far more effective than LH + FSH in stimulating progesterone secretion by cells from actively developing follicles. By contrast, the addition of PGE2 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 (Fig. 24-29).

There was no evidence of any synergism between PGE₂ 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 PGF₂α 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 & K. P. McNatty). Prostaglandins found within the human follicle and corpus luteum could therefore have a major controlling influence on steroid secretion by these structures. Although PGF₂α 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 PGE₂. These studies provide some clues as to the site of action of PGF₂α. Both LH (Channing, 1975) and PGE₂ (Rao, 1973) have specific receptors on the plasma 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 PGF₂α acts directly on the adenylate cyclase system by blocking its activation by LH. Similar in vitro data have been obtained using porcine and granulosa cells (K. M. Henderson & K. P. McNatty, unpublished data) suggesting that the interaction of PGF₂α with this cell type is species independent. These findings however may not be consistent with the hypothesis proposed by Kuehl (1974) since in his model PGE₂ is unlikely to overcome the inhibitory

effect of PGF₂α. Such differences that exist between Kuehl's hypothesis and the present study may depend on the rates at which both PGE₂ and PGF₂α act on the biochemical pathway to progesterone synthesis. Since continued high levels of LH and FSH protect the granulosa cells against subsequent inhibition by PGF₂α it seems likely that the inhibitory effect of PGF₂α is inversely related to the amount of gonadotrophin bound to its receptor; PGF₂α was 200 times less effective when granulosa cells had been exposed to LH + FSH for 6 days before its addition. These findings are in agreement with those of Hichens et al. (1974) who showed that PGF₂α 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 PGF₂α 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.

SUMMARY

Human granulosa cells with differing steroidogenic potential were cultured in vitro. The effects of PGF₂α and PGE₂ on the progesterone output and viability of these cells were investigated.

Prostaglandin F₂α 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 PGF₂α was 200 times less effective when the cells were exposed to LH and FSH for 6 days prior to the addition of the prostaglandin. By contrast PGE₂, 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 PGF₂α. The degree of inhibition by PGF₂α or stimulation by PGE₂ was related to the biosynthetic capacity of the cells.

CHAPTER 11

GENERAL DISCUSSION

The present studies indicate that the hormonal environment within the developing human Graafian follicle may be of considerable importance in determining the subsequent secretory activity of its granulosa cells in the corpus luteum. The precise sequence of endocrine changes occurring in the fluid commences well before ovulation and probably occurs uniquely in the follicle which is destined to ovulate (see discussion, Chapter 5). However, within the ovary at all stages of the menstrual cycle there are a large number of antral follicles present (Block, 1951a). Presumably this is because follicles are continuously leaving the preantral pool, unrelated to the cyclic hormonal changes in plasma (Peters, et al., 1975). Furthermore, Peters et al. (1975) have shown that follicles do not rest at any stage of growth but continue to grow until they either become atretic or ovulate.

Most available evidence indicates that the final stages of follicular development (i.e. from 4 layers of granulosa cells to a mature Graafian follicle) are controlled by the levels of circulating FSH and LH (Mauleon, 1969) and in the rat the time taken for this to occur is between 10 and 17 days (Pedersen, 1970). Follicle-stimulating hormone in women rises to peak concentrations some 12 to 16 days prior to ovulation (Wide, Niliius, Gemzell & Roos, 1973). It is suggested that this rise in plasma FSH coinciding with the spontaneous emergence of a follicle from the pre-antral pool, sets in train a precisely timed sequence of endocrine events within that follicle which leads ultimately to ovulation.

Figure 48 shows some of the factors which may be responsible for the selection of one follicle for ovulation. It is suggested that the follicle leaving the preantral pool during the early follicular

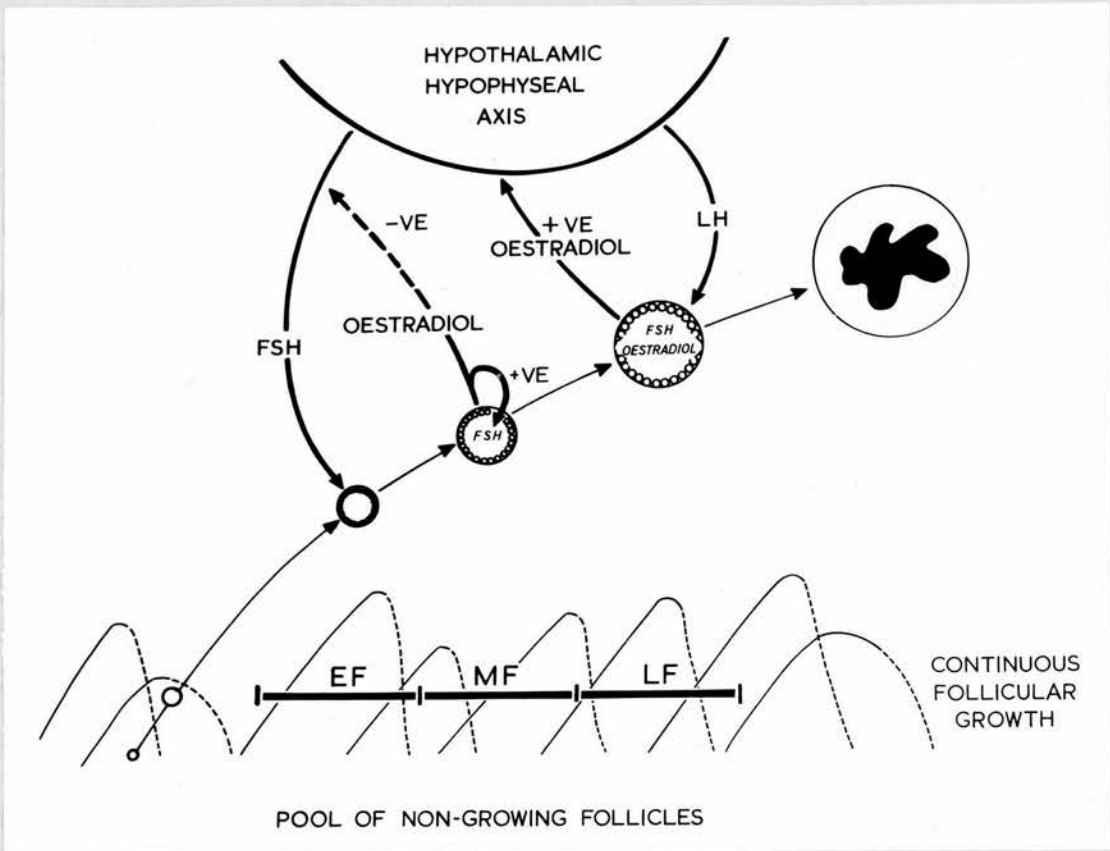


Fig. 48. A summarised dynamic representation of how one follicle is selected for ovulation (see text). Solid lines originating from the 'pool of non-growing follicles' represent follicular growth whilst the dotted lines represent follicular atresia. EF, MF and LF refer to early-, mid-, and late follicular phase of the menstrual cycle. Solid lines originating from the hypothalamic-hypophyseal axis represent the secretions of FSH or LH. The dotted line labelled oestradiol represents the negative feedback effect of oestradiol secretion from the developing follicle on FSH secretion whilst oestradiol entering the follicle (+) indicates intra-follicular stimulation. The solid line from the large developing follicle represents increased secretion of oestradiol and its positive feedback effect on the discharge of LH.

phase of the cycle accumulates high concentrations of FSH.

As the follicle grows it secretes oestradiol into the ovarian vein (Baird, 1971; Baird & Fraser, 1974a) and some accumulates in the follicular fluid (Table 11). During the early to mid-follicular phase (~Day 7) the developing follicle is clearly established as the major source of oestrogen since the concentration of oestradiol is highest in the vein draining the ovary containing the developing follicle (Baird & Fraser, 1974a). This stage of follicular growth is perhaps the most crucial in the process of selecting only one follicle for ovulation; the increasing secretion of oestradiol inhibits FSH (Fig. 48) so that the peripheral concentration of this gonadotrophin falls progressively (Midgely & Jaffe, 1968). Subsequent follicles leaving the preantral pool are therefore unable to accumulate high levels of FSH (Fig. 12 and Table 9).

The high concentrations of FSH and oestradiol within the developing follicle stimulates an increased mitotic rate in the granulosa cells (Delforge et al., 1972; Table 13), an increased steroidogenic potential (Figs. 24 to 29), and an increased number of LH receptors (Zelevnik et al., 1974; Channing, 1975) which prepares the granulosa cells for eventual steroid secretion. The high levels of FSH in the developing follicle are probably maintained despite falling levels in plasma because the high concentrations of follicular oestradiol enhance the incorporation of FSH into that follicle (Goldenberg et al., 1972a). Thus the follicle stimulates its own growth while at the same time inhibiting FSH secretion and hence further maturation of other follicles leaving the preantral pool, which thus become atretic. Finally the rising levels of

oestradiol from the developing follicle stimulates a reflex discharge of LH from the pituitary lasting about 48 hours (Ross et al., 1970; Knobil, 1974); appreciable amounts of this LH enter the follicle (Fig. 13) inhibiting further mitosis (Delforge et al., 1972; Table 13). The presence of LH in the follicle together with prolactin (Figs. 30 and 40) and FSH (Fig. 23) initiates the secretion of progesterone (Figs. 16 and 41). In addition steroidogenesis by the luteinized granulosa cells is probably maintained in the presence of all three pituitary hormones (see Figs. 30, 40, 41 and Table 17).

This hypothesis therefore suggests that following the early entry of FSH into the antral fluid of one follicle and the initiation of oestradiol secretion, this follicle then becomes essentially autonomous, and is in control of its own destiny.

A number of factors may influence the proportion of antral follicles which go on to ovulate. It is reasonable to assume that the uptake of FSH by the developing follicle is important for protecting the follicle against atresia (Peters et al., 1975; Table 17). Thus as more and more follicles accumulate FSH, presumably a greater proportion of antral follicles will go on to ovulate. In anovulatory women where follicular growth and ovulation can be influenced by exogenous gonadotrophin treatment, there is a clearly established relationship between the dose of human menopausal gonadotrophin (HMG), which has FSH and LH activity, and the number of ovulations induced subsequently by human chorionic gonadotrophin (HCG) (Gemzell, 1965; Brown, 1971). Presumably therefore the magnitude of the peak or the duration of elevated concentrations of FSH in plasma may influence the number of follicles which can be stimulated to further development. In

addition to varying the amount of FSH, there may also be variation in the permeability of antral follicles for FSH since there are considerable species differences in the distribution of serum proteins found in follicular fluid (see Edwards, 1974).

Furthermore, the frequency at which follicles leave the non-growing pool independent of gonadotrophic stimuli could also influence the proportion of follicles accumulating FSH during peak concentrations of this hormone in plasma. From studies in the rodent it appears that the age of the animal (Pedersen, 1969), the number of antral follicles (Krarup et al., 1969; Peters et al., 1970) and the number of degenerating follicles (Peters et al., 1973a) may all be factors which influence the rate at which follicles leave the non-growing pool. Thus, differences both between and within species in the number of follicles which go on to ovulate are probably influenced by the hormone concentrations in plasma, the permeability of follicles for pituitary hormones and the intra-ovarian factors which regulate follicular growth.

The present in vitro studies suggest that granulosa cells require a long period (~ 8 - 10 days) of continued exposure to FSH and oestradiol before they will respond maximally to an ovulatory 'surge' of LH (Fig. 24-29). In addition these data also suggest that the premature appearance of LH in the follicle containing FSH and oestradiol may block further granulosa cell maturation. Evidence to support this concept has been demonstrated by Cargille et al., (1973) who showed that a discharge of pituitary LH during the mid-follicular phase resulted in either anovulation or a shortened luteal phase. More recently Friedrich, Kemeter, Sulzer and Breitenacker (1975) administered an ovulatory dose of HCG to women

with previously regular menstrual cycles on Days 1 to 6 after the onset of menstruation; they found that ovulation was either inhibited or postponed and that the granulosa cells within the large follicles were undergoing degenerative changes. They also found that there was no increase in progesterone secretion after the HCG treatment suggesting that granulosa cells were unable to fully luteinize and function normally. These observations strongly support the concept that the hormonal microenvironment is critically important for the maturation of the Graafian follicle.

It is unlikely however that the developing follicle actively excludes LH since this hormone was detected in very low amounts in a pool of antral fluid collected from small follicles during the early follicular phase at a time when there were low concentrations of LH in plasma. The initial appearance of more FSH than LH in the developing follicle probably reflects the differing rates of transfer of these hormones across the blood-follicle barrier.

It is not possible to determine from these studies whether the presence of the α -subunit of LH (or FSH) and/or the β -subunit of LH in follicular fluid reflects the transfer of free subunit across the blood-follicle barrier or whether their presence in antral fluid merely reflects dissociation of intact LH (or FSH) already within the follicle. Nevertheless the in vitro studies strongly suggest that the subunits alone have little or no effect on the steroidogenic potential of human granulosa cells. These findings therefore disagree with those of Nalbandov & Bahr (1974); they found that the α -subunit of ovine LH was a potent stimulator of steroidogenesis in the rabbit when the subunit was injected systemically. The reason for the discrepancy may be that in

Nalbandov's study the α -subunit was injected at a dose of $50 \mu\text{g}$ per kg of rabbit weight which is likely to be a pharmacological dose of free subunit, whereas in the present study the subunits were added to isolated cells which at the lower concentrations are likely to be much more physiological.

There is now substantial evidence to suggest that the concentrations of $\text{PGF}_2\alpha$ and E_2 rise in the preovulatory rabbit follicle immediately prior to rupture (Le Maire, Yang, Behrman & Marsh, 1973; Yang, Marsh & Le Maire, 1973) and that inhibition of prostaglandin synthesis results in ovulatory failure (Armstrong & Grinwich, 1972; Behrman et al., 1972; Grinwich, Kennedy & Armstrong, 1972; Tsafriri, Lindner, Zor & Lamprecht, 1972; O'Grady, Caldwell, Auletta & Speroff, 1972; Orczyk & Behrman, 1972; Tsafriri, Koch & Lindner, 1973). Granulosa cells harvested from preovulatory follicles of rabbits produce large amounts of $\text{PGF}_2\alpha$ in culture medium devoid of C-AMP (Challis et al., 1974). By contrast however human granulosa cells from preovulatory follicles appear to make little of any $\text{PGF}_2\alpha$ (K. P. McNatty, & R. Kelly, unpublished observations).

The role of C-AMP in the initiation of luteinization seems to be uncertain and somewhat variable, for its synthesis by the preovulatory follicle in the rabbit is considerably stimulated by LH for an hour or so but then declines to very low levels (Marsh, Mills & Le Maire, 1973; Le Maire et al., 1973). Following the preovulatory surge of LH in the sheep, Moor et al. (1975) have suggested that C-AMP originates from the theca cells alone and thus acts as a 'distant diffusion activator' to initiate luteinization of the granulosa cells within the Graafian follicle.

However the cellular source(s) of the prostaglandins and C-AMP and also the temporal relationships between LH, C-AMP and the

prostaglandins in the human preovulatory follicle remain obscure.

At the present time it is difficult to establish whether the presence of prolactin in the follicular fluid of a developing follicle is important for maturing the biosynthetic potential of the granulosa cells. Granulosa cell viability is not affected when this hormone was inactivated or removed from the culture medium (Figs. 30 & 40) however it seems that when the cells are steroidogenically competent, just prior to ovulation functional luteinization may not occur without prolactin. Preliminary studies have shown that a s granulosa cells luteinize in vitro (i.e. secrete increasing amounts of progesterone), the concentration of immunoreactive prolactin in the culture medium declines (A. S. McNeilly & K. P. McNatty, unpublished observations); thus the declining concentrations of prolactin in the preovulatory follicle may indeed reflect the increased utilization or metabolism of this hormone by the granulosa cells undergoing luteinization at this time. The marked differences in the concentration of prolactin between plasma and follicular fluid highlight once more the importance of the endocrine micro-environment within the follicle. It is possible that the secretion of oestrogen by the theca cells of the developing follicle are influenced by the concentration of circulating prolactin, since women with galactorrhoea and amenorrhoea appear to have normal levels of LH and FSH in plasma but low levels of urinary oestradiol (Rolland, 1973; Thorner, McNeilly, Hagen & Besser, 1974; McNeilly, 1974). Thus high concentrations of prolactin may inhibit the secretion of oestradiol from the theca cells in a manner similar to that described for granulosa cells in tissue culture (see results, Chapter 6). Failure of the growing follicle to secrete oestradiol

prevents the normal positive feedback to the hypothalamic-pituitary axis (Knobil, 1974) and this in turn would result in anovulation and finally amenorrhoea. Thus the effects of prolactin on the production of oestradiol by theca cells in tissue culture and the biochemical mechanism by which high concentrations of prolactin inhibit steroidogenesis may lead to a greater understanding of the physiological role of prolactin in women.

It appears that high or supra-physiological concentrations of pituitary hormone (either FSH, LH or prolactin) interferes with either normal follicular development, granulosa cell mitoses and/or steroidogenic potential of developing follicles, presumably by abolishing the normal process of follicle selection in addition to disrupting the normal sequence of endocrine changes occurring within the follicles. This may be why ovulation does not normally occur in women during pregnancy when there are sustained high concentrations of HCG and prolactin (Govan, 1968, 1970; Scrimgeour & Baker, 1974).

In women, anovulatory menstrual cycles occur frequently at the extremes of reproductive life, i.e. after the menarche and prior to the menopause (Doring, 1969), and are characterised by the failure of the normal pre-ovulatory LH surge in the presence of an apparently adequate oestrogen secretion by the follicle (Fraser, Michie, Wide & Baird, 1973; Baird, 1974a). Other causes of anovulatory cycles can generally be attributed to a breakdown in the pituitary-ovarian feedback system (Baird, 1974a; Ross & Vande Wiele, 1974). The presence of antibodies in the serum of some patients with Addison's disease which are cytotoxic to granulosa cells in tissue culture provides evidence to suggest

that an immunological mechanism may also contribute to ovulatory failure in a number of cases. The in vitro culture of granulosa cells may prove to be a useful diagnostic aid, to test the sera of women who have primary or secondary amenorrhoea and apparently normal pituitary function for the presence of cytotoxic antibodies.

The present studies using human granulosa cells in culture suggest that PGF₂ α may cause functional luteal regression of the corpus luteum (see discussion, Chapter 10). Recently, PGF₂ α was also shown to inhibit the secretion of progesterone by porcine and bovine granulosa cells grown in culture (Henderson & McNatty, 1975; K. M. Henderson & K. P. McNatty, unpublished data). For effective inhibition of progesterone secretion only minimal amounts of PGF₂ α (50 pg-50 ng) were required (see Table 20; Henderson & McNatty, 1975). This strongly suggests that PGF₂ α is capable of exerting a direct biochemical effect on the luteal cell, thereby inhibiting steroidogenesis.

How is this likely to occur? Behrman et al. (1971b) have suggested that PGF₂ α acts by inhibiting the conversion of cholesterol ester to free cholesterol. However, in view of the central role of C-AMP in stimulating ovarian steroidogenesis (Savard et al., 1965; Dorrington & Kilpatrick, 1967; Robison et al., 1971; Channing & Seymour, 1970; Channing, 1970a; Henderson & McNatty, 1975; ~~see also~~ General Introduction) it is perhaps more likely that PGF₂ α acts directly by inhibiting the synthesis of C-AMP, and so acts on the plasma membrane, the site of C-AMP synthesis; specific receptors for PGF₂ α have in fact been found in the plasma membrane of the human, bovine (Powell et al., 1974a),

and ovine (Powell, Hammarstrom & Samuelsson, 1974b) corpus luteum.

Studies by Rodbell, Birnbaumer & Pohl (1969) on the membrane-bound adenylate cyclase system have led them to propose a three component model of the hormone-responsive membrane-bound adenylate cyclase which is shown in Fig. 49a. 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 PGF₂α acts on the coupling component, either directly or indirectly, to prevent transmission of the signal required to activate the catalytic site (see Fig. 49b). 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, Schwartz & Ulberg (1974) have shown that the adenylate cyclase fails to respond to LH on Days 16 or 17 of the oestrous cycle when PGF₂α secretion is probably maximal. This postulated site of action of PGF₂α can be readily tested both in vitro and in vivo. By acting specifically on the LH coupling unit, PGF₂α should only block the activation by any other hormone having a separate specific regulatory unit linked up to the catalytic site via its own coupling unit; PGE₂ is an example of such a hormone since there is substantial evidence to show that PGE₂ utilises the adenylate cyclase system (Marsh, 1971). When human granulosa cells are cultured in vitro the PGF₂α inhibition of progesterone synthesis can be overcome using PGE₂ (Fig. 46 and Table 21).

Any mechanism of luteal regression must also explain the inability of PGF₂α to induce luteolysis of the newly formed corpus

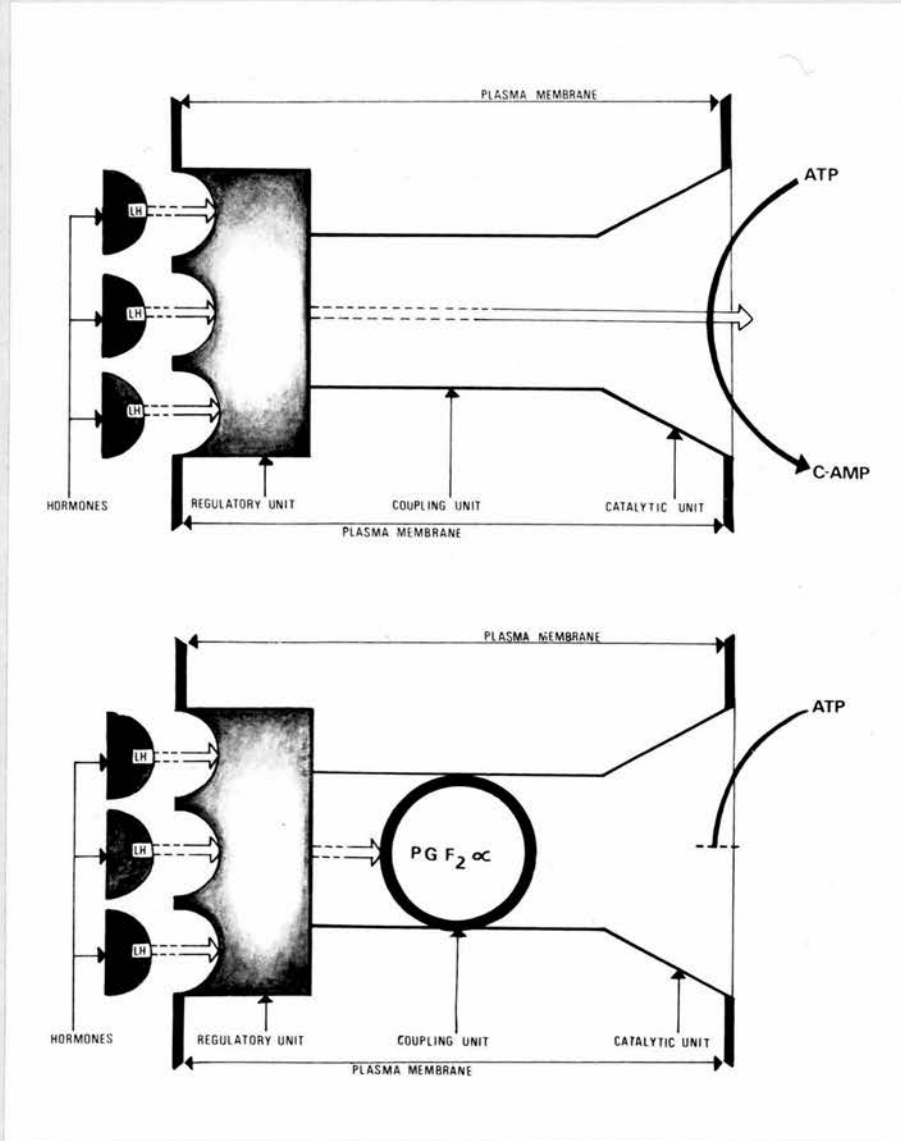


Fig. 49. Three component model of the LH receptor-linked-adenylate cyclase system showing the postulated site of $PGF_2\alpha$ action (see text).

luteum in many animals, e.g. sheep, Day 4 (Hearnshaw, Restall & Gleeson, 1973); cow, Day 4 (Rowson, Tervit & Brand, 1972); horse, Day 4 (Allen & Rowson, 1973) and in particular the pig (Polge, personal communication) 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 preovulatory surge of LH saturates the regulatory units of the luteal cells and it is this bound hormone which protects the young corpus luteum. This hypothesis is supported by the experiments where cultured human granulosa cells, which had previous exposure to high physiological concentrations of LH and FSH, required 200 times more $\text{PGF}_2\alpha$ to effectively inhibit progesterone secretion compared to the amount of $\text{PGF}_2\alpha$ required if it was added at the start of culture (see Figs. 43 and 45).

It is well established that the dissociation of LH from its receptor is a slow process taking several days (Catt, Tsuruhara & Dufau, 1972; Channing & Kammerman, 1973a; Haour & Saxena, 1974; Lee & Ryan, 1974). Moreover only a fraction of these receptors need be occupied to stimulate the adenylate cyclase system maximally (Koch, Zor, Chobsiang, Lamprecht, Pomerantz & Lindner, 1974).

Relative to the levels of LH occurring at the time of the preovulatory surge, when the granulosa cell receptors become saturated with LH those found in peripheral plasma during the luteal phase are very low (Wide et al., 1973): this will encourage the dissociation of LH from its receptor. Thus throughout the luteal phase the LH receptor sites will gradually be vacated, but this will not affect the amount of progesterone being produced by the corpus

luteum. However, as more and more LH dissociates 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" (Singer & Nicolson, 1972), its conformation at any time being the "thermodynamically" most stable form. As the LH receptors on the plasma membrane become full, 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 suitable for 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 hypothesis that PGR_2 may be inducing conformational and/or structural changes within the membrane is strengthened by the recent findings of Hichens et al. (1974), 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 (1975) 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 (Deane et al., 1966) functional regression can be halted by hysterectomy (i.e. removal of PGF2 α) as late as Day 15 (Moor et al., 1970). Massive amounts of PGF2 α (~ 20 ng/ml) are then released from the uterus into the utero-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 (Dingle, Hay & Moor, 1968). Recent studies clearly show that the plasma membrane itself can influence and control lysosomal activity (Szego, 1974). Thus it is possible that low amounts of PGF2 α , 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 PGF2 α .

Although functional regression could arise from a biochemical action of PGF2 α , 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 anoxic damage (Deane et al., 1966; Bjersing et al., 1970). Although PGF2 α will inhibit the secretion of progesterone by luteal cells in vitro, it does not appear to induce structural damage, however if morphological regression is mediated by a vascular effect of PGF2 α , then it is difficult to explain why the newly formed corpus luteum is resistant to PGF2 α .

In conclusion therefore, it is suggested that luteal regression is initiated by a biochemical action of PGF2 α 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 cells. This promotes a conformational change within the plasma membrane facilitating PGF₂α uptake.

2. Under the influence of progesterone, increased amounts of PGF₂α are secreted by the uterus or ovary.

3. PGF₂α 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; cellular C-AMP will diminish, and consequently steroidogenesis will decline.

4. Finally the morphological deterioration of the corpus luteum may result from the subsequent release of larger amounts of PGF₂α, which causes lysosomal activation through further changes in the plasma membrane. However, the possibility of morphological regression being mediated by a vascular effect of PGF₂α cannot be ruled out.

In conclusion, the recent demonstration of PGF₂α receptors in the human corpus luteum (Powell et al., 1974a) and of inhibition of progesterone secretion in culture (see Chapter 10) suggests that PGF₂α may well prove to be the luteolysin in women. The synthesis of 16 aryloxy-prostaglandins (ICI 80996 and 81008) which are 100 to 200 times more potent than PGF₂α in inducing luteolysis in experimental animals and are devoid of the side effects commonly associated with PGF₂α (Dukes, Russell & Walpole, 1974) suggest that the use of long acting synthetic prostaglandins as contraceptive agents should be explored.

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APPENDIX 1

Appendix 1 provides the details relating to the clinical histories of all patients from whom ovaries, or wedge biopsies, peripheral blood samples and endometria were obtained. The day of cycle is the number of days lapsed since the onset of menses. The previous menstrual history indicates the duration of menses in days (top line) and the time from the onset of menses to the commencement of the next menses (bottom line). Parity refers to the number of children born alive plus the number of unsuccessful pregnancies. The term ovary indicates the number of whole ovaries or the number of wedge biopsies (W or 2 X W) received. The size of the wedge varied from a thin slice of cortex to at least one half of the ovary. M-EP, EP, MP, LP, VLP or PP refer to early proliferative - patient still menstruating, early, mid-, late, very late or persistently proliferative phase respectively, whilst ES, MS and LS refer to early, mid- or late follicular phase whilst V-EL, EL, ML and LL refer to very early-, early, mid- and late luteal phase of the menstrual cycle. The stage of the menstrual cycle was assessed from the date since last menstrual period, endometrial biopsy, presence or absence of a corpus luteum, and the concentrations in plasma of LH, FSH, oestradiol and progesterone. - = no specimen obtained and ? = information not recorded. All patients suffered from menorrhagia with the exception of those with stage 0 carcinoma of the cervix.

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora lutea	Follicles	Stage of cycle
1	E.S.	32	19	5/30	2 + 0	2	MS	1	8	ML
2	E.B.	45	8	7/28	3 + 1	W	MP	0	3	MP
3	M.J.	35	14	7/28	2 + 0	W	ES	1	2	EL
4	F.C.	28	19	⁴⁻⁶ 26-28	2 + 0	1	MS	1	2	ML
5	P.H.	30	16	5/28	4 + 0	1	ES	1	6	EL
6	P.McG.	33	5	4/26	1 + 0	1	EP	0	4	EP
7	E.A.	32	11	5/28	3 + 0	2	M-LP	0	11	M-LP
8	S.McD.	31	21	⁴⁻⁵ 28	1 + 0	W	MS	1	4	M-L
9	J.B.	40	1	²¹⁻²⁸ 7	3 + 0	1	E-P	0	2	EP
10	V.C.	43	16	10/28	2 + 0	2	LP-ES	1	8	V.EP
11	C.B.	48	4	6-7/33	4 + 0	W	EP	0	2	EP

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
12	M.F.	42	30	$\frac{2}{30}$	2 + 0	W	LL-E-p	1	3	LL-E-p
13	E.K.	46	13	$\frac{5}{24}$	3 + 0	1	LP	0	1	LF
14	M.A.	48	7	$\frac{6-7}{21-28}$	3 + 0	W	M-p	0	3	MF
15	E.McC.	42	23	Irreg.	11 + 0	1	M-S	1	8	ML
16	G.K.	30	14	$\frac{7}{26}$	0 + 1	1	LF	0	5	LF
17	C.M.	48	9	$\frac{7}{26}$	2 + 0	1	MP	0	1	MF
18	A.B.	35	14	$\frac{8}{28}$	3 ± 1	1	LP	0	2	LF
19	M.B.	40	15	$\frac{3}{28}$	4 + 2	1	LP	0	4	LF
20	A.S.	50	25	$\frac{7}{28}$	3 + 0	W	M-LS	1	1	M-LL
21	M.L.	45	14	$\frac{8-9}{21-24}$	8 + 0	1	ES	2	6	EL
22	M.B.	48	15	$\frac{3}{28}$	4 + 1	2	LP	0	3	LF

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
23	M.B.	43	23	$\frac{7}{24}$	3 + 0	1	M-LS	2	4	M-LF
24	J.McD.	41	21	$\frac{9}{28}$	2 + 1	2	MS	1	3	MF
25	G.N.	48	20	$\frac{2}{28-30}$	9 + 8	1	ML	1	2	ML
26	J.T.	40	4	$\frac{7}{28-32}$	4 + 1	1	EP	0	1	EP
27	M.Q.	49	22	$\frac{3}{28}$	2 + 0	W	MS	1	1	ML
28	F.McG.	46	5	$\frac{10}{26-28}$	4 + 0	1	EP	0	4	EF
29	R.J.	52	16	$\frac{5}{10-28}$	3 + 0	2	ES	1	1	EF
30	C.C.	48	26	$\frac{7-10}{28-32}$	2 + 0	1	LS	1	3	LL
31	A.S.	23	6	$\frac{6}{28}$	0 + 0	2 X W	MP	0	4	MF
32	E.R.	33	9	$\frac{3}{28}$	2 + 0	2 X W	MP	0	5	MF
33	H.J.	41	14	$\frac{7}{28}$	2 + 1	1	LP	0	2	LF

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
34	M.M.	39	14	$\frac{4}{28}$	2 + 0	1	LP	0	1	LF
35	M.S.	44	15	$\frac{10-12}{24}$	5 + 2	1	ES	1	1	EL
36	E.M.	50	19	$\frac{34}{6}$	7 + 0	1	EP	0	1	LF
37	C.P.	38	5	$\frac{7}{21}$	1 + 3	1	MP	0	1	MF
38	J.McD.	32	4	$\frac{7}{21}$	2 + 1	W	EP	0	1	EP
39	M.McL.	42	19	$\frac{4}{21}$	1 + 2	1	LS	1	5	LL
40	I.H.	44	14	$\frac{5}{25-29}$	0 + 0	1	LP-ES	1	3	V.EL
41	D.L.	38	19	$\frac{7}{28}$	3 + 0	1	MP	0	2	P-P
42	B.McL.	43	18	$\frac{5}{25}$	3 + 1	2	LS	1	3	LL
43	H.J.	42	14	$\frac{7}{28}$	0 + 0	2	LP	0	9	LF
44	M.E.	30	16	$\frac{7-14}{28-42}$	2 + 0	2	LP	0	5	LF

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
45	M.B.	40	15	$\frac{7}{28}$	3 + 1	2	LP	0	1	LF
46	D.N.	46	17	$\frac{5}{30}$	2 + 0	2	LP	0	3	LF
47	C.C.	40	17	$\frac{12}{27-34}$	3 + 0	1	ES	1	1	EL
48	D.L.	44	19	$\frac{14}{28}$	1 + 0	1	ES	1	1	EL
49	L.McC.	44	21	$\frac{6}{21}$	2 + 1	1	MS	1	2	ML
50	M.L.	42	13	$\frac{9}{26-28}$	3 + 0	2	LP	0	1	LF
51	I.D.	46	13	$\frac{5}{28}$	3 + 1	1	LP	0	6	LF
52	E.McK.	44	19	$\frac{7-8}{28}$	2 + 1	2	MS	1	1	ML
53	E.S.	31	14	$\frac{7}{28}$	2 + 0	W	LP	0	2	LF
54	D.S.	28	15	$\frac{5}{28}$	3 + 1	W	ES	1	3	EL
55	E.K.	31	6	$\frac{4}{28}$	3 + 0	W	MP	0	6	MP

a) Chronic Pelvic Pain or Dysmenorrhoea

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
56	A.K.	46	13	$\frac{2-4}{28}$	1 + 0	1	LP	0	3	LP
57	J.W.	35	28	$\frac{7}{28}$	3 + 1	W	M-EP	1	2	M-EP
58	A.H.	46	12	$\frac{7}{28}$	4 + 1	W	LP	0	1	LP
59	P.F.	44	28	$\frac{7}{28}$	4 + 0	1	M-EP	1	2	M-EP
60	C.McK.	38	3	$\frac{10}{28}$	3 + 0	1	EP	0	2	EP
61	J.S.	40	10	$\frac{5}{30}$	7 + 10	1	MP	0	7	MP
62	E.A.	33	13	$\frac{5}{28}$	3 + 0	1	LP	0	10	LP
63	G.G.	28	14	$\frac{7}{27}$	4 + 0	1	ES	1	3	EL
64	L.B.	38	15	$\frac{3}{32}$	3 + 0	1	LP	0	5	LP
65	C.R.	36	14	$\frac{3}{28}$	3 + 0	1	LP	0	5	LP
66	F.B.	36	15	$\frac{7}{25-34}$	1 + 0	W	LP	0	3	LP

b) Dysfunctional Uterine Bleeding

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
1	E.R.	33	26	$\frac{3-4}{26-28}$	2 + 0	W	MS-LS	1	1	ML-LL
2	A.F.	41	5 wks since last menses	Grossly irreg.	2 + 0	"	M-P	0	1	P-P
3	L.S.	40	25	$\frac{7-10}{28}$	4 + 0	1	M-P	0	2	P-P
4	K.D.	49	7	$\frac{6-7}{26-31}$	0 + 0	1	E-MP	0	1	E-MP
5	I.M.	47	21	$\frac{7-8}{27-32}$	1 + 1	1	M-S	?	1	ML
6	B.W.	29	29	$\frac{6}{26-34}$	3 + 0	W	L-S	?	7	LL
7	G.S.	23	5 wks since last menses	$\frac{3-4}{28}$	0 + 0	W	P	0	1	P-P
8	B.McV.	35	14	$\frac{10-14}{21-30}$	3 + 0	W	LP	0	1	LP
9	M.B.	40	?	Grossly irreg. last 11 mths	2 + 0	1	MS	1	2	ML
10	M.P.	41	9	$\frac{7}{28}$	2 + 0	1	MP	0	2	MP

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c) Endometriosis

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
1	S.K.	38	8	$\frac{6}{25}$	2 + 0	W	MP	0	1	MF
2	J.C.	34	15	$\frac{4}{28}$	1 + 0	1	LP-ES	1	4	V-EL
3	M.P.	31	19	$\frac{10-15}{30}$	4 + 0	W	LP	0	3	LF
4	A.D.	48	9	$\frac{6-14}{24-28}$	3 + 1	2	LP	0	2	LF
5	A.S.	43	18	$\frac{5}{30}$	5 + 1	1	MS	1	3	ML
6	G.G.	39	17	$\frac{10}{21-28}$	1 + 0	2	LS	1	7	LS
7	M.L.	45		$\frac{5-6}{28-30}$	0 + 0	W	EP	0	5	EP

'primolut'
2 months

c) Endometriosis

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora lutea	Follicles	Stage of cycle
8	M.P.	34	19	$\frac{7}{21}$	2 + 0	1	LS	1	3	LL
9	A.D.	36	9	$\frac{7}{21}$	2 + 0	W	IP	0	1	LF
10	W.P.	40	15	$\frac{5-7}{21-26}$	2 + 0	W	ES	1	5	EL
11	J.H.	36	10	$\frac{5}{28}$	2 + 0	1	MP	0	2	MF
12	M.B.	47	12	$\frac{7}{28}$	3 + 2	1	-	1	2	EL
13	J.J.	36	26	$\frac{5-4}{28}$	1 + 0	1	LS	2	4	LL
14	M.McK.	41	22	$\frac{5-6}{28-30}$	3 + 1	2	MS	1	6	ML
15	E.C.	32	21	$\frac{4-7}{21-28}$	1 + 0	W	MS	1	2	ML

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Partly	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
1	G.L.	44	7	4-5 28	?	2	MP	0	2	MP
2	G.B.	47	17	3-4 24	3 + 0	2	-	1	2	ML
3	J.C.	34	18	2-5 28	1 + 0	W	MS	1	2	ML
4	B.McC.	34	2	Very Irreg.	?	1	EP	1	5	EF
5	H.A.	42	6	3 28	5 + 0	1	MP	0	3	MP
6	J.K.	43	18	3-4 28	3 + 0	2	ES	1	2	EL
7	E.M.	36	19	4-5 28	2 + 0	1	MS	1	2	ML
8	J.R.	42	24	4-5 28	1 + 1	W	MS	1	3	ML

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
9	J.S.	36	10	$\frac{3}{24}$	7 + 1	W	M-IP	0	2	M-IP
10	J.G.	25	20	$\frac{5-6}{28}$	0 + 0	W	MS	2	2	ML
11	M.C.	47	1	$\frac{23-35}{7}$?	1	M-EF	0	1	EF
12	M.B.	43	23	$\frac{4}{28}$	3 + 0	1	LS	1	2	LL
13	E.C.	43	30	Very Irreg.	0 + 0	1	IP-ES	1	3	V-EL
14	A.A.	44	17	$\frac{7}{24-25}$?	W	ES	1	2	EL
15	H.C.	43	28	Very Irreg.	5 + 1	1	MP	0	2	P-P
16	E.McC.	40	10	$\frac{1}{28}$	2 + 0	W	ES	1	1	EL

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Bleeding last 2 mths.	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
17	A.S.	50			Irreg.	3 + 0	2	Menstruating from a proliferative phase	0	2	EP
18	I.S.	43	19		$\frac{7}{28}$	2 + 0	W	LP	0	10	LF
19	C.McK.	47	36		$\frac{6-7}{26}$	0 + 0	1	P	0	4	p-p
20	M.S.	33	15		$\frac{3-4}{28}$	1 + 0	1	LP	0	1	LF
21	M.H.	49	9		$\frac{6-7}{24}$?	1	MP	0	1	MP
22	A.McP.	48	9		$\frac{7}{28}$	3 + 0	W	LP	0	1	LF
23	A.G.	47	28		$\frac{21-28}{8}$?	1	LS	1	1	LL
24	D.McN.	41	36		$\frac{6}{24}$?	1	MS	1	1	ML
25	D.S.	37	16		$\frac{6}{28}$	3 + 1	1	ES	1	1	EL
26	J.McK.	31	13		$\frac{5}{28}$	1 + 0	W	LP	0	6	LF

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
27.	M.F.	42	23	$\frac{4-6}{21-28}$	3 + 0	1	MS	1	4	ML
28	M.McK.	38	23	$\frac{7}{28}$	2 + 0	W	MS	1	3	ML
29	E.McN.	33	27	$\frac{3-4}{26-28}$	2 + 0	1	LS	2	4	LL
30	K.S.	30	9	$\frac{4}{28}$	3 + 0	W	MP	0	3	MF
31	M.F.	50	Bleeding last 2 mths.	Irreg.	2 + 0	1	EP	0	2	EP
32	M.H.	31	8	$\frac{10}{21-28}$	3 + 0	1	MP	0	3	MF
33	F.L.	30	Menstruating	$\frac{10}{28}$	0 + 1	W	M-EF	1	2	EF
34	M.L.	42	13	$\frac{7}{28}$	1 + 1	W	LP	0	1	LP
35	M.B.	52	21	$\frac{7}{28}$	3 + 1	2	MS	1	1	ML
36	E.S.	33	23	$\frac{7}{25}$	3 + 0	1	LS	?	2	LL

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
37	M.P.	35	4	$\frac{7}{21}$	1 + 2	W	EP	0	1	EF
38	K.B.	30	?	Irrreg.	0 + 1	W	EP	0	5	EP
39	D.F.	48	12	$\frac{10}{23}$	1 + 0	1	M-IP	0	1	M-IP
40	K.D.	36	12	$\frac{5}{28}$	3 + 0	1	IP	0	5	IP
41	S.O.	45	25	$\frac{3}{26-28}$	1 + 0	1	LS	1	2	LL
42	K.D.	40	17	$\frac{5}{21}$	1 + 2	1	MS	1	1	ML
43	J.R.	40	25	Irrreg.	0 + 0	2 X W	LS	?	3	LL
44	J.H.	30	15	$\frac{4}{21}$	0 + 1	W	ES	?	1	EL

d) Fibroids

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
45	J.McL.	29	13	$\frac{3-5}{28}$	0 + 2	W	ES	1	1	EL
46	C.B.	26	14	$\frac{7}{28}$	1 + 0	W	LP	0	1	LF
47	S.W.	43	27	$\frac{4-6}{26-32}$?	1	LS	?	3	LL
48	K.D.	39	10	$\frac{7}{30-32}$?	W	MP	0	3	MF
49	E.C.	43	7	$\frac{6}{24}$	2 + 0	1	MP	0	1	MF
50	B.W.	42	29	$\frac{4}{28}$	1 + 0	1	LS	1	3	LL
51	H.McL.	31	16	$\frac{5}{21}$?	W	ES	1	1	EL
52	J.B.	36	13	$\frac{4}{28}$?	W	LP	0	2	LF

e) Stage 0 Carcinoma of the Cervix

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora lutea	Follicles	Stage of cycle
1	E.S.	28	9	$\frac{7-10}{28}$	1 + 0	1	MP	0	4	MF
2	J.G.	49	14	$\frac{5}{26-30}$	4 + 1	W	LP	0	1	LF
3	E.R.	35	24	$\frac{4}{28}$	3 + 4	1	MS	1	8	V-ML
4	A.K.	39	16	$\frac{2-3}{28}$	4 + 1	W	VLP-ES	1	1	V-EL
5	H.F.	38	7	$\frac{4}{28}$	2 + 0	W	EP	0	2	EF
6	H.V.	39	28	$\frac{6}{28}$	3 + 0	W	M-EP	1	4	M-EP
7	M.McT.	36	23	$\frac{4}{28}$	3 + 0	W	MS	2	3	ML
8	E.F.	?	1	$\frac{4}{28}$?	2	EP	0	1	EF
9	M.B.	34	3	$\frac{5-7}{26-28}$?	2	EP	0	2	EP
10	M.S.	26	6	$\frac{4}{28}$?	2	EP	0	2	EP
11	G.G.	31	10	$\frac{4-5}{25-26}$?	2	LP	0	2	LP

e) Stage 0 Carcinoma of the Cervix

Specimen No.	Patient's Initials	Age (years)	Day of cycle	Previous menstrual history	Parity	Ovary	Endometrium	Corpora Lutea	Follicles	Stage of cycle
12	M.L.	34	14	$\frac{5}{21-23}$?	2	ES	1	2	EL
13	E.M.	32	8	$\frac{4}{28}$	2 + 1	W	MP	0	3	MF
14	H.McK.	45	26	$\frac{7}{28-30}$	1 + 0	W	LS	1	3	LL
15	M.C.	30	20	$\frac{7}{31}$?	2	MS	1	1	ML
16	J.H.	36	23	$\frac{4}{28}$	1 + 0	W	MS	1	2	ML
17	T.O'H.	33	8	$\frac{7}{25-26}$	0 + 0	W	MP	0	3	MF
18	J.K.	35	13	$\frac{4}{28}$	2 + 0	W	LP	0	3	LF
19	M.F.	36	19	?	?	W	MS	1	4	ML
20	E.B.	46	?	Grossly Irreg.	2 + 0	2 X W	MP	0	1	MF
21	D.C.	40	20	$\frac{5}{28}$	3 + 0	W	LS	1	1	LL
22	C.W.	33	15	$\frac{5}{28}$?	W	ES	1	1	EL

CHANGES IN THE CONCENTRATION OF PITUITARY AND STEROID HORMONES IN THE FOLLICULAR FLUID OF HUMAN GRAAFIAN FOLLICLES THROUGHOUT THE MENSTRUAL CYCLE

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SUMMARY

The concentrations of FSH, LH, prolactin, oestradiol and progesterone were measured in peripheral plasma and follicular fluid of women throughout the menstrual cycle. With the exception of prolactin, concentrations of pituitary and steroid hormones in follicular fluid correlated with those in peripheral plasma.

Follicle-stimulating hormone was present in a greater number of small follicles (< 8 mm) during or just after the peaks of FSH in peripheral plasma. During the mid-follicular phase the concentration of both FSH and oestradiol in fluid from large follicles (≥ 8 mm) was high. During the late follicular phase the large follicles (≥ 8 mm) contained high amounts of progesterone in addition to oestradiol, low physiological levels of prolactin, and concentrations of LH and FSH about 30 and 60% respectively of those found in plasma. By contrast no large 'active' follicles (≥ 8 mm) were found during the luteal phase although many contained both LH and FSH. Luteinizing hormone was present in a proportion of small follicles (< 8 mm) during the late follicular and early luteal but not at other stages of the menstrual cycle.

It is suggested that a precise sequence of hormonal changes occur within the microenvironment of the developing Graafian follicle; the order in which they occur may be of considerable importance for the growth of that follicle and secretory activity of the granulosa cells both before and after ovulation.

INTRODUCTION

There is considerable evidence to suggest that pituitary gonadotrophins are responsible for the later stages of follicular growth, maturation of the oocyte and ovulation (see reviews of Baker, 1972; Biggers & Scheutz, 1972; Greep, 1973). Although it is possible to correlate changes in gonadotrophins in peripheral plasma

with the production of steroids from the ovary, the actions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on the follicle remain obscure. It has been suggested that gonadotrophins within the follicular fluid itself may have important consequences for follicular steroidogenesis (Channing, 1970, 1972; Edwards, 1974; McNatty, Sawers & McNeilly, 1974). growth of the follicle, oocyte maturation and evolution (Edwards, 1974).

The changing concentration of peripheral plasma oestradiol during the follicular phase of the human menstrual cycle provides an index of follicle growth (Baird, 1971). Oestradiol in the peripheral plasma during this phase of the cycle is derived almost solely from the actively growing follicles (Baird & Fraser, 1974) which also contain high concentrations of oestradiol (Smith, 1960; Short & London, 1961; Giorgi, 1965; Edwards, Steptoe, Abraham, Walters, Purdy & Fotherby, 1972; Baird & Fraser, 1975; Sanyal, Berger, Thompson, Taymor & Horne, 1974). Furthermore, during the immediate preovulatory phase there is an increase in the concentration of progesterone in follicular fluid (Edwards *et al.* 1972; McNatty *et al.* 1974; Sanyal *et al.* 1974) which is also reflected in peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970).

In the present study the concentrations of LH, FSH, prolactin, oestradiol and progesterone were measured in samples of peripheral plasma and follicular fluid collected from women at varying stages of the menstrual cycle. It was hoped in this way to investigate the relationships between the concentrations of pituitary and steroid hormones in follicular fluid and to relate them to concentrations in plasma, follicle size, and the stage of the menstrual cycle.

MATERIALS AND METHODS

Subjects

Ninety-seven subjects (aged 21–48 years) who were at varying stages of the menstrual cycle were undergoing hysterectomy for various gynaecological conditions. The indications for surgery were stage 0 carcinoma of the cervix (17), menorrhagia due to fibroids (35), dysfunctional uterine bleeding (10), endometriosis (9), or chronic pelvic pains or dysmenorrhoea (26). Those with stage 0 carcinoma of the cervix had regular menstrual cycles (21–32 days) and were considered to be endocrinologically normal (Baird & Fraser, 1974). The previous menstrual cycles of the group with menorrhagia varied in length from 21–34 days and about 50% of these subjects had ovulated in the cycle under study, as indicated by the presence of a secretory endometrium and of at least one corpus luteum at the time of the operation. The remaining subjects were in the proliferative phase as assessed by their endometrial histology and date of the last menstrual period.

Dating the menstrual cycle

An endometrial biopsy and a peripheral blood sample (collected before oophorectomy) were obtained during the operation. The endometrium was examined histologically and dated according to the criteria of Noyes, Hertig & Rock (1950). The stage of the menstrual cycle was assessed in all subjects from the date of the last menstrual period, the concentrations in plasma of LH, FSH, oestradiol and pro-

gesterone, endometrial histology and the presence or absence of a corpus luteum. The menstrual cycle was divided into six phases: early follicular, still menstruating (EF); mid-follicular (MF); late follicular (LF); early luteal (EL); mid-luteal (ML) and late luteal (LL).

Ovarian morphology

Ovaries were examined *in situ* to assess gross morphology and to record the presence or absence of a corpus luteum. In some cases excised ovarian specimens were examined by light microscopy after removal of the Graafian follicles to ascertain that no major ovarian pathology existed.

Collection of follicular fluid

The follicular fluid analysed in this study was obtained from ovaries of the 97 subjects described above. Two different methods for the collection of the fluid were used.

In 23 subjects antral fluid was aspirated from 36 Graafian follicles through a 23G needle into a syringe during surgery. It was technically not possible to measure these follicles accurately, consequently they were classified as large (≥ 8 mm) or small (< 8 mm).

In 74 subjects ovarian specimens (whole ovaries or wedge biopsies) were collected into chilled Medium 199 containing Hanks' salts and HEPES buffer (20 mM) (Flow Laboratories) and all follicles which were ≥ 4 mm diameter (153) were dissected out within 2 h of surgery. The diameter of each isolated follicle was measured and the antral fluid aspirated through a 27G needle into a 500 μ l Hamilton syringe.

Fluid collected by either method was frozen at -20 °C until assayed. No attempt was made to recover the oocyte.

Follicles without a lining of granulosa cells, irrespective of size, were defined as 'cystic'. Subsequent data reported for the cystic follicles are considered separately from those obtained for normal follicles.

Radioimmunoassay of pituitary and steroid hormones

Luteinizing hormone and follicle-stimulating hormones

The radioimmunoassays for LH and FSH in plasma and follicular fluid were based upon those previously described (Hunter, Edmond, Watson & McLean, 1974) with modifications for the antral fluid in order to accommodate the small volumes. The original concentrations of standards, tracer and antiserum were retained but the incubation volume was reduced to 150 μ l of which 50 μ l were neat (or diluted) follicular fluid or standard. The counting time was increased proportionately so that in this respect assay precision was undiminished. The following standards were used: LH, MRC 68/40 assumed 77 units/ampoule; FSH, MRC 68/39 assumed 32.8 units/ampoule (MRC National Institute for Biological Standards and Control). The concentrations of LH and FSH are expressed as μ u./ml, where 1 μ u. LH \equiv 11.6 ng LER 907 and 1 μ u. FSH \equiv 44.6 ng LER 907. The assays would normally measure LH and FSH respectively over the ranges 0.8–12.8 and 0.4–6.4 μ u./ml in undiluted follicular fluid. With large follicles, ≥ 8 mm diameter, assays were in general carried

out in duplicate, whilst those for follicles < 8 mm diameter were single determinations. A number of LH assays were also carried out on larger volumes of pooled fluid from small follicles.

Prolactin

Prolactin in peripheral plasma and follicular fluid was measured using a specific double antibody radioimmunoassay (RIA) previously described (McNeilly, 1973; McNeilly & Hagen, 1974). Purified human prolactin for standards and labelling with ^{125}I was generously supplied by Dr H. G. Friesen, University of Manitoba, Winnipeg, Canada. The concentration of prolactin is expressed as ng Friesen prolactin/ml of which $1 \text{ ng} \equiv 20 \mu\text{U}$. MRC 71/222. The minimum detectable level of prolactin was 1.5 ng/ml for both follicular fluid and plasma. Assays were carried out in duplicate in both specimens of plasma and fluid.

Progesterone

Progesterone was measured in peripheral plasma using a radioimmunoassay similar to that described by Thorneycroft & Stone (1972), and in follicular fluid by the method of Neal, Baker, McNatty & Scaramuzzi (1975). Follicular fluid (2–10 μl) was diluted 100- to 1000-fold in phosphate-buffered saline (0.1 mol/l, pH 7.0) and 0.1 ml samples were assayed directly without extraction. The precision and accuracy of the assay was similar to that described by Neal *et al.* (1974). The progesterone antiserum (RI-4) was raised in a rabbit against progesterone-11 α -hemisuccinate conjugated to bovine serum albumin and the specificity was similar to that previously reported (Dighe & Hunter, 1974). The assays were conducted in duplicate and the minimum detectable level of progesterone was 300 pg/ml in plasma and 10 ng/ml in follicular fluid.

Oestradiol

Antiserum for the assay of oestradiol was raised in a rabbit to a conjugate of oestradiol 6-carboxymethyl-oxime and bovine serum albumin. Cross-reactions of other steroids using the routine assay conditions were: oestrone, 3%; oestriol, 0.4%; 6-oxo-oestradiol, 100%; testosterone, 0.003%; progesterone, 0.0002%.

For estimations of oestradiol in plasma an ether extract was evaporated to dryness, equal volumes of 0.05 M-NaOH and carbon tetrachloride were added and shaken together with the residue. Samples of the aqueous phase were then neutralized in a slightly acid diluent for incubation in the RIA system as described by A. Bolton and F. Rutherford (personal communication). Oestradiol in follicular fluid was assayed without prior extraction. The assays were carried out in duplicate and the minimum detectable level of oestradiol was 8 ng/ml in follicular fluid and 20 pg/ml in plasma.

RESULTS

Concentrations of hormones in plasma and follicular fluid in subjects with stage 0 carcinoma of the cervix were compared with those of patients with menorrhagia and were not found to be significantly different. The results from all patients have therefore been pooled.

Concentrations of FSH, LH, oestradiol and progesterone in the antral fluid of

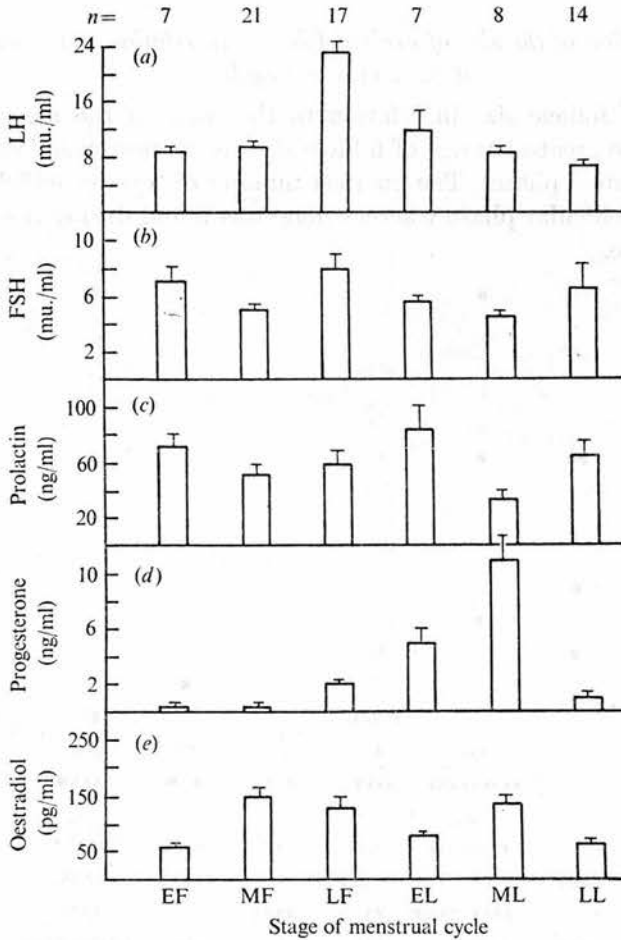


Fig. 1. Mean concentrations of (a) LH, (b) FSH (c) prolactin, (d) progesterone and (e) oestradiol in peripheral plasma during the menstrual cycle. Vertical lines represent +s.e.m. EF, MF and LF refer to early (still menstruating), mid- and late follicular phases respectively, while EL, ML and LL refer to early, mid- and late luteal phases respectively. Numbers of observations are given at the top of the figure.

follicles aspirated *in situ* were not significantly different from those aspirated *in vitro* ($P > 0.2$). The results obtained for each hormone from the two methods of collection have therefore been pooled. Concentrations of prolactin in the antral fluid of follicles aspirated *in situ* are not reported. These samples were stored at -20°C for at least 12 months before being assayed. The immunological and biological activity of prolactin in physiological fluids declines with prolonged storage (H. G. Friesen, personal communication).

Concentration of luteinizing hormone, follicle-stimulating hormone, prolactin, progesterone and oestradiol in peripheral plasma

Mean values (\pm s.e.m.) obtained for the concentrations of LH, FSH, prolactin, progesterone and oestradiol in peripheral plasma with respect to the stage of the menstrual cycle are shown in Fig. 1.

Distribution of the size of excised follicles in relation to the stage of the menstrual cycle

A scatter plot of follicle size in relation to the stage of the menstrual cycle is shown in Fig. 2. The greatest range of follicle sizes (4–20 mm) was found in the late follicular and late luteal phases. The greatest number of 'cystic' follicles (45%) was found in the early follicular phase whereas none was found during the late follicular or early luteal phase.

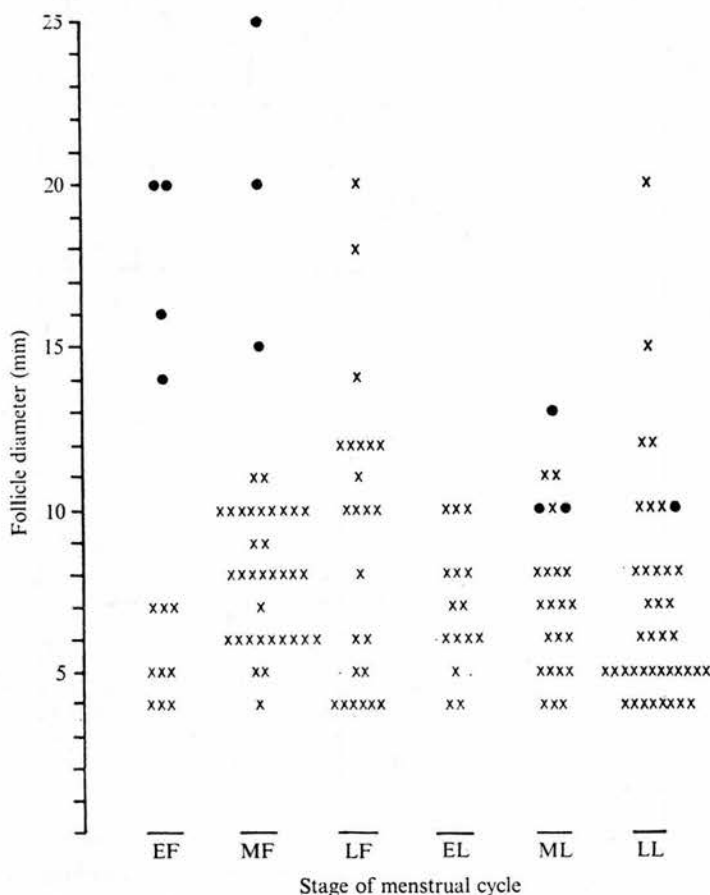


Fig. 2. Distribution of size of excised follicles in relation to the stage of the menstrual cycle. Follicles < 4 mm were not collected; ●, 'cystic' follicles; x, 'non-cystic' follicles. See Fig. 1 for abbreviations.

The concentrations of pituitary and steroid hormones in the follicular fluid of individual Graafian follicles

Follicle-stimulating hormone

There were no significant differences in the concentration of FSH in follicular fluid from follicles aspirated *in situ* when compared with those aspirated *in vitro* ($P > 0.2$). The mean concentration of FSH in all antral fluids in relation to size and the stage of the menstrual cycle is shown in Fig. 3a. The minimum detectable level of FSH in the

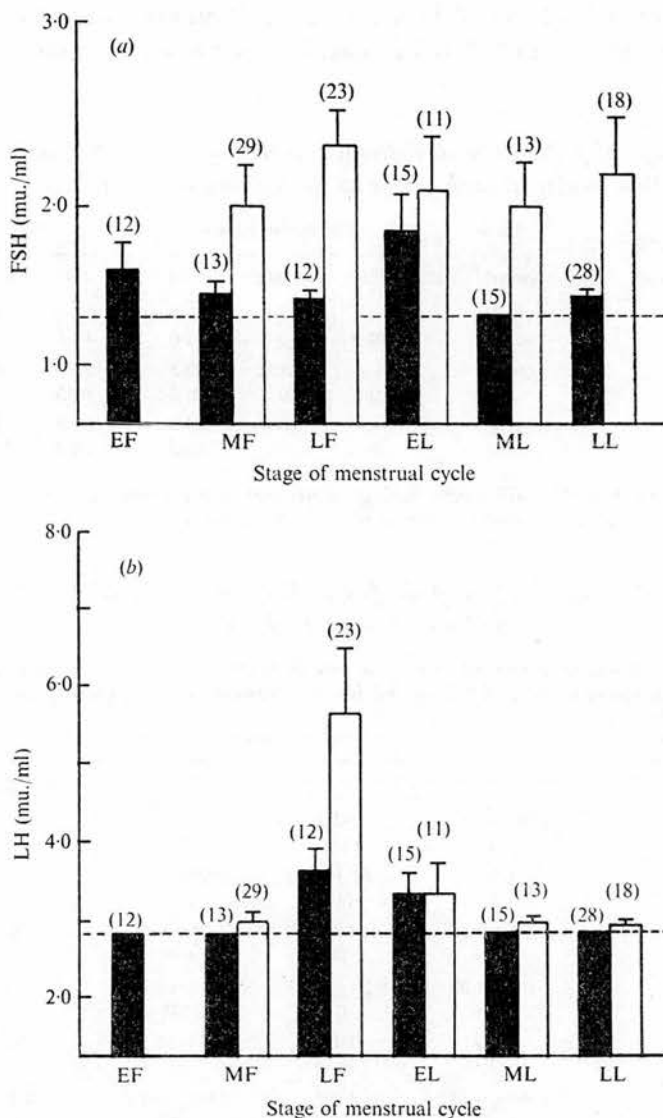


Fig. 3. Concentrations of (a) FSH and (b) LH in antral fluid in relation to follicle size and phase of the menstrual cycle. The vertical lines represent +s.e.m. White bars, mean concentration in follicles $\geq 8\text{ mm}$; black bars, mean concentration in follicles $< 8\text{ mm}$. Broken line = minimum detectable level of (a) FSH (1.3 mu./ml), (b) LH (2.8 mu./ml). See Fig. 1 for abbreviations. Number of observations in parentheses.

smallest follicles (4 mm) was 1.3 mu./ml, which was therefore chosen as the limit of detection for all follicles. The percentage of follicles with detectable FSH at each stage of the cycle is shown in Table 1. In general, the greatest proportion of small follicles with detectable FSH are found either during or immediately after the increase in levels in the plasma (Fig. 1 and Table 1). In contrast, the concentration of FSH in some large follicles was high when the plasma concentrations were low (Fig. 1 and Fig. 3). Follicle-stimulating hormone was detectable in a proportion of the

smallest follicles examined (see Tables 1 and 2). However, at no time during the cycle was the concentration of FSH in follicular fluid more than 60% of the levels found in plasma.

Table 1. *Percentage of follicles with detectable levels of LH, FSH or LH + FSH in relation to size at each phase of the human menstrual cycle*

Pituitary hormone (limit of sensitivity)	Follicle size (mm)	Follicular phase			Luteal phase		
		EF	MF	LF	EL	ML	LL
FSH (1.3 mu./ml)	≥ 8	—	38.0	82.5	45.5	38.5	28.0
	< 8	33.0	15.5	25.0	46.5	0	3.5
LH (2.8 mu./ml)	≥ 8	—	11.0	69.5	25.0	14.0	22.0
	< 8	0	0	16.5	16.5	0	0
LH + FSH	≥ 8	—	10.0	69.5	25.0	14.0	22.0
	< 8	0	0	16.5	16.5	0	0

EF, MF and LF refer to early (still menstruating), mid- and late follicular phase, respectively, while EL, ML and LL refer to early, mid- and late luteal phase respectively.

Table 2. *Concentration of FSH in fluid from different sized follicles at each phase of the human menstrual cycle*

(Values are means ± s.e.m. in mu./ml. Samples less than the detection limit were assumed to have a concentration of 1.3 mu./ml for the purposes of the group mean.)

Stage of menstrual cycle*	Follicle diameter (mm)			
	6	6-8	9-12	12
EF	1.50 ± 0.09 (6)	2.30 (3)	—	—
MF	1.30 (3)	1.40 ± 0.05 (18)	2.30 ± 0.40 (13)	—
LF	1.76 ± 0.26 (8)	1.68 (3)	2.20 ± 0.26 (10)	3.20 (3)
EL	1.66 ± 0.23 (8)	2.10 ± 0.50 (9)	2.10 (3)	—
ML	1.30 (7)	1.30 (11)	1.30 (3)	—
LL	1.46 ± 0.13 (20)	1.30 (12)	3.00 ± 1.10 (8)	3.28 (2)

* For abbreviations see Table 1. Number of observations in parentheses.

Luteinizing hormone

The concentration of LH in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0.2$). The mean concentration of LH in all antral fluids examined with respect to follicle size and the stage of the menstrual cycle is shown in Fig. 3b. The percentage of follicles containing detectable concentrations of LH at various stages of the cycle is shown in Table 1; the lowest detectable level in the smallest follicles examined (4 mm) was 2.8 mu./ml, which was therefore chosen as the limit of sensitivity. There was a greater proportion of large follicles with detectable LH during the late follicular phase (Table 1), when the

concentrations were higher than at any other stage of the cycle (Fig. 3*b*). Luteinizing hormone was not detectable in any follicle of < 8 mm during the early and mid-follicular and mid- and late luteal phases. Fluids from 20 early and mid-follicular phase follicles were pooled to give a total volume of 50 μ l, and the concentration of

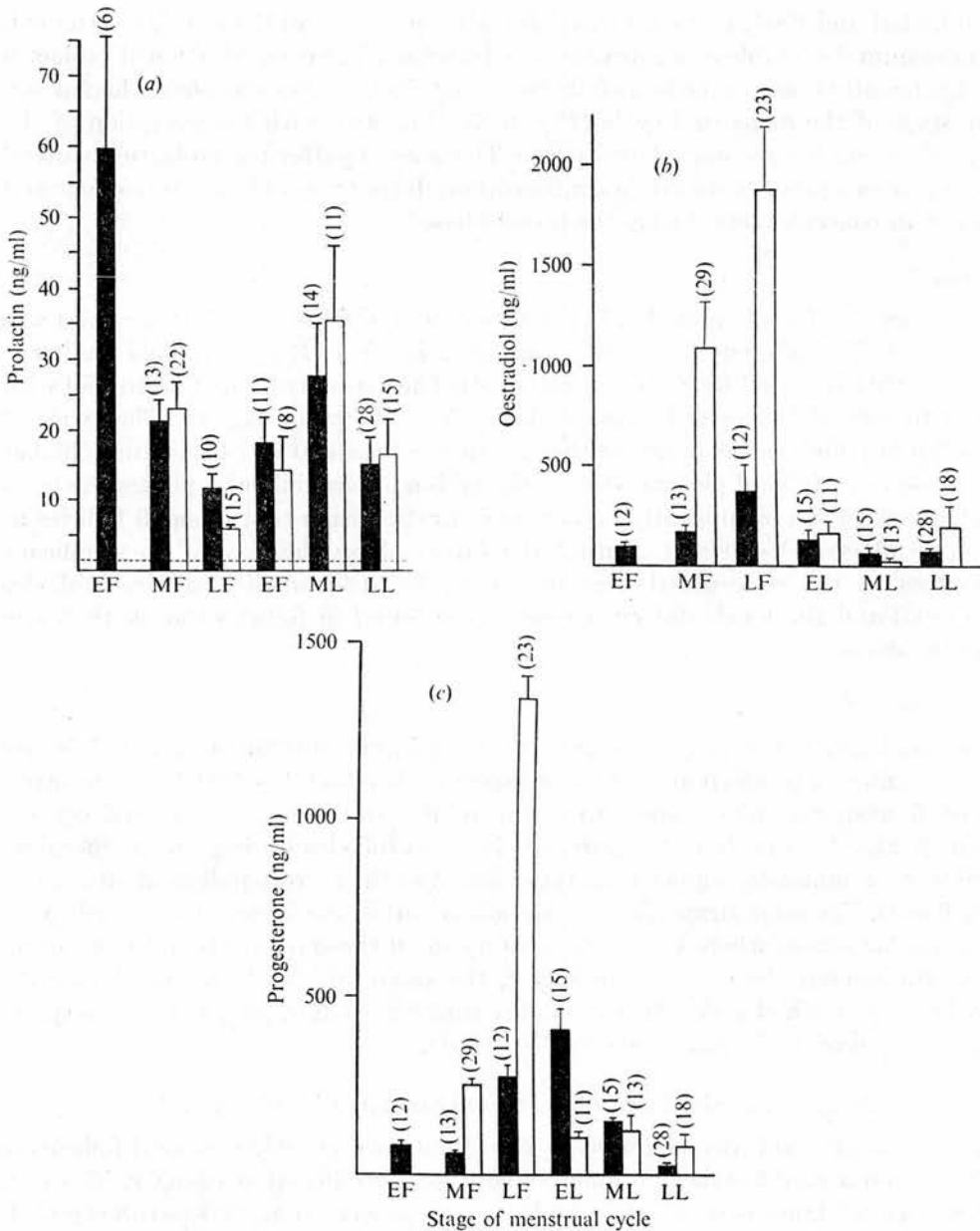


Fig. 4. Concentrations of (a) prolactin, (b) oestradiol and (c) progesterone in follicular fluid in relation to follicle size and stage of the cycle. Vertical lines represent +1 s.e.m. White bars, mean concentration in follicles \geq 8 mm; black bars, mean concentration in follicles < 8 mm. Minimum detectable level of prolactin (1.5 ng/ml) is indicated by the broken line. See Fig. 1 for abbreviations. Numbers of observations in parentheses.

LH was 0.96 mu./ml (detection limit 0.8 mu./ml). Throughout all stages of the cycle the fluid concentration of LH was < 30% of that found in peripheral plasma. Furthermore, LH was only found in those follicles that also contained FSH (Table 1).

Prolactin

Unlike LH and FSH, prolactin was detectable in almost all the follicles examined. The minimum detectable concentration was 1.5 ng/ml. The concentration of prolactin was significantly lower in the large follicles during the late follicular phase than in any other stage of the menstrual cycle ($P < 0.05$) (Fig. 4a), with the exception of the large follicles during the early luteal phase. The general pattern of prolactin in antral fluid indicates a progressive fall in concentration during the follicular phase followed by a rise in concentration during the luteal phase.

Oestradiol

The concentration of oestradiol in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0.2$). This finding is similar to that reported by Sanyal *et al.* (1974). The concentration of oestradiol with respect to size of follicle and stage of the cycle is shown in Fig. 4b. The levels of oestradiol in follicles at all stages of the cycle are between 40 and 40 000 times higher than those in peripheral plasma. During the mid- and late follicular phases the large follicles contained a significantly higher concentration than that in small follicles at the same phase ($P < 0.001$). During the luteal phase there were no significant differences in the concentration of oestradiol between small and large follicles ($P > 0.05$) and the levels did not exceed those found in follicles during the early follicular phase.

Progesterone

The concentration of progesterone in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0.2$). The concentration of progesterone with respect to size of follicle and stage of menstrual cycle is shown in Fig. 4c. Levels of progesterone in large follicles during the proliferative phase were significantly higher than those found in the corresponding small follicles ($P < 0.001$). The most dramatic increase was found in the large follicles during the late follicular phase, where the levels were up to 20 times higher than in any other follicle throughout the cycle. By contrast, the small follicles (< 8 mm) during the early luteal phase had a significantly higher concentration of progesterone than any other follicle during the luteal phase ($P < 0.001$).

Hormones in the fluid of recently ruptured and 'cystic' follicles

Haemorrhagic fluid was aspirated *in vitro* from two recently ruptured follicles in subjects whose endometria still showed very late proliferative changes. The concentrations of hormones in the fluids were: progesterone, 8600–10800 ng/ml; oestradiol, 335–210 ng/ml; LH, 9.6–7.6 mu./ml; FSH 7.9–6.8 mu./ml; prolactin, 14.6–12.1 ng/ml. The levels of LH and FSH were similar to those in peripheral plasma whereas the concentrations of prolactin in follicular fluid were about half the peripheral plasma concentrations. The steroid concentrations were 100- to 1000-fold

higher than the concentrations of oestradiol and progesterone in peripheral plasma. The concentration of progesterone in the fluid was also four to eight times higher than in late follicular phase fluid, but the concentration of oestradiol was at least ten times lower.

Table 3. *Hormone levels in human cystic follicles*

(Samples less than the detection limit were assumed to have a concentration of 1.3 mu./ml, 2.8 mu./ml, 1.5 ng/ml for FSH, LH and prolactin respectively for the purposes of the group means.)

Stage of cycle*	Follicle size (mm)	LH (mu./ml)	FSH (mu./ml)	Prolactin (ng/ml)	Oestradiol (ng/ml)	Progesterone (ng/ml)
EF	20	2.8	2.9	13.8	9	55
	16	6.0	1.7	39.0	205	20
	14	2.8	1.4	25.2	17	20
	20	2.8	1.2	47.0	40	15
MF	20	2.8	6.6	19.9	18	90
	15	12.0	1.3	23.0	230	17
	25	2.8	2.2	28.2	36	58
ML	10	2.8	1.3	23.0	150	90
	13	10.0	1.3	21.0	104	70
	10	2.8	1.3	21.0	30	80
LL	10	14.0	2.4	3.0	19	48

* For abbreviations see Table 1.

Table 4. *Concentrations (a) of oestradiol in the follicular fluid with or without FSH and (b) of progesterone in follicular fluid with or without LH (values are means \pm S.E.M. in ng/ml)*

	Stage of menstrual cycle*					
	EF	MF	LF	EL	ML	LL
(a) Oestradiol concentrations						
FSH ($>$ 1.3 mu./ml)	300 \pm 34 (4)	2120 \pm 410 (13)	2180 \pm 310 (22)	135 \pm 40 (12)	33 \pm 26 (5)	211 \pm 200 (5)
FSH (\leq 1.3 mu./ml)	103 \pm 22 (8)	196 \pm 60 (29)	140 \pm 55 (13)	102 \pm 58 (14)	24 \pm 11 (23)	60 \pm 38 (41)
(b) Progesterone concentrations						
LH ($>$ 2.8 mu./ml)	†	760 (2)	1720 \pm 280 (18)	715 \pm 60 (4)	196 (2)	320 \pm 40 (4)
LH (\leq 2.8 mu./ml)	110 \pm 31 (12)	96 \pm 33 (40)	352 \pm 84 (17)	66 \pm 11 (22)	111 \pm 15 (26)	100 \pm 20 (42)

* For abbreviations see Table 1. Number of observations in parentheses. Limit of detection for FSH is 1.3 mu./ml and for LH is 2.8 mu./ml.

† No follicles containing LH.

Data relating to the hormone levels in cystic follicles are shown in Table 3. All these follicles contained low levels of oestradiol and progesterone irrespective of the stage of the cycle, suggesting that they were 'inactive' (Baird & Fraser, 1975).

Relationship between follicular concentrations of hormones

Those follicles which had detectable levels of FSH during the follicular phase without exception contained significantly higher concentrations of oestradiol than those with undetectable FSH (Table 4a) (EF, MF, LF; $P < 0.01$, $P < 0.001$,

$P < 0.001$, respectively). Conversely, follicles with undetectable FSH contained low levels of oestradiol irrespective of the stage of the cycle. During the luteal phase these correlations no longer held, since follicles with or without FSH had consistently low levels of oestradiol.

A significantly higher concentration of progesterone was found in follicles containing LH (Table 4*b*) (MF, LF, EL, ML, LL; $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.01$ respectively). All the follicles containing LH and a high concentration of progesterone were ≥ 8 mm diameter and also contained FSH.

During the mid-follicular, late follicular and late luteal phase of the cycle the concentration of oestradiol in follicular fluid was correlated with that of progesterone ($r = 0.44$, $n = 43$, $P < 0.01$; $r = 0.88$, $n = 33$, $P < 0.001$; and $r = 0.49$, $n = 37$, $P < 0.01$, respectively).

DISCUSSION

There was no difference in the concentration of hormones in fluid collected from follicles *in vitro* from those collected *in vivo* suggesting that there was little if any production or metabolism of hormones in the time between ovariectomy and aspiration of fluid (cf. Giorgi, Addis & Colombo, 1969; Sanyal *et al.* 1974). Although patients were undergoing surgery for a variety of reasons, the general pattern of hormones in peripheral plasma (Fig. 1) was similar to that described in normal women during the menstrual cycle (e.g. Robyn, Delvoeye, Nokin, Vekemans, Badawi, Perez-Lopez & L'Hermite, 1973). However, the concentration of prolactin in peripheral plasma was up to sixfold higher than that in normal women (McNeilly, Evans & Chard, 1973; McNeilly & Chard, 1974). This is probably because the samples were collected under the stress of surgery which is known to stimulate the release of prolactin (Robyn *et al.* 1973). The overall mean concentration of prolactin in follicular fluid (20 ± 5 ng/ml, $n = 189$) was much lower than that in peripheral plasma. In the absence of information about the rate of exchange of prolactin between plasma and follicular fluid *in vivo* it is impossible to determine whether this difference is of physiological importance or whether it is an artifact due to the elevated prolactin concentration in peripheral plasma as a result of stress. Since all the samples were collected under similar conditions, the striking change in follicular fluid concentration at different stages of the cycle (Fig. 4*a*) is probably not an artifact.

The distribution of follicle size in relation to the stage of the cycle is similar to that previously described in women (Block, 1951) and rhesus monkeys (Koering, 1969). Large follicles (≥ 8 mm) were much commoner during the mid- and late follicular and late luteal phases than at any other stage of the cycle. Although it is difficult to draw conclusions about the dynamics of follicle growth from these cross-sectional observations, it is tempting to speculate that these large follicles represent the end result of two waves of follicular development initiated by the peaks of secretion of FSH occurring at the onset of menses and again at mid-cycle. The minimum time necessary to develop a follicle from the antral to the mature preovulatory stage in women is probably 6–10 days (Gemzell & Johansson, 1971; Bertrand, Coleman, Crooke, Macnaughton & Mills, 1972). The concentration of FSH in small follicles reaches a maximum during the early follicular, late follicular or early luteal phases of the cycle (Table 2) which is either during or just after the peak FSH concentration in peri-

peripheral blood. Furthermore, the concentration of FSH in these follicles apparently increases as they develop into mature preovulatory follicles in spite of the falling levels of FSH in peripheral blood (cf. Table 2 and Fig. 1). Some factor may increase the affinity of these follicles for FSH. Oestradiol, the concentration of which is extremely high in the mid- and late proliferative phase follicles, is known to increase the sensitivity of the ovary to FSH (Goldenberg, Vaitukaitis & Ross, 1972). The high levels of oestradiol in the large follicles during the mid- and late follicular phase are similar to those previously reported by other workers in normal (Smith, 1960; Short & London, 1961; Sanyal *et al.* 1974; Baird & Fraser, 1975) and gonadotrophin-stimulated ovaries (Short, 1964*a*; Edwards *et al.* 1972). Follicles which have detectable levels of FSH during the follicular phase also have a high concentration of oestradiol whereas those follicles with undetectable levels of FSH have significantly lower levels of oestradiol (see Fig. 3*a* and Table 4*a*).

When the concentration of FSH in peripheral plasma was high (early follicular phase and at mid-cycle) FSH was detectable in only a minority of small follicles (diameter < 8 mm) (Tables 1 and 2). Presumably follicles in which the concentration of FSH is high are those which are stimulated to further development (Table 4*a*). It is not known how this minority of small antral follicles are selected although it has been suggested that oocytes are 'programmed' for development in the order in which they are formed during foetal life (Henderson & Edwards, 1968).

About 17% of the small follicles (diameter < 8 mm) in the early luteal phase of the cycle had measurable amounts of FSH and LH (Table 1), presumably as a result of the preovulatory peaks of the gonadotrophins. In contrast to the follicular phase of the cycle, none of the follicles in the luteal phase were functionally 'active', as indicated by the persistently low concentrations of oestradiol. From the time of the LH 'surge' there is an abrupt fall in the mitotic activity of granulosa cells (Delforge, Thomas, Roux, Caneiro de Siqueiro & Ferin, 1972). Thus the presence of LH in some of these small follicles (Table 1) may interfere with their normal orderly development and consequently their steroidogenic potential (Tables 4*a* and *b*). The marked increase in cystic follicles (Fig. 2) from the mid-luteal phase is probably a consequence of this. It may be that an ordered sequence of gonadotrophins, e.g. FSH alone followed by FSH and LH, is necessary for normal follicular development.

Luteinization of the granulosa cells of the preovulatory follicle begins some 24–36 h before ovulation in response to the mid-cycle LH surge (Hertig, 1967; Delforge *et al.* 1972). After exposure to LH, the preovulatory follicle secretes increasing amounts of progesterone as indicated by the rise in concentration in ovarian (Mikhail, 1970; Lloyd, Lobotsky, Baird, McCracken, Weisz, Pupkin, Zanartu & Puga, 1971) and peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970). The relatively high concentration of progesterone in follicular fluid of preovulatory follicles is similar to that found by other workers in normal (Sanyal *et al.* 1974) and gonadotrophin-stimulated ovaries (Short, 1964*a*; Edwards *et al.* 1972), and it is likely that the luteinizing granulosa cells are the source of this steroid (Channing, 1969, 1970; Edwards *et al.* 1972).

The large preovulatory follicles were characterized by a highly vascular appearance (Short, 1964*b*). The granulosa cells were very loosely attached to one another; the walls were slimy and mucoid, and the follicular fluid was bright yellow and

viscous. When the granulosa cells from large follicles collected during the pre-ovulatory phase were cultured *in vitro* they secreted maximal amounts of progesterone in response to minimal physiological concentrations of gonadotrophins in the medium (Channing, 1970; K. P. McNatty & R. S. Sawers, unpublished observations). The granulosa cells are stimulated *in vivo* by the relatively high concentrations of LH in blood and follicular fluid. Presumably the metabolic requirement of these actively secreting cells is high and may account for the relatively low oxygen tension within the follicle (Fraser, Baird & Cockburn, 1973). The relatively low concentration of prolactin may also reflect utilization or metabolism of the hormone by the follicle. It may also play a key role in controlling steroid synthesis, for the production of progesterone by human granulosa cells *in vitro* is inhibited when the concentration of prolactin in the medium exceeds 30 ng/ml (McNatty *et al.* 1974).

It is apparent that despite the presence of FSH in antral fluid the granulosa cells do not secrete progesterone in the absence of LH. Furthermore, the concentration of progesterone in follicular fluid of small follicles during the luteal phase is significantly lower than in preovulatory follicles, even though they contain both FSH and LH. The failure of the granulosa cells in small follicles to secrete substantial amounts of progesterone when exposed to LH suggests that these cells require time before they are capable of responding fully. The latter finding is consistent with the observation that granulosa cells harvested from preovulatory follicles in the pig have a significantly greater number of receptors for LH when compared with the cells harvested at other stages of the oestrous cycle (Channing & Kammerman, 1973; C. P. Channing, personal communication).

The concentration of oestradiol in the haemorrhagic fluid of recently ruptured follicles was very much lower than that found in the large preovulatory follicles. Although most of the fluid is lost at ovulation the low concentration of oestradiol in the haemorrhagic fluid is consistent with the low plasma levels during the immediate post-ovulatory period (Fig. 1) (Moor, 1974). In contrast, however, the levels of progesterone were higher than those found in the preovulatory follicle and this is consistent with the increased plasma levels indicating the growing secretory capacity of the luteinizing granulosa cells.

Steroid levels in the cystic follicles were low irrespective of the stage of the cycle and in many the pituitary gonadotrophin concentrations were high and similar to those found in plasma. These findings suggest that cystic follicles are functionally inactive and are incapable of secreting oestradiol or progesterone even in the presence of gonadotrophins.

These data suggest that a precise sequence of hormonal changes occurs within the microenvironment of the developing Graafian follicle; the order in which the changes occur may well be of considerable importance for the growth of that follicle and the secretory activity of the granulosa cells both before and after ovulation. Figure 5 shows the possible inter-relationships between the concentrations of pituitary hormones in plasma and follicular fluid with respect to the steroidogenic activity and growth of a follicle during the follicular and luteal phase of the menstrual cycle. The changes in the concentrations of pituitary hormones in follicular fluid throughout the menstrual cycle are related to those which occur in peripheral plasma. The presence of FSH in the smallest follicles appears to be important for their development. The

presence of FSH and oestradiol in the follicular phase follicles prepares them for the preovulatory production of progesterone under the influence of LH. The very low concentrations of prolactin in these follicles may be related to the upsurge in metabolic activity within the follicle during this time. The antral fluid of the preovulatory follicle contains relatively large amounts of oestradiol and progesterone, low physiological levels of prolactin, and concentrations of LH and FSH approaching 30 and

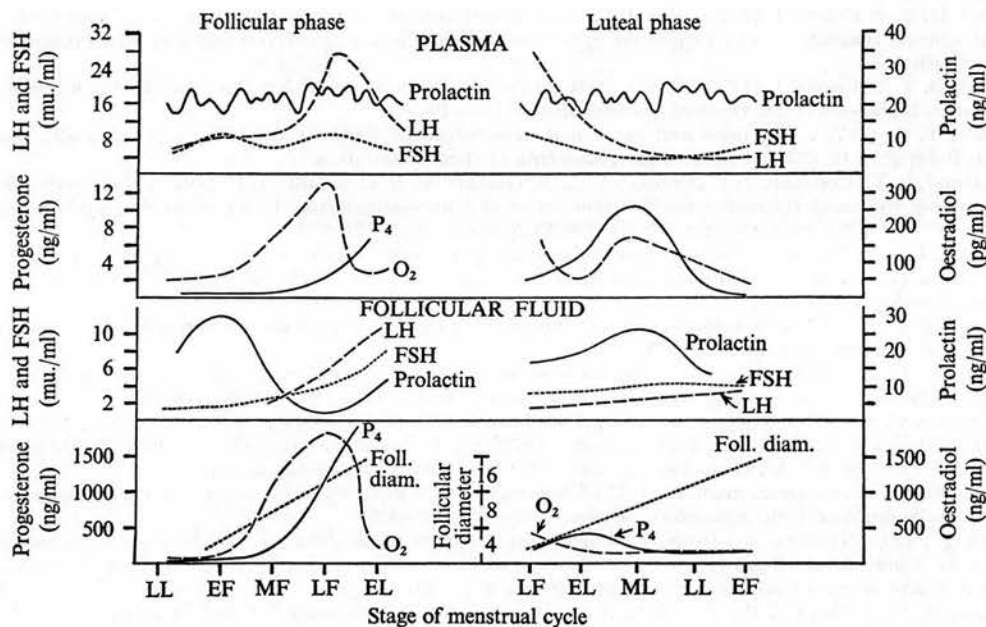


Fig. 5. The postulated relationships between the concentrations of pituitary and steroid hormones in peripheral plasma and in the follicular fluid of a developing follicle during either the follicular or luteal phase of the menstrual cycle (see text). O₂ = oestradiol, P₄ = progesterone. See Fig. 1 for abbreviations.

60% respectively of those found in plasma. These changes in the growth of a follicle destined to ovulate are different from those proposed for the pig (Channing, 1972). Such differences that exist will possibly depend on the permeability of a Graafian follicle in different species to protein hormones (see Edwards, 1974, for review).

Furthermore, it is suggested that the large inactive follicles found during the luteal phase are a consequence of LH interference during the growth of these follicles under the influence of FSH. The consequence of these findings may prove to be important in the treatment of amenorrhoeic or anovulatory patients with exogenous gonadotrophins.

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