A STUDY OF SOME ENDOCRINE ASPECTS OF FOLLICULOGENESIS IN SHEEP

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A THESIS SUBMITTED FOR THE DEGREE OF

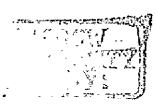
DOCTOR OF PHILOSOPHY

IN THE

FACULTY OF VETERINARY MEDICINE

THE UNIVERSITY OF GLASGOW

APRIL, 1976



ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor Sir William Weipers and to Professor D.D. Lawson for granting me the facilities to carry out this work, and to Mr. P.G. Hignett and Dr. T.A. Douglas for their supervision and advice. I am particularly indebted to Mr. C.D. Munro for constant help and encouragement throughout these studies.

I am grateful to Dr. B. Cook of the Department of Steroid Biochemistry, Glasgow Royal Infirmary for advice concerning the oestrogen radioimmunoassay, and to Dr. R.B. Land and Mr. W.R. Carr of the Animal Breeding Research Organisation, Edinburgh for help in the estimation of luteinising hormone.

I also wish to thank Miss N. Burns and her staff for the preparation of histological sections, Mr. A. Finnie and his staff who prepared the photographs contained in this thesis and Mr. D. McLaren of the Department of Statistics, University of Glasgow who carried out some statistics used in Chapter 4. I would also like to thank the stockmen who cared for the experimental animals used during these studies.

I am grateful to the Meat and Livestock Commission for a scholarship (S2.16.01) to pursue these studies. Finally I would like to thank Mrs. M. Christie for typing this thesis.

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SUMMARY

Some aspects of follicular development and function were studied in sheep, during the neonatal period and during the oestrous cycle. The number and appearance of large follicles (4 mm or greater in diameter), ovarian weight and volume, uterine horn diameter and pituitary weight were recorded at laparotomy or slaughter. The preparation of histological sections allowed total ovarian oocyte counts to be carried out and uterine epithelial height to be measured. Peripheral plasma levels of total unconjugated oestrogens were determined by a sensitive radioimmunoassay. Two breeds of sheep, Finnish Landrace and Scottish Blackface, were used throughout the studies in order to investigate the physiological factors responsible for variations in litter size. Mean litter sizes of 2.37 and 1.29 were produced by the Finnish and Blackface flocks respectively.

In mature cycling ewes a peak in the peripheral plasma level of oestrogens was recorded during prooestrus. The peak levels were not significantly different between the two breeds. Elevated levels of oestrogens occurred on the day before and on the day of oestrous onset in the Finnish ewes, whereas the level was elevated only on the day before the onset of oestrus in the Blackfaces. These differences may be related to the greater number of ovulating follicles and the longer duration of oestrus reported for the more fecund breed.

In the neonatal lambs, of both breeds, an increase in ovarian volume between 7 and 35 days of age was accompanied by an

increase in the number of small vesicular follicles (generally 2 mm or less in diameter) visible on the ovarian surface. Uterine horn diameter and uterine gland development also increased during the same period. The peripheral plasma level of oestrogens remained basal in control lambs at all ages. At 35 days of age the number of oocytes in vesicular follicles was significantly greater in the Finnish lambs than in the Blackfaces.

Follicular development was stimulated in neonatal lambs by the administration of PMSG. In a proportion of lambs at 7, 21 and 35 days of age the ovaries were refractory to PMSG-treatment. Among the lambs that were stimulated, the response, in terms of the development of large follicles, was extremely variable. The greatest range in the response occurred in lambs of 49 days of age and less, and with the higher doses of PMSG. Uterine horn diameter and epithelial height were significantly greater in the treated lambs than in controls. PMSG-treatment was associated with a significant elevation in the peripheral plasma level of oestrogens. The concentrations increased progressively until the administration of HCG, 4 days after PMSG, resulted in an immediate decline in the concentration. HCG also induced ovulation or luteinisation in a proportion of the large follicles. Spontaneous ovulation of the stimulated follicles did not occur in lambs of 49 days of age and less. The peak level of oestrogens was significantly correlated with the number of large follicles.

The use of methallibure (I.C.I. 33,828) was investigated as a possible means of suppressing the endogenous secretion of gonadotrophins in the neonatal lamb. In both breeds, the

parenteral administration of methallibure daily from birth had no effect on ovarian size, uterine structure and plasma levels of oestrogens at 35 days of age. However the number of oocytes in primordial follicles was greater in methallibure-treated animals. Pituitary weight, as a percentage of bodyweight, was significantly reduced in the treated Finnish lambs and significantly greater in the treated Blackface lambs. Methallibure-treatment resulted in a smaller and delayed release of LH following LH-RH stimulation, at 45 days of age, in both breeds.

In both normal and methallibure-treated animals the response to PMSG, in terms of follicular development and levels of oestrogens, was not significantly different between the Finnish and Blackface lambs. Therefore it was concluded that, in lambs of 7-49 days of age, ovarian sensitivity was similar in the two breeds. This implies that extra-gonadal factors are responsible for the different ovulation rates reported for mature Finnish and Blackface ewes.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Reproductive performance, of which litter size is a major component, is an important factor affecting the profitability of sheep production (Wallace, 1955). The number of lambs in a litter is largely controlled by the number of oocytes shed by the ovaries at ovulation, other factors such as embryonic death having less influence on the final litter size (Packham and Triffitt, 1966). Although environmental variables, such as age and level of nutrition, may affect ovulation rate there is also evidence for genetic variation among breeds and strains of sheep (Bradford, 1972). There have been many attempts to improve the reproductive performance of sheep by selecting for an increased incidence of multiple litters (Turner, 1969). A knowledge of the physiological factors controlling ovulation rate might produce a method of predicting the reproductive performance of female sheep which would be of value in selection programmes.

At ovulation the oocytes are released following the rupture of the follicles in which they were contained within the ovary. Providing a suitable environment for the developing oocytes is one function of ovarian follicles. Another equally important function is the secretion of steroid hormones, the theca interna of preovulatory follicles being the primary site of oestrogen production in the non-pregnant ewe (Seamark, Moor and McIntosh, 1974). Oestrogens secreted by preovulatory follicles serve several important functions, such as conditioning the

female to accept the male, stimulating the release of pituitary hormones concerned with the ovulatory process and creating an environment within the reproductive tract that is suitable for both gamete transport and fertilisation, and for subsequent development of the fertilised ova (Emmens, 1969). There is evidence to suggest that the circulating level of oestrogens may be related quantatively to the number of ovulating follicles present in the ovaries (Saumande and Pelletier, 1975).

The present studies were designed to provide further information on the physiological factors controlling ovulation rate in sheep. Following the importation of a selected group of Finnish Landrace ewes into Britain, Donald and Read (1967) reported an average litter size of 3.0. This compares with an average litter size of 1.85 for Scottish Blackface ewes maintained under similar conditions (Wiener, 1967). Wheeler and Land (1973) have reported ovulation rates of 2.94 and 1.26 for Finnish and Blackface ewes that were of a similar order of magnitude as their litter sizes. Due to the well-established differences in prolificacy between Finnish Landrace and Scottish Blackface sheep these two breeds were selected for use throughout the present studies. Ovarian activity, both naturally and in response to exogenous gonadotrophins, has been compared in the two breeds. Two main criteria have been used throughout to assess ovarian activity, follicular development and the peripheral plasma level of oestrogens.

1.2 OVARIAN FOLLICLES IN THE SHEEP

Several authors have recorded their observations on the morphology of the ovaries in sheep (Marshall, 1904; Grant, 1934; Cole and Miller, 1935; McKenzie and Terrill, 1937). According to Grant (1934) the ovaries are roughly bean-shaped in form, about 2 cm long and each weighing about 1.5 g. McKenzie and Terrill (1937) recorded greater variation in size, with ovarian weights ranging from 1.9 to 4.0 g and the length ranging from 1 to 3 cm. Along one edge the ovary is attached, by means of the mesovarium, to part of the broad ligament of the uterus, and to part of the ampulla of the oviduct, although the ampulla does not surround the ovary in the ewe (Grant, 1934).

The structure of the ovary consists of an outer cortex surrounding an inner medulla. The medulla is composed of loose fibrous tissue containing numerous large blood vessels, lymphatics and nerves merging with the vascular connective tissue of the mesovarium at the hilum. The cortex, which forms the major part of the ovary, consists of connective tissue, often fibrous, smooth muscle cells and interstitial cells, with numerous blood vessels, lymphatics and bundles of nerve fibres. The superficial tissues of the cortex are differentiated into the tunica albuginea, which is only thin in the sheep, and the ovarian surface is covered by a continuous layer of germinal epithelium in this species (Grant, 1934).

Follicles, in all the stages of their development, are found within the cortex of the ovary (Plate 1.1). Although

there is little information on follicular development in the sheep, the uniformity of follicular morphology among mammalian species (Brambell, 1956) suggests that the general information also applies to the sheep. Complete classifications of ovarian follicles, according to their size and morphology, have been proposed from work in mice (Mandl and Zuckerman, 1950; Pederson and Peters, 1968). A simpler classification, grouping follicles according to morphology, is more frequently used. Throughout the present studies follicles have been placed in one of three groups, primordial, growing or vesicular, corresponding to the types 1 to 3b, 4 to 5b and 6 to 8 respectively of Pederson and Peters (1968).

(a) Primordial follicles

The smallest follicles in the ovary are distributed in the periphery of the cortex. They consist of an oocyte surrounded by a single layer of flattened epithelial cells (Plate 1.2). The number of primordial follicles reaches a maximum at the end of the oogenetic period, which occurs during foetal life in the sheep (Mauleon, 1969; see also Chapter 4, Section 4.1). Subsequently each given follicle and enclosed oocyte can remain in the "non-proliferating pool" or begin to grow, thereby entering the "proliferating pool" (Schwartz, 1974).

(b) Growing follicles

Growing follicles are those that have left the "non-proliferating pool" and begun to develop but do not possess an antrum (Plate 1.3). The first indication of follicular growth is multiplication of the flattened epithelial cells surrounding the oocyte. Initially they are still organised in a single layer around the

oocyte, although they change from flattened to cuboidal or columnar cells. These epithelial cells become separated from the oocyte by the deposition of the zona pellucida. This occurs discontinuously at first but eventually results in a continuous investment around the oocyte. Further multiplication of the epithelial cells leads to the oocyte being surrounded by several layers of cells. At an early stage the growth of the follicle is accompanied by the gradual formation of a definite concentric sheath of dense stromal tissue around it which constitutes the theca. Although the theca has begun to develop in the growing follicles it has not differentiated into inner and outer layers, the theca interna and externa.

(c) Vesicular follicles

Vesicular follicles are those in which an antrum is present (Plate 1.4). The first sign of the antrum is the development of irregular lacunae among the cells of the membrana granulosa which become filled by the secretion of liquor folliculi. Progressive accumulation of fluid induces distension and coalescence of the lacunae, bringing about the formation of the antrum. In the mature vesicular follicle the antrum is bounded by a peripheral zone of epithelial cells, the membrana granulosa, which is several cells thick and extremely uniform. The oocyte remains surrounded by several layers of epithelial cells, the cumulus oophorus, which merges with the membrana granulosa at one point and projects into the antrum.

When the antrum starts to form, the theca begins to differentiate into the theca interna and theca externa. The theca

externa is distinguished from the cortical stroma by its denser nature and by the concentric arrangements of the cells, which are a mixture of fibroblasts and myoid cells. The latter resemble smooth muscle cells and may have a contractile function (O'Shea, 1973). The theca interna consists mainly of elongated fibroblast-like cells interspersed with a few larger epithelioid-type cells and numerous collagen fibrils (Hay and Moor, 1975b). A thin membrana propria separates the theca interna from the membrana granulosa.

In the mature vesicular follicle, prior to ovulation, the membrana granulosa is completely avascular. Numerous blood vessels and lymphatics are present in the theca externa and communicate with a fine plexus of vessels in the theca interna. When the secretion of oestrogens by the preovulatory follicle reaches a maximum (see Section 1.3) the theca interna becomes more highly vascularised and there is a concomitant increase in the number and size of the large epithelioid cells in this layer (Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short and Younglai, 1972). As the follicle approaches ovulation the attachment of the cumulus oophorus to the wall of the follicle begins to break down leaving the inner cells of the cumulus radially arranged around the oocyte as the corona radiata (Cole and Miller, 1935). About 12 hours before ovulation degenerative changes become apparent in the thecal cells (Moor, Hay and Seamark, 1975).

The process of ovulation in the ewe has been described in detail by McKenzie and Terrill (1937). Conspicuous changes in

the appearance of the follicle were confined to the 4 hours prior to ovulation. Before this the preovulatory follicles varied from 4-10 mm in diameter and were not markedly distended. They were usually darker and more vascularised than other follicles. During the final 4 hours, the follicle destined to ovulate began to protrude from the surface of the ovary, becoming conical in shape. As the follicle grew larger the membranes over the surface became thinner and more transparent. About an hour before rupture a small round clear area appeared near the centre of the follicle and gradually increased in height and diameter. In most cases, a few minutes before rupture one or more tiny cones or projections, often from 1-3 mm in height, swelled out from this clear area. Ovulation occurred at the apex of one of these cones and the follicular fluid was released from this point. The liquor folliculi usually was released slowly although in a few animals an initial spurt was seen. The fluid was free from blood unless the rupture point involved a blood vessel. A gelatinous plug, usually blood-streaked, formed at the rupture point. The size of the follicle at ovulation varies greatly but is most commonly 9-10 mm (Grant, 1934). Follicles may rupture at any point on the ovarian surface except near the attachments.

During ovulation the oocyte, surrounded by the corona radiata, and the liquor folliculi are expelled causing the collapse of the follicle and folding of the follicular wall. After ovulation blood cells, from ruptured vessels in the theca interna, are to be found scattered in the membrana granulosa, although in the sheep very little haemorrhage remains within the

cavity of the follicle (Marshall, 1904). The luteal cells in the sheep develop from the hypertrophy and hyperplasia of the membrana granulosa cells. Ingrowths from the theca interna and externa provide a connective tissue network between the epithelial cells, resulting in breakdown of the membrana propria. The development of a connective tissue network is accompanied by a corresponding proliferation of blood vessels within the developing corpus luteum, originating from the theca. The development and fate of the corpus luteum has been reported by Marshall (1904) and Warbritton (1934) and will not be described further here since these studies were limited to the development of the follicle up to and including ovulation.

Of the follicles present in the ovaries at birth only a very small number will ultimately ovulate. The majority are destined to undergo atresia at some stage of their development. Atretic follicles are to be found in the ovaries throughout life; however atresia occurs more commonly at certain periods than at others (Brambell; 1956). A wave of atresia has been recorded in several species soon after birth. In the adult atresia is also more frequent at certain times such as during pregnancy and lactation, and at the beginning of anoestrus in seasonal breeders.

Atresia can affect all sizes of follices and usually results in degeneration of both the oocyte and the follicle (Brambell, 1956). Marshall (1904) has described the characteristics of atresia in larger follicles in the sheep. The ovum, which is retained in the follicle, degenerates and eventually disappears altogether. The granulosa cells often become scattered

in the liquor folliculi, but they degenerate and eventually disappear. When the granulosa cells are in an advanced state of degeneration there is a loose ingrowth of connective tissue to fill the cavity. Brand and de Jong (1973) distinguished two further forms of atresia in the sheep, obliterative atresia, which was characterised by folding of the follicular wall, and cystic atresia in large follicles, which was characterised by a thinning of the follicular wall. The atresia of mature follicles may be accompanied by the rupture of the blood vessels of the theca and haemorrhage into the antrum, forming a haemorrhagic cyst (Heape, 1905). These may persist for some time but are eventually reabsorbed. However, Marshall (1904) reported only very slight haemorrhage in the atretic follicles of the sheep.

The atresia of follicles is accompanied by the degeneration and regression of granulosa cells. However, in some species, the theca interna and some surrounding stromal elements hypertrophy to form interstitial gland tissue (Guraya, 1973). In addition to this thecal origin of interstitial gland tissue in adult animals, a "primary type" has been described in immature mice which arises from the differentiation of stromal mesenchymal cells (Stegner, 1970).

Although there is no report of the presence of interstitial gland tissue in sheep ovaries specifically, its presence has been demonstrated in the ovaries of many mammalian species (Brambell, 1956; Guraya, 1973). However the degree of development and the distribution varies widely in the ovaries of different species. In some mammals, such as the human, cow and

rhesus monkey, the cells are of a very transient nature, soon reverting back to the stromal tissue from which they were originally derived, resulting in no accumulation of interstitial gland tissue. In other mammals, such as the rabbit, ferret, stoat, mole and bat, they begin to accumulate to form a major proportion of the total ovarian structure. Furthermore interstitial gland tissue has been shown to undergo changes in size and in histological characteristics during the oestrous cycle and during pregnancy (Harrison, 1962).

1.3 OESTROGENS IN THE SHEEP

Oestrogen is a collective term for all substances that produce oestrus, induce growth of the vagina, uterus and mammary glands and control the female secondary sexual characteristics (Allen, Hisaw and Gardner, 1939). By 1900 it had been established that the ovaries produced a substance that was essential for the maintenance of normal reproductive function. It was shown by Knauer (cited by Corner, 1943) that removal of the ovaries terminated sexual function and also produced atrophy of the uterus. This effect could be overcome by the transplantation of ovarian fragments anywhere in the body, implying that the ovary exerts an influence through the medium of an internal secretion. During the early 1900's several investigators (cited by Corner, 1943) found that ovarian extracts would also prevent the atrophy of the uterus that resulted from removal of the ovaries.

An important advance came with the discovery that the condition of the reproductive organs, and also oestrus, could be predicted by vaginal smear changes (Stockard and Papanicolaou, 1917). Using this test, Allen and Doisy (1923) showed that the liquor folliculi from sows' ovaries contained a substance that would stimulate changes typical of oestrus in spayed rats. They went further in purifying the hormone than had previous investigators and also suggested that the hormone was produced by mature follicles within the ovary.

The subsequent discovery of a rich source of oestrogenic activity in the urine of pregnant women (Ascheim and Zondek, 1927)

and pregnant mares (Zondek, 1930) was valuable in providing an abundant supply of material for extraction studies. Shortly after this, several oestrogens were isolated and their structural formulae determined.

The first oestrogenic compound to be isolated in crystalline form was oestrone from human pregnancy urine (Thayer, Veler and Doisy, 1930). Shortly afterwards a different compound was isolated from human pregnancy urine (Marrian, 1930) and human placenta (Browne, 1933) which was later known as oestriol. This was followed by the isolation of oestradiol-17β from pregnant mares' urine (Wintersteiner, Schwenk and Whitman, 1935) and the same hormone was obtained from the follicular fluid of sows' ovaries (MacCorquodale, Thayer and Doisy, 1935). Subsequently oestradiol-17α and 17-dihydroequilenin were isolated from pregnant mares' urine (Hirschmann and Wintersteiner, 1938). Mares' urine has also been shown to contain three other oestrogens, equilenin, equilin and hippulin (Girard, 1933). More recently 16-epioestriol (Marrian and Bauld, 1955), 16α-hydroxyoestrone, 16-oxo-oestradiol-17β, 16β-hydroxyoestrone (Layne and Marrian, 1958) and 18-hydroxyoestrone (Loke, Marrian and Watson, 1959) have been isolated from human pregnancy urine. Since then a number of other oestrogens have been isolated from biological sources (Preedy, 1968). A wide range of synthetic non-steroidal compounds have been produced which appear to have little chemical similarity to the natural oestrogens but have effects in the animal similar to those produced by natural oestrogens (Dodds, 1955).

In the female the main sites of production of oestrogens

are the ovary, the adrenal glands and the placenta. In the sheep, oestrone, but no oestradiol-17β, were detected in samples of adrenal venous blood collected from an animal with a transplanted adrenal gland (Baird, 1968). During pregnancy, an increase in the level of total unconjugated oestrogens has been recorded, with the greatest increase in concentration occurring during the last 48 hours of gestation (Challis, Harrison and Heap, 1971). Higher levels were detected in ovarian venous blood than in jugular blood. Oestrone was the oestrogen present in the greatest concentration at parturition with smaller amounts of oestradiol-17β and oestradiol-17α (Robertson and Smeaton, 1973).

However, in the non-pregnant female the primary source of oestrogens is the ovary. In the sheep oestradiol-17β has been shown to be the predominant oestrogen secreted by the ovary (Short, McDonald and Rowson, 1963; Lindner, Sass and Morris, 1964; Moore, Barrett, Brown, Schindler, Smith and Smyth, 1969). By perfusing the autotransplanted ovary in the ewe with radioactively labelled testosterone and androstenedione (Rado, McCracken and Baird, 1970), it was confirmed that oestradiol-17β was the principal oestrogen secreted by the ovary, with smaller amounts of oestrone and no oestradiol-17α, oestriol or conjugated oestrogens. From studies using autotransplanted ovaries it was estimated that the secretion rate of oestradiol-17β was about five times that of oestrone (Baird, Goding, Ichikawa and McCracken, 1968).

The urinary and faecal metabolites of oestrogen in the non-pregnant ewe are chiefly in the form of oestradiol-17α (Wright, 1962; Velle, 1963a). It is likely that oestradiol-17α is

formed by conversion within the body from other oestrogens. Liver tissue slices from sheep can convert both oestradiol-17β and oestrone into oestradiol-17α (Lyngset and Velle, 1968). Washed erythrocytes from ovine blood have been shown to convert oestradiol-17β to oestrone and oestrone to oestradiol-17β, although oestradiol-17α was never isolated (Lunaas and Velle, 1960). The metabolic pathway, oestradiol-17β $\rightleftharpoons$ oestrone $\longrightarrow$ oestradiol-17α has been suggested for the sheep (Velle, 1963a).

The actual site of production of oestrogens within the ovary has been the subject of many investigations. On the grounds that oestrogenic changes, such as cornification of the vagina, were associated with the preovulatory growth of the follicle, Allen and Doisy (1923) regarded this structure as the main site of oestrogen production. Since then oestrogens have been isolated from the follicular fluid of many species (MacCorquodale et al., 1935; Short, 1962a; Short, 1962b). Thayer and Doisy (1928) found that, although the concentration of the hormone was greatest in the liquor folliculi of cow and pig ovaries, there was also activity in the residual ovarian tissue. From a review of the early literature, Corner (1938) concluded that, although oestrogenic activity had been attributed to the corpus luteum and interstitial gland tissue of the ovary, the usual site of oestrogen production was the theca interna of follicles of all sizes. There have been many histochemical observations implicating the cells of the theca interna as a source of production of oestrogens (Parkes and Deanesly, 1966).

However Falck (1959) found that it was necessary to

transplant both rat granulosa cells and thecal cells into the anterior chamber of the eye of spayed rats to obtain normal oestrogen secretion; granulosa cells transplanted alone secreted only progesterone. He therefore proposed a functional relationship between the theca interna and granulosa cells in the production of oestrogens. Contrary to this theory a "two-cell type" hypothesis of steroid synthesis in the ovary has been developed by Short (1962b) from work in the mare. He postulated that the theca interna cells have all the enzyme systems necessary for the synthesis of oestradiol-17β from cholesterol, whereas the granulosa cells were forced to produce mainly progesterone due to the relative inactivity of their 17α-hydroxylase and desmolase systems. Furthermore, by injecting radioactive precursors into mare follicles *in vivo*, Younglai and Short (1970) were able to show that progesterone produced by the granulosa cells was unlikely to be an important substrate for theca cells.

To return to the sheep, Moor (1973) has explanted follicles over 2 mm in diameter from the ovaries of cycling animals and shown that only those follicles greater than 4.5 mm in diameter were highly active secretors of oestrogens in culture. The pattern of secretion by the large follicles, during a culture period of 7 days, depended on the stage of the oestrous cycle at which they were removed (Seamark *et al.*, 1974). Follicles explanted on day 14 secreted high levels of oestrogens. The production of oestrogens was reduced in follicles explanted on day 15 and this was accompanied by an increase in the production of androgens and 17α-hydroxylated progestagens. Seamark *et al.*

(1974) concluded that the cells of the theca interna were probably responsible for the synthesis of these steroids. Hay and Moor (1975a) have studied the distribution of Δ^5-3α-hydroxysteroid dehydrogenase (3β-HSD) in ovarian follicles as a general indication of steroid biosynthesis. During the 72 hours preceding ovulation 3β-HSD activity was demonstrated histochemically in the theca interna of large follicles, where the intensity of the reaction increased as ovulation approached. 3β-HSD activity was not observed in the cells of the membrana granulosa prior to ovulation, but within a few hours of ovulation 3β-HSD activity was present in the luteinised granulosa cells. Steroid production by follicles explanted at oestrus was characterised by almost no oestrogens or androgens after the first day in culture, whereas large amounts of progesterone and 20α-hydroxy-pregn-4-en-3-one were secreted (Seamark et al., 1974). Cultures of granulosa cells have produced only progesterone, 20α-hydroxy-pregn-4-en-3-one and pregnenolone, with no detectable amounts of oestrogens, androgens or 17α-hydroxylated progestagens. These findings in the sheep were taken to be compatible with the two-cell theory of Short (1962b) and indicate a transfer of steroid synthetic capacity from the theca interna to the membrana granulosa at ovulation.

Circulating steroid levels in the sheep have been related to events in the ovaries. The concentration of oestrogens in ovarian venous plasma reaches a maximum during prooestrus (Scaramuzzi, Caldwell and Moor, 1970; Bjersing et al., 1972). The highest levels of oestrogens were obtained from the ovary containing the largest non-atretic follicle and coincided with the

maximal development of the theca interna of this follicle (Bjersing et al., 1972). A decline in oestrogen secretion occurred before the first observable degenerative changes in the thecal cells and the level of oestrogens was low by the time ovulation occurred.

The production of androgens by preovulatory follicles in vitro is supported by the work of Baird et al. (1968) who isolated testosterone and androstenedione from the venous blood of autotransplanted ovaries in the ewe. The secretion rate of androstenedione in particular followed the pattern of secretion of oestradiol-17β very closely. There is no conclusive evidence in the sheep for the secretion of progesterone before ovulation. Bjersing et al. (1972) failed to detect a rise in the secretion of progesterone around the time of oestrus. Wheeler, Baird, Land and Scaramuzzi (1975) detected a small transient increase in the secretion rate of progesterone coincident with the preovulatory LH peak. However they did not determine whether the preovulatory follicle was the source of this progesterone secretion.

There is evidence to suggest that steroid hormones are secreted also by the interstitial gland tissue of the ovary in many species (Guraya, 1973). Oestrogens (Falck, 1959) and androgens (Falck, Menander and Nordanstedt, 1962) may be secreted by the interstitial tissue of the rat ovary. Human ovarian stroma has been shown to synthesise a number of steroid products, although principally androgenic steroids (Rice and Savard, 1966). However, in the sheep, Roche, Karsch, Foster and Dziuk (1974) found that the destruction of follicles by X-irradiation, leaving only stromal

tissue in the ovaries, resulted in serum LH levels that were indistinguishable from the elevated levels of LH in ovariectomised ewes. They suggested that ovarian stroma in the sheep does not secrete sufficient steroids to inhibit the release of LH. Confirming these findings, Moor, Hay and Seamark (1975) found that sheep ovarian stroma maintained in culture secreted only small amounts of steroids.

1.4 CONTROL OF LITTER SIZE IN SHEEP

Sheep are intermediate, with respect to litter size, between strictly litter-bearing species, such as the pig, and species, such as the horse and cow, which normally have a single off-spring. It is usual for sheep to have one or two young at a time but they may give birth to as many as six or seven in a litter. Litter-size has several factors contributing to its variation, the number of eggs shed by the ovary at ovulation, the number of monovular twins, the number of eggs fertilised and the proportion of embryos which develop to term. Monovular twins occur only rarely in sheep (Morley, 1948) and from a review of the literature Edey (1969) concluded that prenatal mortality was usually 20-30% in sheep. Several authors have compared numbers of corpora lutea observed at laparotomy (Packham and Triffitt, 1966) or at slaughter (Marshall, 1904) with the normal lambing performance for the breed involved and concluded that the fecundity of sheep is mainly influenced by the number of eggs shed from the ovaries at ovulation.

In the individual ewe ovulation rate is sensitive to several environmental sources of variation. The number of lambs in a litter increases with age until around 5-6 years of age with a gradual decline thereafter (Reeve and Robertson, 1953). Body-weight and the level of nutrition before and at the time of mating can influence ovulation rate (Coop, 1966). Ovulation rate has also been shown to decline as the breeding season progresses (Land, Dickinson and Read, 1969). In addition to these environ-

mental effects there is good evidence for genetic variation in litter size. Several authors have reviewed the reported differences in mean litter size among various breeds and strains of sheep (Reeve and Robertson, 1953; Bradford, 1972). Bradford (1972) concluded that under uniform environmental conditions there were at least two-fold differences in litter size among different breeds.

In view of the genetic variation in litter size and therefore ovulation rate among different breeds of sheep it would be of value to understand the factors controlling ovulation rate. There have been many investigations into whether the source of control lies within the ovary itself or in the level of gonadotrophic stimulation. Both factors have been shown to contribute to overall variation in the number of eggs shed, but their relative importance has differed among the breeds.

Several workers have observed differences in plasma and pituitary gonadotrophin concentrations among breeds and strains of sheep of differing fecundity. Land, Crighton and Lamming (1972a) estimated the pituitary Luteinising hormone (LH) content during the oestrous cycle in Finnish x Blackface, Merino x Blackface and purebred Blackface ewes. Pituitary LH content was higher in the Merino x Blackfaces at mid-cycle and it was postulated that more LH may be released at this time by the more fertile Finnish-cross ewes, producing a higher level of gonadotrophic stimulation of the ovaries. Others have investigated gonadotrophin concentrations in lambs of various breeds and strains as a possible early indicator of fecundity. Thimonier, Pelletier and Land (1972) observed

significant differences in plasma LH concentrations and in the oestrogen induced LH discharge among lambs of various breeds and concluded that some aspects of the release of LH in lambs are related to the ovulation rate of their breed type. Both plasma and pituitary gonadotrophin levels have been measured by Trounson, Chamley, Kennedy and Tassell (1974) in 5 month old lambs from groups of Merinos selected for and against multiple births. The lambs from the more fecund strain had higher plasma LH levels and there was a similar trend, although not significant, for pituitary LH and Follicle-stimulating hormone (FSH) levels suggesting that both synthesis and release of gonadotrophins had been affected by selection. Bindon (1973) also observed a significantly higher plasma LH level at 30 days of age in a more fecund strain of Merinos. Bindon and Turner (1974) have shown a transient episodic LH release pattern occurring earlier in life in both male and female lambs belonging to a more fecund strain of Merinos, which they suggested may have contributed to the greater ovulation rate displayed by these lambs after puberty.

There is also evidence that the degree of ovarian sensitivity to endogenous gonadotrophins can account for differences in ovulation rate. A number of studies using laboratory animals (McLaren, 1962; Land and Falconer, 1969; Wolfe, 1971) have attributed genetic differences in ovulation rate to changes in ovarian sensitivity but all their experiments involved intact mice in which endogenous gonadotrophins may have influenced the result. However, Bindon and Pennycuik (1974), using hypophysectomised and intact mice, found a significant increase in the response to

administered gonadotrophins in mice selected for high ovulation rate, therefore concluding that differences in ovarian sensitivity do represent part of the response to selection for ovulation rate.

Several workers have found similar differences in response to exogenous gonadotrophins among various breeds and strains of sheep, although they have all used animals with intact pituitaries. Using groups of Merino ewes selected for and against multiple births, Bindon, Ch'ang and Turner (1971) and Trounson and Moore (1972) obtained a significantly greater ovulation rate in response to various doses of pregnant mare's serum gonadotrophin (PMSG) in the more fecund strain. Bradford, Quirke and Hart (1971) induced superovulation in several breeds of sheep and found that the Finnish Landrace ewes, with the highest natural fecundity, yielded more than double the number of fertilised eggs per ewe than the next best breed. Of course since all these experiments used intact ewes the results could either have been due to differences in ovarian sensitivity or to additive effects between exogenous gonadotrophin and endogenous pituitary activity.

There is therefore considerable evidence to suggest either that ovulation rate in sheep is controlled by the level of circulating gonadotrophins, or alternatively that ovarian sensitivity is the variable factor. The problem will remain unsolved until the ovarian sensitivity can be compared in breeds of differing fecundity in the complete absence of endogenous gonadotrophins.

1.5 OUTLINE OF THE STUDY

Small flocks of pure-bred Finnish Landrace and Scottish Blackface sheep were established to provide experimental animals for use throughout the present studies. Representative examples of mature ewes belonging to the two breeds are shown in Plates 1.5 and 1.6 together with their respective pure-bred off-spring.

The general intention of these studies was to provide further information on the physiological factors controlling ovulation rate. Various aspects of follicular development and function have been compared in Finnish Landrace and Scottish Blackface sheep, both during the neonatal period and in the mature ewe. Peripheral plasma levels of oestrogens were investigated as a possible means of assessing ovarian activity in the intact animal. In order to measure the extremely low levels of oestrogens present in the peripheral circulation of sheep it was necessary to develop a sensitive and specific assay method for the estimation of plasma oestrogens.

In mature Finnish Landrace and Scottish Blackface ewes, plasma levels of oestrogens were estimated daily around the time of oestrus. Neonatal lambs have been used to compare ovarian sensitivity in response to the administration of exogenous gonadotrophins in the two breeds. As noted previously, the investigation of ovarian sensitivity in the intact animal is liable to be complicated by the endogenous secretion of gonadotrophins. For this reason the use of the pituitary blocking agent, methallibure, has been investigated as a possible means of completely suppressing endogenous gonadotrophin secretion in the neonatal lamb.

CHAPTER TWO

A RADIOIMMUNOASSAY FOR OESTROGENS

2.1 INTRODUCTION

Oestrogens can exist in blood in the unconjugated state or in conjugation, principally with sulphuric acid or glucuronic acid (Preedy, 1968). The formation of oestrogen conjugates by the liver allows the hormone to be excreted by the kidney. Both conjugated and unconjugated oestrogens can also be bound to plasma proteins (Sandberg, Slaunwhite and Antoniades, 1957). According to O'Donnell and Preedy (1961) the significance of the protein-binding of oestrogens is not clear. The protein-bound hormone may be in equilibrium with the unbound hormone. In that case protein-binding could constitute a store from which active un-bound oestrogens could be readily mobilised. Ideally the complete determination of plasma oestrogens would require the separation and estimation of each individual oestrogen in all the various states in which it may exist in the blood. In practice, assay methods usually measure either the total amounts of each oestrogen or the amounts of unconjugated oestrogens in the blood.

An early crude indication of the presence of oestrogens in the blood resulted from the modification of bioassay techniques that had been developed for urinary oestrogens (Krichesky and Glass, 1947). The method involved the introduction of pellets of dried blood, with no preliminary purification, into the vaginas of ovariectomised rats. The parameter measured was the appearance of cornified cells in the vaginal smear. Using this technique, Krichesky and Glass (1947) were able to conclude that the blood levels of oestrogens were higher in intact animals

(rabbits and humans) than in castrates.

The first reliable estimations of blood levels of oestrogens resulted from the development of chemical assay methods. In order to measure amounts of total oestrogens in the blood, complex preliminary procedures were included to denature plasma proteins, thereby releasing the protein-bound oestrogens, and to hydrolise the oestrogen conjugates, before the samples were extracted (O'Donnell and Preedy, 1961). A variety of chromatographic procedures were then employed to resolve the extracted oestrogens into the individual hormones which were then estimated. In 1958 Diczfalusy and Magnusson published a modification of the method of Brown (1955), for the estimation of urinary oestrogens, which made use of the Kober (1931) colour reaction. According to O'Donnell and Preedy (1961) although the use of large volumes of blood compensated for the insensitivity of the Kober colorimetric reaction, interfering substances in the blood made the method relatively non-specific, despite the Allen (1950) correction for non-specific colours. The method of Preedy and Aitken (1961) involved the determination of total blood oestrogens by sulphuric acid fluorescence. Although this method was more specific than that of Diczfalusy and Magnusson (1958) it had the disadvantage of being laborious to perform (O'Donnell and Preedy, 1961). The above methods were satisfactory for the determination of the increased levels of oestrogens in women during late pregnancy but were not sufficiently sensitive for the detection of plasma oestrogens during the menstrual cycle.

Roy (1962) further modified the estimation method of Brown

(1955) by the incorporation of the fluorescent reaction developed by Ittrich (1958). The resulting improved sensitivity allowed the levels of oestrone to be determined in samples of whole blood during the menstrual cycle. Svendson (1960) described a method that involved the extraction of oestrogens from plasma using chloroform and their estimation by an isotope derivative procedure. This was sufficiently sensitive to determine the levels of unconjugated oestrone and oestradiol-17β in plasma from menstruating women. A similar sensitivity was obtained in the gas chromatography and electron-capture detection method developed by Wotiz, Charransol and Smith (1967). These workers reported levels of unconjugated and total plasma oestrogens in women. Although these more sensitive chemical assays allowed some estimation of the levels of oestrogens during the oestrous cycle, they still suffered from the disadvantage that relatively large volumes of plasma were necessary which precluded their use in studies where frequent samples from the same subject were required. Furthermore none of the chemical estimation methods was sufficiently specific without elaborate extraction and purification procedures which often made the methods very time-consuming.

Further progress in the development of sensitive and specific assays for the estimation of blood oestrogens came with the isolation of selective oestrogen-binding proteins. The basic procedure in these assays involved the use of a specific protein to ascertain the competition between the compound to be measured and a similar or chemically-related radioactive compound for a limited number of binding sites on the protein. The free and bound

fractions of the labelled compounds were then separated and the partition of the labelled compound between the free and bound fractions evaluated. Since this was a function of the mass of the unlabelled compound present, the mass of the compound present in unknown samples could be calculated by interpolation on a standard curve. Three types of protein were employed, a β-globulin present in plasma (Murphy, 1968), a receptor macromolecule obtained from the uterus (Korenman, 1968) and specific antisera (Abraham, 1969).

The general field of binding of steroids to plasma proteins was originally investigated in the 1950's (Sandberg et al., (1957) and the resulting information utilised to develop estimation procedures for several groups of steroids. However the lack of availability of a suitable, specific, naturally occurring oestrogen-binding protein in plasma meant that the method could not be used to estimate oestrogens until Murphy (1968) reported a sex-hormone binding globulin (S.H.B.G.) in human plasma during late pregnancy with a high affinity for oestradiol and testosterone. A competitive protein-binding assay utilising this material was subsequently developed for oestrogens (Dufau, Dulmanis, Catt and Hudson, 1970). S.H.B.G. lacked specificity due to cross-reaction with both oestrogens and testosterone. This necessitated the preliminary chromatographic purification of plasma extracts to eliminate non-oestrogenic steroids (Dufau et al., 1970). The method was used to determine plasma oestradiol-17β levels throughout the menstrual cycle, oestrone and oestriol showing negligible cross-reaction. The advantages of the method included

the availability and easy preparation of the binding protein, the stability of the material when frozen and the fast reaction time (Murphy, 1970).

The existence of a material in the uterus which could bind oestrogens was first suggested by the retention of tritiated oestradiol-17β in rat uteri long after the plasma was depleted of radioactivity (Jensen and Jacobson, 1960). This information led to the isolation of uterine cytosol from the uteri of pregnant rabbits (Korenman, 1968) and its use in a specific and sensitive assay for oestrogens (Korenman, Tulchinsky and Eaton, 1970; Corker, Exley and Naftolin, 1970). Unlike the S.H.B.G. the uterine cytosol showed no cross-reaction with non-oestrogenic steroids present in plasma, but significant binding of all oestrogens made it necessary to carry out a separation procedure. The method involved a simple extraction procedure, using ether, followed by the chromatographic separation of the oestrogens on celite columns prior to assay (Korenman et al., 1970; Corker et al., 1970). This system gave a measure of hormonal activity rather than concentration since the activity of the uterine cytosol in binding oestrogens was in proportion to their biological activity (Korenman et al., 1970). Shutt (1969) successfully applied this assay method to the estimation of unconjugated oestradiol-17β in the peripheral plasma of women or ovarian vein plasma in sheep. The chief problems associated with the use of tissue proteins were concerned with their separation from other non-specific low-affinity tissue proteins and their lack of stability (Murphy, 1970). The original rabbit preparation was stable for only two

weeks when stored in solid CO_2 (Korenman, 1968); however by freeze-drying the material (Corker et al., 1970) or using ovine uteri (Shutt, 1969) stability was maintained for several months.

Low molecular weight organic molecules, such as steroids, are not inherently immunogenic. However, as Landsteiner (1921) demonstrated, if these low molecular weight haptens were covalently coupled to a protein molecule the resulting conjugate was immunogenic, allowing antibodies specific for the hapten to be produced. Antibodies against steroids have been produced by the active immunisation of sheep and rabbits with steroids covalently coupled to albumin (Lieberman, Erlanger, Beiser and Agate, 1959; Thorneycroft, Tillson, Abraham, Scaramuzzi and Caldwell, 1970; Goodfriend and Sehon, 1970). Abraham (1969) first reported a radioimmunoassay for plasma oestrogens using an antiserum raised in sheep against oestradiol-17β-hemisuccinate coupled to bovine serum albumin (Ferin, Zimmering, Lieberman and Vande Wiele, 1968). Although it has been possible to raise antisera showing no detectable cross-reaction with non-oestrogenic hormones, antisera raised against the C-3 and C-17 conjugates of oestradiol (Abraham, 1969; Niswender and Midgley, 1970; Thorneycroft et al., 1970) have all cross-reacted significantly with the other oestrogens. More recently attempts have been made to prepare steroid derivatives in such a way that the important functional groups are left free to act as antigens. Using the C-6 position as the site of attachment to the carrier protein, Exley, Johnson and Dean (1971) have raised antisera against oestradiol-17β and oestriol which had low cross-reactivities with other oestrogens, with the exception of

6-keto-17β-oestradiol.

A highly specific antiserum is desirable since it precludes the necessity for preliminary purification of the individual oestrogens in extracts of biological samples. The required amount of purification also depends on the relative concentration of the interfering steroids and the non-specific interference by plasma proteins and lipids. If a steroid exists in a biological fluid in a sufficiently high concentration, as for example oestradiol-17β in follicular fluid (Abraham, Odell, Edwards and Purdy, 1970), it can be measured directly, interference by plasma lipids and proteins being minimised by dilution. However Abraham et al. (1970) have suggested that conjugated oestrogens present in the plasma may cross-react with the antiserum. More commonly, radioimmunoassay methods for oestrogens have included ether extraction (Abraham, 1969; Wu and Lundy, 1971; Abraham et al., 1970). Diczfalusy and Lindkvist (1956) have shown that conjugated oestrogens are not extracted from plasma by ether and therefore only unconjugated plasma oestrogens are estimated by this method. Separation of the individual oestrogens in the ether extract has been achieved by a variety of chromatography procedures, such as celite microcolumns (Abraham and Odell, 1970); Sephadex LH-20 columns (Mikhail, Chung, Ferin and Vande Wiele, 1970) and silica gel chromatography (Hotchkiss, Atkinson and Knobil, 1971).

Two kinds of radioactively labelled steroids have been used in radioimmunoassays, gamma emitting isotopes and beta emitters. Commercial preparations of steroids are available labelled with ^{3}H or ^{14}C and therefore, although these beta emitters have specific

activities considerably lower than the gamma emitters, they have been used by most investigators. Niswender and Midgley (1970), investigating the use of ^{131}I and ^{125}I labelled steroids, claimed greater sensitivity than with ^{3}H labelling and indicated the advantage of using tritiated steroids to determine recoveries of individual samples while using radio-iodinated steroids in the assay.

A variety of methods has been employed to separate the free from the antibody-bound steroid during the radioimmunoassay of oestrogens. Midgley, Niswender and Ram (1969a) described a double antibody method for the precipitation of the protein-bound complex which had the disadvantage of requiring an incubation period of several days. Antibody polymerisation has been reported (Mikhail et al., 1970) but this added a high-speed centrifugation step. The use of a solid-phase system (Abraham, 1969; Abraham and Odell, 1970) accomplished incubation and the separation of free from antibody-bound steroid in a single step, but required routine and reproducible coupling of antibody to a solid support. Dextran-coated charcoal (Abraham, Hopper, Tulchinsky, Swerdloff and Odell, 1971; Wu and Lundy, 1971) has been used widely to precipitate the free steroid, since it is simple to use and gives a high precision to the assay (Abraham, 1974). Polyethylene glycol precipitation of the antibody-bound steroid has been claimed by Schiller and Brammall (1974) to be as simple and precise as dextran-coated charcoal, with the additional advantage that no dissociation of the antigen-antibody complex occurs.

The use of antibodies as binding proteins for steroids

has provided further potential for increasing the specificity and sensitivity of oestrogen estimations. Radioimmunoassays have been used to measure the levels of unconjugated oestrogens in the peripheral circulation throughout the menstrual cycle in women (Mikhail et al., 1970) and during the oestrous cycle in cattle (Henricks, Dickey and Hill, 1971) and sheep (Pant, Hopkinson and Fitzpatrick, 1972). Unfortunately most antisera produced so far show some cross-reaction with the different oestrogens, making it necessary to carry out a preliminary chromatography procedure in order to estimate the oestrogens individually. This will only become unnecessary with the production of completely specific antisera. A disadvantage of radioimmunoassays is that antiserum production is laborious taking several months, and precisely the same antiserum composition will never be obtained twice. However, if a suitable batch of high titre is obtained it may last for some time due to the stability of the material (Thorneycroft et al., 1970).

During the present studies a sensitive assay method was required for the estimation of oestrogens in the peripheral circulation of sheep. Radioimmunoassay methods have already proved sufficiently sensitive for this purpose. The method reported by Hotchkiss et al. (1971) has been adapted for use in these studies.

2.2 MATERIALS

Phosphate buffered saline (P.B.S.)

The assay buffer was prepared from the following reagents, all of which were Analar Grade.

Sodium chloride	B.D.H. Chemicals Ltd., Poole, England.
Sodium dihydrogen orthophosphate	"
Disodium hydrogen orthophosphate	"
Thimerosol (Ethylmercurithiosalicylate)	Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, England.

40 g of sodium chloride and 0.5 g of Thimerosol were dissolved in glass distilled water. 34 ml of 0.5 M sodium dihydrogen orthophosphate and 68 ml of 0.5 M disodium hydrogen orthophosphate were added and the volume made up to 5 litres using glass distilled water. The pH was adjusted to 7.0 if this was found to be necessary and the buffer stored at 4°C.

P.B.S. - 0.1% gelatin

Gelatin	B.D.H. Chemicals Ltd.

A 0.1% solution of gelatin was prepared by dissolving 1 g gelatin in P.B.S., heating slightly to dissolve the powder and making up to 1 litre. The solution was stored at 4°C.

Diethyl ether

Pronalys grade	May and Baker, Ltd., Poole, England.

Antibody

Sheep antiserum (No. 029-18) was raised against oestradiol-17β-hemisuccinyl-bovine serum albumen by Dr. B. V. Caldwell, (Department of Obstetrics and Gynaecology, Yale University, New Haven, Connecticut 06510). It was supplied in 0.5 ml aliquots diluted 1:100 in P.B.S. - 0.1% gelatin and was stored at -15°C. The working solution used in the assay was the dilution of antiserum that bound 50% of oestradiol-17β-^{3}H (see Section 2.4.6). This was freshly prepared each week by further dilution with P.B.S. - 0.1% gelatin and stored at 4°C.

Oestradiol-17β, cold standard

B.D.H. Chemicals Ltd.

Serial dilutions of oestradiol-17β in ethyl alcohol (Burrough's) were prepared and stored at -15°C. The standard solutions contained 50, 100, 200, 500, 1000 and 2000 pg/ml of oestradiol-17β in order that a uniform volume, 0.1 ml, of each could be delivered for each individual point on the standard curve.

Oestradiol-17β, radioactive

2,4,6,7 (n) ^{3}H oestradiol	The Radiochemical Centre,
250 μCi in 250 μl of	Amersham,
Benzene:Ethanol 95:5	Buckinghamshire,
Specific activity 85 Ci/mmol	England.

The stock solution was prepared by making the contents of the vial up to 25 ml with ethyl alcohol. This gave a solution with an activity of 10 μCi/ml and a concentration of 32 ng/ml. It was stored at -15°C. To obtain a working solution 0.2 ml of the stock solution was evaporated to dryness and redissolved in 10 ml

of P.B.S. - 0.1% gelatin giving a final concentration of 64 pg/ 0.1 ml and 10,000 cpm/0.1 ml. This was freshly prepared for each assay.

Dextran-coated charcoal suspension

Activated charcoal	Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, KT2 7BH.
Dextran T70	Pharmacia Fine Chemicals AB Uppsala, Sweden.

The charcoal was washed three times using methyl alcohol and dried in a rotary evaporator. 2.5 g of washed charcoal and 0.25 g of Dextran T70 were suspended in 1 litre of P.B.S. A fresh suspension was prepared monthly and stored at 4^{o}C.

Liquid scintillation counting materials

Liquid scintillator ELS93	Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

The scintillator contained Toluene:Triton X in the proportion 2:1 and 0.4 g PPO and 0.2 g POPOP per litre.

Disposable plastic scintillation vials	Koch-Light Laboratories Ltd.

Nuclear Chicago Mark I Scintillation Counter

The scintillation counter had a counting efficiency of 49% for Tritium.

Glassware

Disposable 100 mm x 15.5 mm soda glass test tubes for use in the radioimmunoassay were soaked in Decon 90 (Decon Laboratories Ltd., Ellen Street, Portslade, Brighton) and washed well in tap water followed by glass distilled water before being dried in an oven prior to use. Disposable Pasteur pipettes and Eppendorf tips were not washed before use. All other glassware, including 15 ml glass-stoppered extraction tubes, was non-disposable and therefore soaked in Decon 90 for at least 12 hours before being washed and dried as above.

2.3 METHODS

2.3.1 Collection and treatment of blood samples

Blank plasma was obtained from an ovariectomised ewe which had received 20 mg dexamethasone acetate (Dexamethasone, Berk Pharmaceuticals Ltd., Godalming, Surrey) daily for the previous 3 days in an attempt to suppress adrenal function. The blood was collected into 500 ml heparinised glass bottles through a large bore needle and centrifuged at 4°C at 1,500 g for 30 mins. The plasma was separated and stored in small volumes at -15°C until required.

Blood was also collected from parturient ewes, at which time the level of plasma oestrogens have been shown to be elevated (Challis et al., 1971; Robertson and Smeaton, 1973). Plasma was removed and stored as for blank plasma. By including aliquots of this control plasma in consecutive assays the reproducibility of the assay could be assessed.

A minimum of 10 ml of blood was collected from the jugular vein of experimental animals into heparinised evacuated glass tubes (Vacutainers, Becton and Dickinson, New Jersey, U.S.A.). The samples were maintained at 4°C for not more than 2 hours before being centrifuged at 4°C at 3,200 g for 15 mins. The plasma was separated and the samples stored in small glass vials at -15°C until they were assayed for oestrogens, usually within a few months of being collected.

2.3.2 Extraction of plasma samples

A maximum of 30 samples was included in each assay.

Volumes of 0.1 to 4.0 ml of plasma were used depending on the expected level of oestrogens in the sample. Within each assay the same volume of plasma was used throughout. Also included in duplicate in each assay were samples of blank plasma, samples of control plasma and aliquots of glass distilled water to provide water blank values. 0.1 ml aliquots of three of the cold oestradiol standards were pipetted in duplicate into extraction tubes (i.e. 20, 50 and 100 pg/tube) and evaporated to dryness. The appropriate volume of blank plasma was added to these tubes and they were allowed to stand at room temperature for 30 mins. before being extracted along with the other plasma samples. These extracted standards provided an estimation of procedural losses.

Ether extraction of the plasma samples was carried out as shown in Fig. 2.1. 2.5 ml of diethyl ether was used for plasma volumes of 1.0 ml or less and 5.0 ml of ether for plasma volumes greater than 1.0 ml. The tubes were mixed for 10 mins. on a mechanical rotary mixer at approximately 30 revolutions per minute. Following centrifugation at 510 g for 10 mins. at 4°C, the lower phase (plasma) was frozen either by standing the tubes in a mixture of solid CO_2 and ethyl alcohol, or by placing them at -15°C. The ether extract was decanted into glass assay tubes and evaporated to dryness at 40°C under a stream of air.

2.3.3 Assay procedure

0.1 ml aliquots of the oestradiol standards were pipetted into duplicate assay tubes, to provide a standard curve (5 to 200 pg), and evaporated to dryness at 40°C under a stream of air. Six

empty tubes were included to provide duplicate values for the total counts used in the assay (T.C. tubes) and quadruplicate values for the amount of binding obtained with the dilution of antibody used (B.C. tubes).

The method used to assay these standards and the extracted plasma samples is shown in Fig. 2.2. 0.5 ml of P.B.S. - 0.1% gelatin was added to each of the standard and sample tubes and they were agitated on a vortex mixer to wash any residue from the walls of the tubes and redissolve the oestrogens. With the exception of the T.C. tubes, 0.1 ml of diluted antiserum was added to each of the tubes. 0.1 ml of the working solution of ^{3}H-oestradiol-17β was then added to all the tubes and, after further thorough mixing on a vortex, incubation was carried out overnight at 4°C.

Separation of the free from antibody-bound oestrogen was carried out entirely at 4°C. 0.5 ml of P.B.S. - 0.1% gelatin was added to the T.C. tubes and 0.5 ml of the dextran-coated charcoal, maintained in suspension by a magnetic stirrer, was delivered into the remainder of the tubes using an Eppendorf pipette. The tubes were agitated on the vortex mixer and allowed to stand for 15 mins. before the charcoal was precipitated by centrifugation at 3,200 g for 10 mins. The supernatant fluid was poured into liquid scintillation vials containing 10 ml of scintillation fluid and mixed thoroughly to give a clear fluid. The radioactivity in each vial was then determined in a liquid scintillation counter.

2.3.4. Calculation of results

The results obtained from this radioimmunoassay were evaluated using a computer programme (Cook, 1972). A standard curve was derived from the results for the oestradiol standards. A cubic equation was fitted with the logarithm of the amount of hormone as the abcissa and the percentage values of protein-bound radioactivity as ordinate. The amount of radioactivity bound to antibody in the B.C. tubes (i.e. in the absence of any added cold oestradiol-17β) was defined as 100% binding. The concentration of oestradiol-17β in the aliquots of unknown samples was then calculated by interpolation on the standard curve.

Further corrections were made to the results by the use of a desk computer. If the mean water blank value was above the sensitivity of the standard curve, it was subtracted from the values obtained for the unknown plasma samples and the samples of control plasma. Using the mean recovery value of extracted standards obtained within each individual assay (Section 2.4.3) the results for unknown samples and control samples were corrected for procedural losses. The results were finally expressed as picograms of total unconjugated oestrogens per ml of plasma.

2.4. RESULTS

2.4.1 Standard curve

The data from six consecutive standard curves are shown in Table 2.1 and a composite standard curve has been plotted from these data (Fig. 2.3). Extra standards were added to extend the standard curve beyond the range of 5-200 pg used during routine radioimmunoassays.

When the results were evaluated on the basis of percentage bound the coefficient of variation was low over the whole standard curve, never rising above 10%. When considered on the basis of mass, the coefficient of variation in the range of 10 to 400 picograms varied from 6.9 to 11.2%. However at the 5 pg level the variation was 20.2% indicating that the curve was not very precise at this level.

2.4.2 Blanks

Values for plasma and water blanks are shown in Table 2.2. The water blank values varied little, being around 7 pg for aliquots of 0.5 or 4.0 ml of glass distilled water. The mean plasma blank values were 15.4 pg for a 4.0 ml sample and 10.9 pg for a 0.5 ml sample.

2.4.3 Accuracy

Within each assay known amounts of standard oestradiol-17β (20, 50 and 100 pg) were added to the appropriate amount of blank plasma and thereafter regarded as unknown samples. The apparent

amounts recovered when these standards were extracted from 0.5 or 4.0 ml of plasma are shown in Table 2.3. Linear relationships were obtained between the amount of oestradiol-17β added and that measured as seen in Fig. 2.4, with correlation coefficients of 0.96 and 0.95 for 0.5 and 4.0 ml of plasma respectively. The coefficient of variation was 12-17% for extraction from 0.5 ml plasma and 12-13% for extraction from 4.0 ml plasma.

To calculate per cent recoveries for individual extracted standards the following formula was used -

$$\% \text{ recovery} = \frac{E_2 - 17\beta \text{ measured} - \text{blank plasma value}}{E_2 - 17\beta \text{ added to blank plasma}} \times 100$$

The blank plasma value was the mean value obtained within an assay. The per cent recoveries for 20, 50 and 100 pg of oestradiol-17β from 0.5 and 4.0 ml of plasma are shown in Table 2.3. To obtain an overall recovery value for an assay the mean of the individual recoveries was calculated. The mean overall recoveries for 0.5 and 4.0 ml assays were 87.4% and 81.1% respectively.

2.4.4 Precision

To evaluate the inter-assay variation, plasma samples were measured in duplicate in consecutive assays. Intra-assay variation was assessed by obtaining several measurements of the same samples of plasma within the one assay. Results for the inter-assay variation are shown in Table 2.4 for two different plasma samples. The coefficient of variation was around 16% between assays. Results for the intra-assay variation are shown in Table 2.5. The coefficient of variation within an assay was 6-8%.

2.4.5 Sensitivity

From the results for the standard curve shown in Table 2.1 it was determined that the mean values for the percentage radioactivity bound at the 2.5 and 5.0 pg level, were significantly different ($P < 0.001$). This implies that the sensitivity of the standard curve was below 5.0 pg. However the coefficient of variation at the 5.0 pg level was 20.2% and therefore the practical limit of sensitivity for the standard curve was set at 5.0 pg.

2.4.6 Antibody titre

Antibody dilution curves were prepared by incubating various dilutions of the antiserum with 64 pg ^{3}H-oestradiol-17β in the presence of 0 pg or 100 pg of oestradiol-17β. The antibody titre has been defined as the dilution of antiserum that binds 50% of the labelled tracer in the absence of cold steroid, within the standard conditions for the assay (Abraham, 1974). Antibody dilution curves produced during March, 1973 and February, 1975 are shown in Figs. 2.5 and 2.6 respectively. During March, 1973 an antibody dilution of 1/30,000 was selected for use in the assay since this gave approximately 50% binding in the presence of 0 pg of cold steroid and good separation of the 0 pg and 100 pg curves also occurred with this dilution. This was an indication that the slope of the standard curve would be steepest with this dilution of antiserum. For the same reasons an antibody dilution of 1/20,000 was selected for use during February, 1975.

2.4.7 Antibody specificity

Some steroids were tested for cross-reaction with the oestradiol-17β antiserum. Known amounts were extracted from 0.5 ml of blank plasma, and estimated within the standard conditions for the radioimmunoassay. The resulting curves are shown in Fig. 2.7. The per cent cross-reaction was calculated as follows: if y picograms of steroid S were required to displace 50% of the ^{3}H-oestradiol-17β bound to the antiserum and x picograms of oestradiol-17β standard were required to displace 50% of the ^{3}H-oestradiol-17β, then the per cent cross-reaction of steroid S = (x/y) x 100 (Abraham, 1969).

The per cent cross-reactions of the steroids tested are shown in Table 2.6. Cortisol, progesterone and testosterone showed less than 0.1% cross-reaction at all quantities up to 1 µg. Oestriol was a weak competitor with 5% cross-reaction, while oestrone showed 52% cross-reaction.

2.5 DISCUSSION

A radioimmunoassay method has been described for the estimation of total unconjugated oestrogens in small samples of peripheral plasma from sheep. The acceptability of any method of hormone estimation is dependent upon two main factors, the reliability and the practicability of the method (Loraine and Bell, 1966; Abraham, 1974). According to Borth (1957), and more recently Midgley, Niswender and Rebar (1969b) and Abraham (1974) referring specifically to radioimmunoassays, the most useful criteria for assessing the reliability of an assay are sensitivity, precision, accuracy and specificity.

Sensitivity has been defined as the smallest single result which can be distinguished from zero (Borth, 1957). It is dependent upon the affinity of the antiserum, the mass of labelled tracer and antibody used in the assay, the volume of the incubation medium and the precision of the assay (Abraham, 1974). The sensitivity of the assay in the measurement of steroid levels in biological fluids is also influenced by the method blank values and the recovery of the steroid (Abraham, 1974).

A practical limit of sensitivity for the standard curve reported here was taken as 5 pg. This compares well with the sensitivities for radioimmunoassays reported by other workers. Wu and Lundy (1971) obtained a sensitivity limit of around 20 pg for a dextran-coated charcoal method. Other methods of separating the free from antibody-bound steroid have produced poorer

sensitivities. A solid-phase method described by Abraham (1969) had a sensitivity of 20 to 30 pg, and the use of polymerised antiserum (Mikhail et al., 1970) produced a sensitivity of approximately 50 pg.

As mentioned previously the sensitivity of the assay is considerably influenced by the blank value, which can be described as the apparent amount of steroid found in a sample which does not contain the steroid. The blank effect can be minimised by the use of a high affinity antiserum (Abraham, 1974) and the addition of gelatin to the assay buffer (Hotchkiss et al., 1971). The method of separation of free from bound steroid also influences the blank value which is less in the dextran-coated charcoal system (Abraham et al., 1971) than in the solid-phase system (Abraham and Odell, 1970). The physiological condition of the person performing the assay may also be an important factor (Abraham, 1974). Many organic solvents used for extraction have given rise to high blank values possibly due to the formation of products in stored solvents which are able to inhibit binding in a non-competitive way (Murphy, 1970).

In the assay reported here the plasma blank values (mean 11 to 15 pg) were above the sensitivity limit of the standard curve. These values may have represented true levels of oestrogens and not merely non-specific interference due to other constituents in the plasma since the blank plasma was obtained from an ovariectomised ewe with intact adrenal glands (Baird, 1968). The results obtained using this radioimmunoassay have not been corrected for plasma blank values.

Water blank values (mean 6 to 7 pg), representing the non-specific effect due to reagents, were often greater than the sensitivity limit for the assay. This was largely due to the high sensitivity of the standard curve but the fact that ether, used for extracting the plasma samples, was not redistilled before use may also have contributed. As Ekins and Newman (1970) have indicated, this non-specific effect upon the primary antigen-antibody reaction will depend on the relative concentration of antigen and antibody in the system. It will therefore vary at different points on the standard curve. Other investigators (Mikhail et al., 1970; Wu and Lundy, 1971) have overcome this by adding the solvent blank to the oestrogen standards so that all points on the standard curve were corrected for this non-specific effect. Murphy (1970) has suggested that solvent blanks are not necessarily subtractable since substances in plasma extracts are able to reduce the blank values of added solvents. In the present report, although the water blank value was only estimated at the zero pg level, the sample results have been corrected for this solvent blank when it was greater than the sensitivity of the standard curve. Many of the unknown plasma samples which were assayed contained low levels of oestrogens which would have been influenced significantly by a high water blank value.

Accuracy has been defined as the extent to which the mean of an infinite number of measurements of a substance agrees with the exact amount of the substance which is present (Midgley et al., 1969b). In practice the accuracy of radioimmunoassays has been tested by recovery experiments. Many investigators (e.g. Abraham,

1969; Wu and Lundy, 1971; Robertson, Smeaton and Durnford, 1972) have estimated the percentage recovery for each individual sample by adding tracer amounts of the radioactive steroid to the unknown plasma samples prior to extraction. Aliquots from the purified extract are counted for recovery estimations. This has the disadvantage of involving more pipetting steps and therefore may introduce further errors into the method. In the present assay, as in that of Henricks, Dickey and Hill (1971), an average recovery for the assay was estimated by adding increasing amounts of the cold oestradiol to aliquots of the blank plasma. The amount of steroid subsequently measured in the assay was then related to the amount added, after taking the effect of the blank plasma into consideration.

An average recovery of 87.5% was achieved from 0.5 ml plasma and a slightly lower recovery of 81.5% from 4.0 ml plasma, due probably to relatively less ether being used for extraction. The recoveries reported by other authors for oestradiol-17β have ranged from 70 to 90% (Mikhail et al., 1970; Wu and Lundy, 1971; Robertson et al., 1972). The recovery of 70% reported by Wu and Lundy (1971) was due to the intentional collection of a limited fraction from the column used to purify the samples. Abraham (1974) has suggested that a high recovery percentage will occur if the oestradiol standards are not allowed to bind to plasma proteins prior to extraction. A minimum of 30 mins. incubation at room temperature was allowed for equilibration between the oestradiol standards and the plasma in the assay described here.

Correlation coefficients for the recovery of 20, 50 and

100 pg of oestradiol-17β from 0.5 ml and 4.0 ml of blank plasma were 0.96 and 0.95 respectively. This suggests that the accuracy of the radioimmunoassay was consistent over the range of values studied. Nevertheless, at all values studied, the coefficient of variation for the amount of oestradiol-17β recovered was 5-6% for 0.5 ml of plasma and 11-14% for 4.0 ml plasma, although the inclusion of blank plasma values in these results will have contributed to this variation.

Precision has been defined (Midgely et al., 1969b) as the extent to which a given set of measurements of the same sample agrees with the mean i.e. the amount of variation in the estimation of unlabelled hormone. Within-assay variation is evaluated by measurements of the same sample in the same assay, and the between-assay variation by measurements of the same sample in consecutive assays. As a general rule the between-assay variation for steroid radioimmunoassays is greater than the within-assay variation (Abraham, 1974).

The within-assay variation for the assay reported here was 6-8% whereas the between-assay variation was around 16%. These values were within the quoted range for steroid radioimmunoassays as reported by Abraham (1974). Error may have been introduced at all stages of the assay, during extraction, at any of the many pipetting steps and during the procedure to separate free from antibody bound steroid. Midgley et al. (1969b) have compared the use of various types of micropipetting equipment and shown that the use of a Hamilton syringe can increase the precision of the assay. Eppendorf automatic pipettes were used throughout the

assays reported here, which may explain the high between-assay variation. Gelatin was included in the assay buffer since this has been shown to improve the precision of the assay (Wu and Lundy, 1971).

The specificity of an assay has been defined as the extent of freedom of interference by substances other than the one being measured (Midgley et al., 1969b). Other steroids must be shown not to interfere significantly in the assay, either because they do not react with the antiserum or their concentration is relatively low compared to the steroid being estimated.

The antiserum used in the present assay, which had been raised against oestradiol-17β, was also shown to cross-react significantly with oestrone (52%) and oestriol (5%). The non-oestrogenic steroids that were tested, cortisol, progesterone and testosterone failed to react significantly at all quantities up to 1 μg. The results of Caldwell (personal communication) for the cross-reaction of the same antiserum are shown in Table 2.6. He has reported 100% cross-reaction with oestrone as well as with oestradiol-17β, and 10% cross-reaction with oestriol. The difference between his results and those reported here cannot be explained. Oestrogen conjugates are a possible source of interference with the antiserum (Abraham et al., 1970); however it has been shown that these are not extracted by ether (Diczfalusy and Lindkvist, 1956). The assay presented here therefore measured the total unconjugated immunoreactive oestrogens present in the plasma, due to the significant cross-reaction with other oestrogens as well as oestradiol-17β.

Using this radioimmunoassay, the plasma samples from experimental animals were generally assayed within a few months of being collected. The effect of long term storage on plasma samples has not been studied although Abraham (1974) has suggested that samples can be stored for up to 2 years at -20°C in air-tight containers. Repeated freezing and thawing produces hydrolysis of steroid conjugates and gives the false impression of high unconjugated steroids (Abraham, 1974).

The working dilution of tritiated oestradiol was prepared freshly for each individual assay and the working solution of antibody was freshly prepared each week. According to Abraham (1974) working solutions of antisera and tritiated steroids are stable for up to 4 weeks at 4°C. Thorneycroft et al. (1970) reported that the stock dilution of antibody could be stored for up to 1 year at -15°C with no loss of binding activity and repeated freezing and thawing had no effect on the activity of their antiserum. The antibody used here lost some binding activity over the period of 2 years; however the standard curve was still usable at the end of this time.

The sodium phosphate buffer used in the present assay had a molarity of 0.1 and pH 7 which has been shown to be optimal for the assay of oestradiol-17β (Tillson, Thorneycroft, Abraham, Scaramuzzi and Caldwell, 1970). Gelatin was added to the buffer at a concentration of 0.1%. Furthermore 0.5 ml of P.B.S. - 0.1% gelatin was added to each assay tube before the antibody and tritiated oestradiol was added. Gelatin has been shown to improve the precision of the assay and produce a steeper standard

curve, as well as improving the stability of the antiserum (Wu and Lundy, 1971).

The use of a dextran-coated charcoal suspension to separate the free from antibody-bound oestrogen has been shown to give a very high precision to the assay with minimal non-specific interference (Abraham, 1974). In the present assay 96-98% of the free steroid was absorbed by the charcoal within 15 mins. The disadvantage of dextran-coated charcoal is that since dissociation of the antigen-antibody complex may occur during the separation procedure (Abraham, 1974) the time of incubation should be consistent within an assay. For this reason an automatic pipette was used to deliver the charcoal to achieve the shortest possible time interval between adding the charcoal to the first and the last tube. However it was still necessary to limit the number of unknown plasma samples to thirty per assay.

Abraham (1974) has described the practicability of an assay as the skill required to perform it, the time involved in its performance and the cost of the assay. The assay reported here was technically easy to perform. Extraction of the plasma samples was carried out using a single aliquot of ether; this achieved a satisfactory recovery of 80% or more. Both the ether extract and the supernatant remaining after the free hormone had been precipitated by charcoal, were decanted by pouring off rather than removing an aliquot by pipette, with no detriment to the precision of the assay. The estimation of recovery values by means of extracted standards rather than the addition of tracer amounts of radioactive oestrogens to all the unknown samples also

contributed to the simplicity of the method, although the accuracy of individual estimations may have suffered. Incubation of the antibody and steroid was carried out overnight. Although others have reported a shorter incubation time, overnight incubation was not inconvenient and may have contributed to the sensitivity of the assay (Abraham et al., 1970). The use of dextran-coated charcoal for the separation of free from antibody-bound steroid had the disadvantage of limiting the number of plasma samples that could be included in the assay. Nevertheless the procedure was rapid and easy to perform and contributed to the practicability of this assay method.

Due to cross-reaction of the antiserum used in this assay with oestrone and oestriol as well as with oestradiol-17β, the method measured total immunoreactive unconjugated oestrogens. The separate estimation of the individual oestrogens would have required the inclusion of a chromatographic step. However this would have increased the blank value and thereby decreased the sensitivity of the method, as well as increasing the time required to perform the assay. The high sensitivity of the present method has allowed the low levels of oestrogens in the peripheral circulation of sheep to be measured in small samples of plasma.

CHAPTER THREE

THE ESTIMATION OF PLASMA OESTROGENS IN ADULT EWES AROUND OESTRUS

3.1 INTRODUCTION.

The ewe is a seasonally polyoestrous animal with a mean cycle length of 16.4 to 17.5 days (Robinson, 1959). In 1900 Heape described the phases of the oestrous cycle as prooestrus, oestrus, metoestrus and dioestrus and these terms have since been defined for the ewe (Grant, 1934; Robinson, 1959). Oestrus has well-defined limits since it is the time when the ewe will stand to be mated by the male. Ovulation in the ewe occurs around 30 hours after the onset of oestrus (Cole and Miller, 1935; Santolucito, Clegg and Cole, 1960). The remaining phases of the cycle cannot be so clearly defined. Following oestrus the ruptured follicle becomes organised into luteal tissue during metoestrus, and dioestrus is the period when the ovary is dominated by the presence of a corpus luteum secreting progesterone. However, as will be described later, follicular growth is also proceeding continuously during dioestrus. Prooestrus covers the period when the corpus luteum is regressing and the follicles that are destined to ovulate are undergoing maturation. Oestrogens secreted by these maturing follicles are the predominant hormones produced by the ovary during this period. The present studies were chiefly concerned with two phases of the cycle, prooestrus and oestrus, and in particular with the secretion of oestrogens by the preovulatory follicles.

In the ewe there have been several different descriptions of the growth of vesicular follicles during the oestrous cycle. From the examination of ovarian structure in ewes killed at different stages of the cycle early investigators concluded that the

follicle destined to ovulate grew continuously throughout the preceding cycle (Grant, 1934; Kammlade, Welch, Nalbandov and Norton, 1952). Later workers examined serial sections from the ovaries of ewes killed at various times during the cycle and recorded all follicles greater than 1 mm in diameter (Robertson and Hutchinson, 1962; Hutchinson and Robertson, 1966). They concluded that the ovulatory follicle developed rapidly during the period 4 hours to 5 days after the onset of the previous oestrus, but increased in volume only slightly from then until the onset of oestrus. More recently it has been suggested that there may be waves of follicular growth during the cycle. By marking the large follicles (4-5 mm) present in the ovaries with Indian ink at 3 different times during the cycle and monitoring their subsequent fate, Smeaton and Robertson (1971) suggested that there were at least three phases of follicular growth and that only large follicles marked at, or just before, the onset of oestrus actually ovulated. Brand and de Jong (1973) followed microscopically the mean volume of follicles (> 2 mm in diameter) throughout the cycle and obtained evidence for two waves of follicular growth. The first wave, from days 1 to 10, resulted in atresia of the largest follicle whereas the second wave, which began on day 6, culminated in the ovulation of the largest follicle(s). There is general agreement that the follicle destined to ovulate undergoes rapid pre-ovulatory growth, beginning after the onset of oestrus and resulting in a follicle about 1 cm in diameter at ovulation (Cole and Miller, 1935).

The development of sensitive assay methods has allowed the levels of oestrogens, during the oestrous cycle of the ewe, to be

determined in ovarian venous blood (Moore et al., 1969; Scaramuzzi et al., 1970; Cox, Mattner and Thorburn, 1971; Holst, Braden and Mattner, 1972; Bjersing et al., (1972) and also in the peripheral circulation (Obst, Seamark and Brown, 1971; Pant et al., 1972; Yuthasastrakosol, Palmer and Howland, 1975). By the simultaneous collection of blood and examination of the ovaries it has been possible to relate the circulating level of oestrogens to follicular morphology.

Oestrogen peaks have been detected between days 2 to 4 (Scaramuzzi et al., 1970; Cox et al., 1971; Holst et al., 1972, days 6 to 8 (Scaramuzzi et al., 1970; Obst et al., 1971) and during the prooestrous phase of the cycle (Moore et al., 1969; Scaramuzzi et al., 1970; Pant et al., 1972; Bjersing et al., 1972; Yuthasastrakosol et al., 1975). The peaks occurring during the luteal phase were smaller than the main peak during prooestrus (Scaramuzzi et al., 1970). It has been suggested that these minor oestrogen peaks may be associated with the waves of follicular growth described previously.

During prooestrus the highest ovarian vein levels of oestrogens were invariably obtained from the ovary containing the largest non-atretic follicle (Bjersing et al., 1972). By culturing follicles in vitro (Moor, 1973) it was demonstrated that only those greater than 4.5 mm in diameter secreted high levels of oestrogens; smaller follicles produced low levels of oestrogens throughout the cycle. The concentration of oestrogens in the vein draining the ovary with the largest developing follicle, started to increase on

day 13 of the cycle (Bjersing et al., 1972) reaching a peak prior to the onset of oestrus and declining to a low level before ovulation (Moore et al., 1969; Scaramuzzi et al., 1970; Bjersing et al., 1972). This pattern of oestrogen secretion was confirmed by in vitro studies (Seamark et al., 1974) in which large follicles explanted on day 14 of the cycle produced the highest levels of oestrogens; oestrogen production declined rapidly in follicles explanted just before oestrus, and was very low in follicles explanted at oestrus.

The factors controlling the growth of follicles and the follicular production of oestrogens during the oestrous cycle of the ewe remain uncertain. Changes in the amounts of FSH and LH in the pituitary gland have been determined throughout the cycle (Santolucito et al., 1960; Robertson and Hutchinson, 1962). The pituitary content of LH increased from the 2nd to 15th day of the cycle, with an overall increase in FSH during the same period. A massive decrease in the content of both gonadotrophins occurred during the period before ovulation. It was found later that the release of FSH began approximately 8 hours before the onset of oestrus whereas the release of LH did not start until after the onset of oestrus (Robertson and Rakha, 1966).

Estimations of plasma LH in the ewe (Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969; Wheatley and Radford, 1969) showed that the concentration remained low during most of the cycle with a dramatic increase occurring shortly after the onset of oestrus. This confirmed the release of LH by the pituitary at this time. The reports of plasma FSH levels however

are contradictory. Pant, Fitzpatrick and Hopkinson, (1973) recorded a rise in the concentration of FSH coinciding with the LH peak, a second peak occurring 25 hours later. Bjersing et al. (1972) were unable to show a significant rise in blood FSH concentration during oestrus, and McCracken, Baird and Goding (1971) also quoted several unpublished studies which showed no consistent changes in plasma FSH concentration during the oestrous cycle.

Since no significant alteration in the plasma level of either gonadotrophin has been recorded during the cycle, when waves of follicular growth occur and the secretion of oestrogens is initiated, the stimulus for these events remains unknown. It is possible that a low continuous release of gonadotrophins from the pituitary controls the development of follicles. Bjersing et al. (1972) have suggested that the secretion of oestrogens could be brought about by a change in ovarian sensitivity to pre-existing gonadotrophin levels. It is interesting that although there is a significant secretion of oestrogens while the concentration of progesterone is still high, the maximal secretion of oestrogens always follows immediately after the decline in progesterone (Moor et al., 1969; Scaramuzzi et al., 1970; Obst et al., 1971; Bjersing et al., 1972). Brand and de Jong (1973) have suggested that the secretion of progesterone by the corpus luteum may actually inhibit the secretion of oestrogens by the follicles.

Oestrogens produced by the preovulatory follicle are thought to provide the stimulus for the release of LH in the ewe. Administration of oestradiol-17β regularly produced a typical oestrous peak of LH secretion in anoestrous ewes (Goding et al.,

1969; Symons, Cunningham and Saba, 1973). The administration of exogenous progesterone has been shown to inhibit the release of LH stimulated by exogenous oestradiol-17β in the ovariectomised ewe (Cumming, Brown, Blockey and Goding, 1971a). Furthermore exogenous oestradiol-17β was unable to evoke a rise in plasma LH during the luteal phase of the cycle. McCracken et al. (1971) have suggested that progesterone secreted by the corpus luteum prevents the release of LH which otherwise might be evoked by the minor peaks of oestrogens which occur during the luteal phase. Following regression of the corpus luteum, and the resulting decline in progesterone levels, oestrogens stimulate the preovulatory surge in LH secretion. Ovulation has been shown to occur regularly between 21 and 26 hours after LH release (Cumming, Brown, Blockey, Winfield, Baxter and Goding, 1971b).

Another function of oestrogens produced by the preovulatory follicle is to induce oestrous behaviour (Fletcher and Lindsay, 1971). However oestrogens are not solely responsible for oestrous manifestation in the ewe. Robinson (1959) found that a period of progesterone priming prior to the administration of oestradiol benzoate was required for regular cyclic oestrous behaviour in ovariectomised ewes. Furthermore the concentration of androstenedione in ovarian vein plasma is very high just before ovulation in the ewe (Baird et al., 1968). Although a very weak androgen, androstenedione may be metabolised peripherally to testosterone (Baird, Horton, Longcope and Tait, 1969) and androgens may play an important part in female libido (Everitt and Herbert, 1969).

After reviewing the early breeding records for various breeds of sheep, Asdell (1964) concluded that the mean length of oestrus was from 30 to 36 hours with little difference between breeds. Robinson (1959) considered however that there were extreme differences in the duration of oestrus, both between and within breeds, ranging from 3 to 84 hours. He suggested that many factors affected the oestrous period such as age, relative stage of the breeding season, nutritional status and libido of the ram. An association between prolificacy and the duration of oestrus has been noted in the Merino (Dunlop and Tallis, 1964), between the Finnish Landrace and British breeds of sheep (Land, 1970b) and between the Romanov and Solognote (Land, Pelletier, Thimonier and Mauleon, 1973). Land (1970b) has reported a significant correlation between duration of oestrus and litter size in Finnish Landrace ewes and he suggested this was mediated through ovulation rate and the pattern of secretion of oestrogens around the time of ovulation. Ovariectomised ewes, pretreated with progesterone, have been used to demonstrate a relationship between the duration of induced oestrus and the dose of oestradiol-17β administered (Scaramuzzi, Lindsay and Shelton, 1971; Fletcher and Lindsay, 1971; Land, Thompson and Baird, 1972b). This study reports on peripherally circulating levels of oestrogens in relation to the natural fecundity of Finnish Landrace and Scottish Blackface ewes.

3.2 MATERIALS AND METHODS

The work was carried out during the breeding season of 1974, using purebred Finnish Landrace and Scottish Blackface ewes. The 24 Finnish ewes, ranging in age from ewe lambs to 7 year old ewes, and 17 cast Blackface ewes, approximately 7 years old, were run with their respective breed flocks. All the ewes were kept outdoors with the grazing supplemented by hay and concentrates in the winter. During the first week in November vasectomised rams were put with the ewes for about 3 weeks to determine an oestrous date for each ewe. The rams were fitted with harnesses and marking crayons which have been shown to be an effective method for the detection of mating in sheep (Radford, Watson and Wood, 1960). For the following cycle entire rams, also equipped with marking crayons, were run with the ewes, 2 Finnish rams with the Finnish flock and 2 Border Leicester rams with the Blackface flock.

Examination of the ewes for signs of oestrus was carried out between 9-11 a.m. daily. The onset of oestrus, when the ewes were first marked by the ram, was designated day 0 of the cycle. Using the information obtained from the vasectomised rams, blood samples were collected daily from the ewes, beginning 4 days before the anticipated onset of the fertile heat and continuing until 5 days after the actual onset of heat. Ten ml of heparinised blood were removed from the jugular vein between 9-11 a.m. each day. Plasma was separated from the blood samples and stored as described in Chapter2(Section 2.3.1). Total unconjugated

oestrogens were determined in 4 ml aliquots of plasma, using the radioimmunoassay method described in Chapter 2.

Individual litter sizes for the experimental Finnish ewes were determined when they lambed the following spring. Due to the fact that the Blackface ewes would not be available at lambing, their litter sizes were determined by X-ray during the 4th month of pregnancy. Litter sizes are also reported for the entire Finnish flock over a period of 3 years and for the Blackface flock over a period of 2 years.

3.3 RESULTS

3.3.1 Litter sizes

Litter sizes for the Finnish Landrace and Scottish Blackface ewes are given in Table 3.1. Although there was some variation in the mean litter size recorded for the 2 breeds in different years, the mean overall value of 2.37 for the Finnish flock was significantly greater than that of 1.29 for the Blackface flock ($P < 0.001$). The mean litter size of 2.61 for the group of experimental Finnish ewes and that of 1.08 for the Blackface group were also significantly different ($P < 0.001$). The litter sizes for the experimental groups were similar to those obtained for the whole flocks of each breed.

3.3.2 Plasma levels of oestrogens

The mean levels of oestrogens in the peripheral plasma of the experimental Finnish Landrace and Scottish Blackface ewes are shown in Table 3.2 and the individual values for all animals given in Appendix 1. The different patterns of secretion of oestrogens are depicted in Fig. 3.1. Results were not available for some ewes during the first few days of the sampling period as they came into oestrus earlier than anticipated. Mean base levels of oestrogens for each breed were calculated from the values obtained on days -4 and -3. There was no significant difference between the base levels of 5.1 ± 1.7 and 5.3 ± 1.7 pg/ml for the Finnish and Blackface ewes respectively.

An increase above the base level occurred in both breeds

around the onset of oestrus. In the Finnish ewes the concentration of oestrogens was significantly elevated between days -2 and -1 of the cycle ($P < 0.01$) and between days -1 and 0 ($P < 0.05$). The mean peak concentration in the Finns, reached on day 0, was 11.4 pg/ml. On the succeeding day the concentration had dropped again to a low level. In the Blackface ewes the mean peak concentration of oestrogens, 9.1 pg/ml, which occurred on day -1, was significantly greater than the base level for the breed ($P < 0.05$) although on a between-day basis there was no significant elevation in the level. The concentration had dropped again to a low level on day 0. There was no significant difference between the mean peak levels of oestrogens for the two breeds.

3.4 DISCUSSION

Finnish Landrace sheep were introduced into Britain in 1962 and have since been shown to differ from Scottish Blackface sheep in many aspects of ovarian function. Average litter sizes have been reported for Finnish sheep kept in Scotland of 2.0, 3.0 and 3.4 for ewes aged 1, 2 and 3 or more years respectively (Donald and Read, 1967). Scottish Blackface sheep kept under similar conditions in Scotland were found to produce mean litters of 1.9 (Wiener, 1967).

The flock of Finnish Landrace ewes used in the present study produced mean litter sizes of 2.64, 1.96 and 2.55 for the years 1973, 1974 and 1975 respectively. The poor response in 1974 reflected the large number of ewe lambs in the flock at this time. It is well recognised that the number of lambs born to a ewe increases steadily until 5-6 years of age with a gradual regression thereafter (Reeve and Robertson, 1953). The mean litter sizes of 1.42 and 1.13 for the flock of Blackface ewes during 1973 and 1974 respectively were rather low for the breed due to the flock containing predominantly old ewes (Reeve and Robertson, 1953). Nevertheless the mean litter sizes of 2.37 and 1.29 obtained overall for the Finnish and Blackface ewes respectively, in conjunction with the reports of other workers, demonstrate the higher natural fecundity of Finnish Landrace ewes compared to Scottish Blackface ewes.

The experimental ewes, in which plasma concentrations of oestrogens were estimated during the breeding season of 1974, showed

similar litter sizes as their respective breed flocks. For managemental reasons, outwith this study, the experimental Blackface ewes were sired by Border Leicester rams. However it has been shown that the breed of the ram does not affect the number of lambs in litters sired (Barker and Land, 1970). The mean litter sizes of 2.61 and 1.08 for the experimental Finnish Landrace and Scottish Blackface ewes were significantly different.

The circulating levels of oestrogens were determined in the peripheral plasma of the experimental ewes around the time of oestrus. The mean base levels of 5.1 and 5.3 pg/ml for the Finnish and Blackface ewes respectively were similar to that of 5.2 pg/ml reported by Yuthasastrakosol et al. (1975) and 7.0 pg/ml reported by Obst et al. (1971). A slightly higher value of 11.9 pg/ml has been reported by Pant et al. (1972). In the present study the base levels of oestrogens in cycling ewes were higher than the mean value of 15.4 pg recorded for 4 ml of plasma, from an ovariectomised ewe, used as blank plasma in the oestrogen radioimmunoassay (Chapter 2, Section 2.4.2). This implies that the ovaries were contributing towards the basal secretion of oestrogens in the intact ewe in addition to an extra-gonadal source, probably the adrenals (Baird, 1968), which is responsible for the oestrogens present in the ovariectomised ewe. Ovarian stroma, maintained in culture, has been shown to secrete only small quantities of steroids (Moor et al., 1975). Therefore the theca interna of developing follicles may be contributing to the basal levels of oestrogens in cycling ewes.

A significant increase in the concentration of oestrogens

was detected in the ewes immediately prior to the onset of oestrus. The mean peak concentrations of 11.4 pg/ml for the Finnish ewes and 9.1 pg/ml for the Blackface ewes were approximately twice the base level. Yuthasastrakosol et al. (1975) reported a similar peak value of 13.3 pg/ml in the peripheral blood before oestrus. From the secretion rate of oestrogens by transplanted ovaries, Goding, Baird, Cumming and McCracken (1972) have calculated that the peak level of oestrogens in the ewe should not exceed 12.0 pg/ml. Pant et al. (1972) reported a slightly higher peak value of 21.2 pg/ml but this was also approximately twice their base value. Obst et al. (1971) reported considerably higher peak values of 30-140 pg/ml which probably represented a difference in assay technique. It is clear that in comparing different reports of circulating hormone levels, in any particular species, the assay techniques employed must be taken into consideration.

The results obtained in this study have allowed comparisons to be made between the prooestrous peaks of oestrogens in Finnish Landrace and Scottish Blackface ewes. There were certain similarities in that the rate of increase in oestrogen levels was similar and the peak levels reached in each breed were not significantly different. However significantly elevated levels of oestrogens occurred over a period of 2 days in the Finnish ewes and only 1 day in the Blackfaces.

By observing numbers of corpora lutea at slaughter (Marshall, 1904) or during laparotomy (Packham and Triffitt, 1966) it has been shown that the number of lambs is mainly influenced by ovulation rate, other factors playing a smaller role.

Wheeler and Land (1973) have reported ovulation rates of 2.94 and 1.26 for Finnish and Blackface ewes respectively that are of a similar order of magnitude as the litter sizes for the two breeds. It seems likely that the presence of mature follicles in the ovaries of ewes during prooestrus is related to the increase in the circulating concentration of oestrogens at this time. Moor (1973) found that large follicles, greater than 4.5 mm in diameter, explanted from the ovaries of ewes during prooestrus, produced considerably greater levels of oestrogens in culture than all smaller follicles explanted at the same time. The different pattern of secretion of oestrogens prior to oestrus in the Finnish ewes compared to the Blackfaces in this study may be attributed to the larger number of mature follicles in the ovaries of the Finnish ewes during prooestrus.

There are several possible explanations for the different patterns of secretion of oestrogens in the Finnish Landrace and Scottish Blackface ewes. Firstly, if the quantity of oestrogens secreted per follicle was greater in the Blackfaces, this would result in similar levels of oestrogens in the two breeds even though there were a larger number of follicles developing in the ovaries of the Finnish ewes. Evidence is presented in Chapter 4 to suggest that, when neonatal lambs belonging to the two breeds were stimulated by exogenous gonadotrophins, the quantity of oestrogens secreted per follicle was greater in the Blackface lambs than in the Finnish lambs. An alternative explanation for the similar peak levels of oestrogens in the ewes belonging to the two breeds is that the larger number of follicles

in the ovaries of the Finnish ewes developed slightly out of phase with one another. This would obviously result in lower peak levels of oestrogens in this breed than if the follicles developed completely in synchrony.

The longer duration of secretion of oestrogens in the Finnish ewes can also be explained by the follicles developing out of phase in this breed. There are however other possible explanations. Firstly if oestrogens were metabolised at a faster rate in the Blackface ewes this would result in a more rapid disappearance of oestrogens from the circulation in this breed. Secondly the secretion of oestrogens may be terminated earlier in the Blackface ewes. Moor (1974) has suggested that the preovulatory surge of LH is responsible for terminating the secretion of oestrogens by the preovulatory follicles in the ewe. In ovariectomised Finnish and Blackface ewes the release of LH in response to an oestrogenic stimulus, has been shown to occur later in the Finnish ewes (Land, personal communication). However the temporal relationship of the two events in intact Finnish and Blackface ewes is not known.

It is clear that more information is required concerning the growth and function of follicles during prooestrus in Finnish Landrace and Scottish Blackface ewes before their different patterns of secretion of oestrogens at this time can be fully explained.

As well as having different ovulation rates Finnish and Blackface sheep have been shown to differ in other aspects of their reproductive function. Finnish Landrace ewes have been found to

have an oestrous period of 48 to 72 hours (Donald and Read, 1967), which is much longer than that of 23 hours (Hafez, 1952) or 43 hours (Land 1970b) reported for Scottish Blackface ewes. It has been demonstrated that oestrous behaviour in the ewe is controlled by the action of oestrogens on specific regions in the hypothalamus (Radford, 1967). Land (1970b) suggested that the greater duration of oestrus in the Finnish ewes reflected a different endogenous pattern of secretion of oestrogens around the time of oestrus in this breed compared to Blackface ewes.

Ovariectomised ewes have been used to demonstrate a relationship between the dose of oestradiol benzoate or oestradiol-17β administered and the length of oestrus induced by this treatment (Scaramuzzi et al., 1971; Fletcher and Lindsay, 1971; Land et al., 1972b). An increase in the dose was found to result in a longer behavioural oestrus. Fletcher and Lindsay (1971) postulated that the duration of oestrus was related to the amount of oestrogens accumulated in the hypothalamus which would increase as the dose of oestrogens was increased. Land et al. (1972b), using Finnish Landrace and Scottish Blackface ewes, were able to show that in both breeds the duration of oestrus was increased following a doubling of the dose of oestradiol-17β and also that this increase was greater when the higher dose was given as two injections, 24 hours apart. This suggested that both the intensity of the oestrogen stimulus and the duration of the stimulus were involved in controlling the duration of oestrus. The present studies, using intact Finnish and Blackface ewes, showed that the levels of oestrogens reached a similar height in the two breeds and varied

only in that the duration of an elevated level was longer in the Finnish ewes. This suggests that the duration, rather than the intensity, of the oestrogen stimulus is more important in controlling the length of oestrus in the normal ewe.

Another explanation for the longer duration of oestrus in the Finnish ewes than in the Blackface ewes would be a greater hypothalamic sensitivity to oestrogens in the Finnish breed. Land et al. (1972b) observed a greater mean response, in terms of duration of oestrus, following the same treatments in ovariectomised Finnish ewes compared to Blackfaces, indicating that the Finnish ewes were more sensitive to oestrogens.

The interval between the oestrogen stimulus and the onset of oestrus has also been investigated in ovariectomised ewes (Scaramuzzi et al., 1971; Fletcher and Lindsay, 1971). With an increase in the dose of oestradiol benzoate the time to onset of oestrus was shortened. Fletcher and Lindsay (1971) suggested that the onset of oestrous behaviour was controlled by the rate of accumulation of oestrogens in the hypothalamus which increased as the dose of injected oestrogen was increased. In the present studies the interval between the initial rise in the level of oestrogens and the onset of oestrus was similar in the Finnish and Blackface ewes. According to the theory of Fletcher and Lindsay (1971) this was because the rate of increase in the level of oestrogens was similar in the two breeds, resulting in a similar rate of accumulation of oestrogens in the hypothalamus.

These studies therefore have contributed further information on the action of oestrogens in determining both the

time of onset and duration of oestrus. They suggest that the duration of oestrus in intact ewes is controlled by the duration of the oestrogen stimulus with variations in hypothalamic sensitivity to oestrogens being an additional factor involved among breeds of differing fecundity. The time to onset of oestrus is more likely to be influenced by the rate of increase in the circulating level of oestrogens.

As well as controlling the duration and time of onset of oestrus, the different pattern of secretion of oestrogens in the Finnish ewes may also influence other events during the cycle. The prooestrus oestrogen peak has been shown to be the stimulus for the preovulatory discharge of LH by the anterior pituitary in the ewe (Goding et al., 1969; Symons et al., 1973). Using breeds of varying fecundity Land et al. (1973) have shown that the duration of the interval between the onset of oestrus and the start of discharge of LH was greater in the more fecund Romanov breed. They postulated that the timing of the LH discharge may be controlled by the pattern of secretion of oestrogens during prooestrus. In the present study it was shown that the only difference in the prooestrous secretion of oestrogens between Finnish and Blackface ewes was the longer duration of hormone secretion in the Finnish ewes, the peak levels being similar in the two breeds. Alternatively Land et al. (1973) suggested that the timing of the LH release was controlled by the sensitivity to oestrogens of the hypothalamic centre responsible for the release of LH. This second hypothesis has been supported by the finding, in female lambs of 11 weeks of age, that the time interval between the injection of oestradiol

benzoate and the subsequent release of LH was greater in lambs of a high prolificacy breed than in those of a breed with lower prolificacy (Thimonier, Pelletier and Land, 1972). Similarly it has been shown that a delayed LH release occurred in ovariectomised Finnish ewes compared to Blackface ewes in response to the administration of oestrogens (Land, personal communication). It is possible that a decrease in the sensitivity of the hypothalamus in more fecund breeds of sheep requires an oestrogen stimulus of longer duration, as recorded in this study for Finnish ewes, in order to bring about the release of LH.

In conclusion, the larger litter sizes and longer duration of elevated levels of oestrogens, observed in the Finnish ewes compared to the Blackfaces in the present study, may both be a consequence of a larger number of pre-ovulatory follicles in the ovaries of the Finnish ewes during prooestrus. It is likely that the different patterns of secretion of oestrogens are responsible, at least partly, for variations between the two breeds, both in the duration of oestrus and in the timing of the LH peak.

CHAPTER FOUR

NORMAL AND INDUCED FOLLICULAR DEVELOPMENT IN NEONATAL LAMBS

4.1 INTRODUCTION

The total reserve of primordial follicles and their enclosed oocytes is established in the ovaries of sheep before birth (Mauleon, 1969). In all vertebrates that have been investigated primordial germ cells originate extra-gonadally and migrate across the dorsal mesentery to become established in the embryonic genital ridge (Franchi, Mandl and Zuckerman, 1962). The resulting primitive gonad consists of the germinal epithelium, containing the germ cells, enclosing a central core of mesenchymal tissue. Further development of the gonad involves two proliferations of the surface epithelium which invade the underlying mesenchymal tissue (Franchi et al., 1962). In the female the first proliferation results in the production of the sex cords which give rise to the ovarian medulla. Germ cells may be included in this first proliferation but eventually degenerate. The second proliferation of the germinal epithelium gives rise to the ovarian cortex. The majority of the germ cells remain situated in the peripheral cortex of the ovary and their mitotic division, together with proliferation of the epithelial cells, results in the cortex being the dominant layer in the primitive female gonad.

When the primitive gonad has differentiated into an ovary the primordial germ cells can be termed oogonia. Further mitotic divisions of oogonia result in their transformation into primary oocytes. In the sheep oogonal mitoses occur between days 30 and 90 of foetal life (Mauleon, 1969). The primary oocytes so formed must then undergo meiotic division to yield firstly a secondary

(haploid) oocyte and first polar body and secondly by division of the secondary oocyte to yield an ovum and a second polar body. The first appearance of meiotic prophase occurs at around 52 days of foetal life in the sheep (Mauleon, 1969). Development of the oocyte proceeds until the diplotene stage of meiosis is reached. The oocytes remain arrested at this stage of development, known as the dictyate state, until ovulation is imminent. In the sheep, although meiosis is resumed before ovulation, the first polar body is not separated until immediately after the oocyte is shed from the ovary (Cole and Miller, 1935). Therefore in the sheep, in which the oogenetic period is completed in the foetus, the ovary at birth contains only oocytes in the dictyate state of meiosis.

Soon after they arrive in the genital ridges the primordial germ cells acquire a covering of flattened cells. It is generally believed that these cells are derived from the surface epithelium although, since the germ cells are associated with both the surface epithelium and with subepithelial mesenchyme, Witschi (1951) has proposed that follicle cells may arise from either tissue. Follicular development (as described in Chapter 1, Section 1.2) is proceeding within the ovaries of the foetus and the first follicles with an antrum appear 50-60 days after the end of the oogenetic period (Mauleon, 1969). Large numbers of vesicular follicles have been observed in the ovaries of lambs at birth (Land, 1970a; Kennedy, Worthington and Cole, 1974). Kennedy et al. (1974) recorded an increase in the number of both growing and vesicular follicles between birth and 4 weeks of age. This was reflected in ovarian weights, at 4 and 8 weeks of age,

that were 7 and 11 times, respectively, the ovarian weight at birth. Foster, Roach, Karsch, Norton, Cook and Nalbandov (1972a) also observed a seven-fold increase in ovarian weight between day 148 of gestation and day 18 post-partum. By 12 weeks of age ovarian weight had declined due to atresia of the large numbers of growing and vesicular follicles seen at earlier ages (Kennedy et al., 1974). Thereafter the ovary resembled the adult condition in that one or two follicles were generally much larger than the rest, and ovarian weight remained constant until 33 weeks of age. The diameter of the largest follicle in each ovary increased steadily from birth to 33 weeks (Kennedy et al., 1974).

Since Velle (1963b) identified oestradiol-17α in the tissues of foetal sheep there have been several reports of the presence of oestrogens in the foetus. Oestrogens have been detected in the blood of foetal lambs from 70 days of gestation onwards (Findlay and Cox, 1970) with the highest levels occurring during late gestation (Findlay and Cox, 1970; Rawlings and Ward, 1972; Alexander, Britton, Corker, Naftolin and Nixon, 1973). Findlay and Cox (1970) identified oestradiol-17β, oestradiol-17α and oestrone in foetal plasma, chiefly in the conjugated state. Placental tissues have been shown to be capable of synthesising the range of oestrogens present in the foetus (Findlay and Seamark, 1971) implicating the placenta as the source of oestrogens in the sheep at this time.

Kennedy et al. (1974) detected oestradiol-17α and oestradiol-17β in the urine of lambs at birth. However they were not able to detect oestrogens in the urine of older animals until

33 weeks of age. Uterine epithelial height in their lambs was greater at birth and at 4 weeks of age than in older lambs which they related to the urinary oestrogens detected in new-born animals. However Liefer, Foster and Dziuk (1972) have shown that ovariectomy at 5 days of age resulted in significantly lighter uteri compared to controls when the lambs were killed at 44 days of age. This implied that sufficient ovarian steroids were being secreted in the neonatal lamb to influence the growth of the uterus.

Indirect evidence has also been produced to suggest that the ovaries of neonatal lambs may not be secreting oestrogens. Circulating levels of LH in female lambs have been shown to increase significantly during the first 2 weeks of life whereas in male lambs the levels of LH remained low over the same period (Foster, Cook and Nalbandov, 1972e). Castration of the male lambs caused serum LH levels to rise whereas ovariectomy of the female lambs had no effect on serum LH levels. The authors suggested the difference was due to the negative feedback of gonadal steroids on the hypothalamo-hypophyseal system becoming established earlier in the male than in the female. It has been demonstrated that the negative feedback system can function in the neonatal female lamb since the administration of oestrogens suppressed both the synthesis and release of LH (Liefer et al., 1972). This implied that the ovaries were not producing sufficient amounts of an inhibitory steroid. It is not until 9 weeks of age that the ovaries begin to have an inhibiting effect on the pituitary secretion of LH (Foster, Jaffe and Niswender,

1975).

Neonatal lambs have been used to investigate the physiological factors underlying differences in ovulation rate among breeds and strains of sheep. Scottish Blackface and Welsh lambs have been shown to have a significantly larger number of oocytes, but a smaller proportion of oocytes in large follicles with an antrum, than their Finnish Landrace crosses at birth (Land, 1970a). Similarly lambs from a group of Merino sheep selected against multiple births had a larger number of primordial follicles in their ovaries at birth and 7 days of age compared to lambs from a group selected for a high incidence of multiple births (Trounson et al., 1974). Plasma LH levels were found to be greater in lambs belonging to the more fecund strain of Merino sheep at 30 days (Bindon and Turner, 1974) and 5 months of age (Trounson et al., 1974).

The present studies were designed to compare ovarian sensitivity in neonatal Finnish Landrace and Scottish Blackface lambs. In an attempt to stimulate further follicular development and ovulation, it was necessary to administer an exogenous hormonal stimulus. There have been many reports of the use of gonadotrophins to stimulate follicular development in the sheep. In general the hormones used have been pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG).

In hypophysectomised female rats PMSG has been shown to stimulate follicular development as a quantitative response related to the dose of hormone administered (Cole, Pencharz and Goss, 1940). Ovulation did not occur following small doses of

PMSG although, by increasing the dose, luteinisation was achieved. Ovulation has been stimulated by administering PMSG to rats previously primed with PMSG (Zarrow, Caldwell, Hafez and Pincus, 1958). Nevertheless the ovulation that occurred when the hormone was administered to intact immature rats was attributed to a synergism with endogenous gonadotrophin secretion (Rowlands and Williams, 1941). Therefore although PMSG has a predominantly follicle-stimulating effect it can also show luteinising activity. Half-lives of 6 days in the gelding (Catchpole, Cole and Pearson, 1935) and 21 hours in the sheep (McIntosh, Moor and Allen, 1975) have been reported for PMSG. Connell (1965) has attributed the slow disappearance from the blood-stream to the high molecular weight of PMSG. It has also been suggested that the long half-life is compatible with the fact that a single injection is as effective as multiple injections (Cole, Guilbert and Goss, 1932).

The actions of HCG have been described by Evans and Simpson (1950). In immature rats, rabbits and mice HCG stimulated follicular development, ovulation and luteinisation. However the only effect on the ovaries of hypophysectomised females was to stimulate interstitial cell development with no follicular development or corpus luteum formation. The follicular development that occurred in intact animals was attributed to a synergism with endogenous pituitary FSH.

Cole and Miller (1933) were the first to demonstrate the possibility of controlling ovarian activity in the ewe by the use of PMSG. There have since been many reports of the use of this hormone to increase the ovulation rate and thereby to improve

fecundity during the breeding season (Wallace, 1954; Palsson, 1962) and to induce ovulation during anoestrus (Dutt, 1953; Robinson, 1954). A single injection of PMSG stimulated ovulation in the anoestrous ewe (Cole and Miller, 1933) although a period of progesterone priming was found to be necessary for the induction of oestrus as well as ovulation (Robinson, 1959). During the breeding season a single injection of PMSG on the 12th or 13th day of the oestrous cycle (Wallace, 1954; Palsson, 1962) successfully increased the lambing percentage. A dose response relationship has been reported with an increase in the dose of PMSG resulting in an increase in the number of ovulations (Palsson, 1962). Wallace (1954) concluded that a dose of 650 to 1000 i.u. PMSG was most effective in improving fecundity without resulting in superovulation.

Exogenous gonadotrophins have also been administered to prepubertal sheep to investigate the possibility of decreasing the generation interval in sheep, either by advancing the age at which puberty is attained or by producing large numbers of ova for transfer to other animals. The ovaries of new-born lambs are refractory to exogenous gonadotrophins, but by 3-4 weeks of age follicular growth can be stimulated (Mansour, 1959; Mauleon, 1969). Using a combination of 1000 i.u. PMSG and 500 i.u. HCG, Mansour (1959) achieved follicular development and ovulation from 4 weeks of age onwards. The use of PMSG alone did not produce ovulation until 16 weeks of age. Land and McGovern (1968) carried out a similar experiment in 9 week-old lambs using 700 or 350 i.u. PMSG followed by 500 i.u. HCG. They achieved a greater degree of

follicular development with the higher dose of PMSG and also suggested that,by shortening the interval between PMSG and HCG treatment, both ovulation rate and fertilisation rate may be increased. Other workers (Foote and Bennett, 1968; Burfening and van Horn, 1970; Riera, Matthews, Svejda and Foote, 1970) using various doses of PMSG (600 to 800 i.u.) obtained ovulation and also pregnancy in prepubertal lambs ranging from 5-12 months of age.

Due to the proven ability of PMSG and HCG to stimulate follicular development and ovulation in neonatal lambs, and also because these hormones are readily available commercially, they were used in the present studies. The response to PMSG was compared in Finnish and Blackface lambs and related to the normal development of ovarian morphology and function at this age.

4.2 MATERIALS

4.2.1 Animals

Experimental Finnish Landrace and Scottish Blackface female lambs were obtained from purebred breeding flocks maintained on the premises. The sheep were kept at pasture, with supplementary feeding when necessary, and were brought indoors only at lambing. To avoid any effect of season on reproductive function the two flocks were mated simultaneously, ensuring that lambs belonging to both breeds were available for experimentation at the same time of year. The lambs were born during March and April. Male littermates were removed at birth, allowing all the female lambs to be reared on their mothers. Not more than three lambs had to be reared by any ewe, although the original litter sizes in the Finnish flock were often larger than this. To supplement the number of experimental Blackface lambs during the study, purebred lambs with known birth-dates were purchased from a neighbouring farm. These animals were born at the same time of year as the home-bred lambs and were acquired at 1-3 days of age. They had received maternal colostrum and thereafter were reared artificially on Denkavit Lamb milk powder (R.H.M.).

The experimental lambs used during each of the 3 years of the study were as follows:

(a) 1973

All Finnish lambs born during 1973 joined the breeding flock and were not available therefore for use in experiments. The Blackface lambs born during 1973 were used for preliminary

studies.

(b) 1974

During 1974 all home-bred Finnish and Blackface lambs were used as experimental animals. The number of Blackface lambs was supplemented by purchased animals.

(c) 1975

All Finnish lambs used during 1975 were home-bred, whereas all the Blackface lambs were bought in.

4.2.2. Drugs

(a) Folligon (Pregnant Mare's Serum Gonadotrophin)	Organon Labs. Ltd., Crown House, Morden, Surrey.
(b) Chorulon (Human Chorionic Gonadotrophin)	Organon Labs. Ltd.

Both hormone preparations were stored at $4^{o}C$. The same batch was used for all the experiments carried out in any one year. However a different batch was used during each of the 3 years of the study since the manufacturers advise that the potency of the freeze-dried material is retained for only 2 years at low temperature. The freeze-dried powder was reconstituted immediately before use in the sterile water provided.

4.3 METHODS

4.3.1 Experimental procedure

(a) 1973

Since there were no Finnish lambs available for experimentation in the spring of 1973, the Blackface lambs were used for preliminary investigations into the stimulation of follicular development during the neonatal period. At birth they were assigned to groups, each containing 3 animals, and, at approximately monthly intervals, various hormone treatments were administered. With one exception each group was treated only once. Two control lambs were included at 69 and 130 days of age and one control animal at 30 and 94 days of age; these animals received injections of sterile water in place of hormone treatments.

Three groups with an average age of 30 days (range 28-33) received either 1000, 500 or 250 i.u. PMSG by subcutaneous injection on day 0 followed by an intravenous injection of 500 i.u. HCG on day 4. At an average age of 69 days (range 63-71) two groups received 500 i.u. PMSG subcutaneously on day 0 followed on day 4 by 500 i.u. HCG intravenously. One of these groups had received the same treatment at 30 days of age. At an average age of 94 days (range 93-95) one group of lambs received 500 i.u. PMSG subcutaneously on day 0. Finally at an average age of 130 days (range 123-137) two groups received either 1000 or 500 i.u. PMSG subcutaneously followed by 500 i.u. HCG intravenously on day 3.

10 ml samples of heparinised blood were collected twice daily from the jugular vein of each lamb between 9-10 a.m. and

4-5 p.m. for the period of an experiment. Plasma was separated from these blood samples and stored as described in Chapter 2 (Section 2.3.1). On treatment days the hormones were administered between 9-10 a.m., immediately after the collection of blood. Examination of the ovaries in both test and control lambs at 30, 69 and 130 days of age was carried out by laparotomy 5-7 days after the administration of PMSG. At 94 days of age the ovaries were examined when the lambs were killed on day 3, by an intravenous injection of pentobarbitone sodium (Euthetal; May & Baker Ltd., Dagenham, Essex).

(b) 1974

As a result of the preliminary work of 1973 investigations during 1974 were limited to the first two months of age, during which time the greatest response to PMSG had been obtained. At birth the lambs were assigned to four groups, each containing 4 test and 1 control animal from each breed. The groups were treated only once at 7, 21, 35 or 49 days of age. 750 i.u. PMSG were administered subcutaneously on day 0 to the test animals, and the control lambs received injections of sterile water. Blood samples were collected as described above, between 9-10 a.m. daily, for 7 days after treatment or until the lamb was killed. In every age group ovarian examination in two of the test animals from each breed was carried out by laparotomy on day 4. The reproductive tracts from the remaining test animals and the controls were examined following slaughter on day 4. The lambs were killed by the intravenous injection of pentobarbitone sodium.

(c) 1975

From the results of investigations during 1974 it appeared that the small group size may have prevented the production of statistically significant results. Therefore during 1975 all Finnish and Blackface lambs were studied at a single age. Furthermore the dose of PMSG was reduced in an attempt to prevent the excessive ovarian stimulation seen in some lambs during 1974. HCG was also given to allow the comparison of both follicular development and ovulation rates between the two breeds.

Fifteen Blackface and 13 Finnish lambs were treated at 35 days of age. 500 i.u. PMSG were given subcutaneously on day 0 followed on day 4 by 500 i.u. HCG administered intravenously. Five Blackface and 5 Finnish lambs were included as control animals and received injections of sterile water in place of hormone treatments. Blood samples were collected as described above between 9-10 a.m. daily. Examination of the reproductive tracts was carried out on day 6 following slaughter of the test and control lambs by intravenous administration of pentobarbitone sodium.

4.3.2 Laparotomy

Laparotomy, to examine the reproductive tract of experimental lambs, was performed under general anaesthesia. In animals younger than about 1 month of age, anaesthesia was induced and maintained on a mixture of Halothane (Fluothane, I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire) and oxygen delivered through

a malleable rubber mask moulded to the muzzle of the lamb. In older animals anaesthesia was induced using pentobarbitone sodium (Nembutal, Abbott Laboratories Ltd., Queenborough, Kent), administered intravenously; endotracheal intubation was performed using a laryngoscope and anaesthesia maintained on a mixture of Halothane and oxygen in a closed circuit apparatus.

The animals were secured in a supine position on an adjustable operating table and the head end of the animal was lowered to prevent the inspiration of saliva or of regurgitated rumenal content. The operation site was cleared of wool and the skin prepared by scrubbing with a solution of Savlon Hospital Concentrate (I.C.I. Ltd.) and then swabbing with Pevidine Surgical Scrub (Berk Pharmaceuticals Ltd., Catteshall Lane, Godalming, Surrey).

A ventral mid-line incision was made immediately anterior to the mammary gland, passing through skin and linea alba. By lowering the anterior end of the animal the abdominal viscera fell forward, clearing the pelvic area and allowing the reproductive tract to be seen in its position below the bladder. After examination of the reproductive tract it was carefully replaced beneath the bladder taking care not to rupture any mature follicles. Using a simple continuous suture of chromic catgut, the linea alba was reunited and the subcutaneous tissues were brought together in a similar manner. Simple interrupted sutures of nylon were used to reunite the skin. An injection of penicillin and streptomycin was given intramuscularly to provide antibiotic cover.

The lambs were placed in a recovery pen until they had regained consciousness when they were returned to their pen. Skin sutures were removed approximately 7 days after the operation.

4.3.3 Macroscopic examination of reproductive tract

(a) Ovaries

During the course of a laparotomy operation observations of ovarian changes were recorded and ovarian measurements were noted using a sterile pair of small callipers. Measurements were taken of the length from pole to pole, the width from the attachments to the outer edge, and the width at right angles to this measurement. It was assumed that the shape of an ovary most closely resembled that of a solid ovoid, the volume of which is given by the formula -

$$V = \frac{4\pi \cdot \frac{a}{2} \cdot \frac{b}{2} \cdot \frac{c}{2}}{3}$$

Using this formula the volume, V, of an ovary was calculated when a, b and c were the three measurements noted for that ovary.

Ovarian follicles were defined as having been stimulated by the hormone treatment if their surface diameter was 4 mm or greater. The number and diameter (measured using the callipers) of these follicles were noted as well as the number of follicles showing signs of ovulation or luteinisation. Ovulation was defined by the presence of an ovulation point. Follicles were described as luteinised when they appeared collapsed, solid and discoloured; an ovulation point was not present although internal haemorrhage had occurred in many of these follicles. Where an

ovary had been excessively stimulated and it was impossible to count the follicles accurately, an estimated number was recorded.

Following the slaughter of the lambs their ovaries were dissected free from the mesovarium before measurements were recorded and follicles counted. The ovaries were blotted free of surface moisture before being weighed to an accuracy of 10^{-3}g.

(b) Uterus

The uterine horn diameter was measured by placing the pair of callipers across the right horn immediately anterior to the bifurcation.

4.3.4 Microscopic examination of reproductive tract

(a) Ovaries

The ovaries from the control Finnish Landrace and Scottish Blackface lambs during 1975 were fixed in acetic-formol-alcohol. One ovary of each pair was taken at random, double-embedded in celloidin and paraffin wax, and serially sectioned at 5-7 μ. Every 20th section from ovaries less than 1 cm in diameter and every 40th section from ovaries greater than 1 cm in diameter was mounted and stained with Heidenhain's Iron Haematoxylin (Culling, 1974).

The number of oocytes was counted in every section stained, using the nucleus as a marker. Oocytes were grouped as (1) those with no more than one layer of follicular cells, (2) those with more than one layer of cells but lacking an antrum and (3) those with an antrum. The total number of oocytes within each group was obtained for the whole ovary by multiplying the sum

of the counts for the stained sections by the number of sections each represented.

A correction was then applied to these counts, as described by Mandl and Zuckerman (1951), in order to allow for the possibility that the same nucleus may have been counted twice. This will happen when the diameter of the nucleus is greater than the thickness of the section. In every 5th section counted the nuclear diameter was measured (using an eyepiece micrometer) in 5 oocytes selected at random from each morphological group. When there were fewer than 5 oocytes present, they were all counted. The mean nuclear diameter for each morphological group was used to correct the overall count for that group using the following formula -

$$\text{True population} = \frac{\text{thickness of section}}{\text{thickness of section} + \text{mean nuclear diameter}} \times \text{No. of oocytes counted}$$

As the ovarian sections were examined, observations were recorded on the degree of atresia present among the vesicular follicles. The criteria used for the identification of atresia were those described by Brand and de Jong (1973). Early signs included degeneration of the granulosa cells, with invagination of the thecal tissue into the membrana granulosa and interruption of the membrana propria. Advanced degenerative changes, involving obliterative and cystic atresia, were also observed.

(b) Uterus

A portion of the right uterine horn, immediately anterior to the external bifurcation, was taken from all the slaughtered

lambs during 1974 and 1975 and fixed in formol saline. The tissues were single-bedded in paraffin wax and cross-sections, 5-7 μ thick, were taken. Tissue sections were stained with haematoxylin and eosin, using Mayer's Haemalum (Culling, 1974).

Uterine epithelial height was measured, using an eyepiece micrometer, at three positions in both the caruncular and intercaruncular regions of the uterine wall. The mean epithelial height was calculated for each region.

4.3.5 Estimation of plasma oestrogens

Total unconjugated oestrogens were estimated in the samples of plasma collected from the lambs using the radioimmunoassay described in Chapter 2. Estimations were usually carried out on 0.5 ml samples. However when the plasma level of oestrogens was found to be high the sample size was reduced to 0.1 ml.

4.4 RESULTS

4.4.1 1973

The details regarding bodyweights at slaughter, ovarian volumes and number of entire and ovulated or luteinised follicles for the Blackface lambs during 1973 are shown in Table 4.1. Mean plasma levels of oestrogens for the groups of lambs are shown in Figs. 4.1 and 4.2, and the individual values for all animals are given in Appendix 2.

(a) Controls

In the control lambs ovarian volume was quite variable and showed no obvious increase with advancing age. With one exception no large follicles were observed in the ovaries of the control lambs; lamb No. 12 had a 5 mm diameter follicle on the surface of one ovary. The levels of oestrogens in the plasma of control lambs remained at a consistently low level, never rising above 12 pg/ml and with no detectable peaks.

(b) 250 i.u. PMSG

In the group of lambs that received 250 i.u. PMSG at 30 days of age there was a single ovulated follicle in the ovary of one lamb. Ovarian volumes in all three lambs were similar to those found in the control lambs. In lamb No. 24, which developed a single large follicle, a slightly higher level of oestrogens of 20 pg/ml was recorded on day 4. However all other levels of oestrogens in this lamb and in the other two in the group were no higher than the levels found in the control lambs.

(c) 500 i.u. PMSG

Among the lambs that received 500 i.u. PMSG at 30, 69, 94 and 130 days of age the response ranged from 1 to 7 large follicles. There was no significant difference in the response at different ages although each of the three lambs at 130 days of age produced only one follicle. A proportion of the follicles in some of the lambs showed signs of luteinisation or ovulation. In most of the treated lambs ovarian volumes were similar to those found in the controls. However, when there were several large follicles present, this was reflected by an increase in ovarian volume. The level of oestrogens in some of the lambs treated with 500 i.u. PMSG were considerably higher than those found in the control lambs. At 30 days of age elevated levels occurred in all three lambs reaching a mean peak of 50.0 $\pm$ 37.5 pg/ml on day 4, falling again thereafter. At 69 days of age an elevated level was recorded in only one out of six lambs, resulting in an apparent mean peak of 18.8 $\pm$ 23.0 pg/ml on day 4. At 94 days of age the lambs were killed on day 3, probably before the level of oestrogens had reached a peak. Nevertheless the mean level of 25.0 $\pm$ 18.5 pg/ml on day 3 showed an elevation over the levels found in control lambs. At 130 days of age all three lambs had low levels of oestrogens similar to those found in control lambs.

One group of lambs (Nos. 31, 22 and 33R) was treated with 500 i.u. PMSG on two occasions, at 30 and 69 days of age. Although two of the lambs responded, on the second occasion, by the development of a similar number of large follicles as other lambs at the same age, in the third lamb (No. 22) laparotomy at 69 days revealed

the ovaries covered in adhesions, making an assessment of the response impossible. For this reason, and also because there was no way of knowing what effect treatment at one age would have on the response at later ages, it was decided to use animals on one occasion only in subsequent years.

(d) 1000 i.u. PMSG

A dose of 1000 i.u. PMSG stimulated follicular development in all lambs to which it was given. At 30 days of age the response ranged from 9 to an estimated 50 large follicles, whereas at 130 days of age the response ranged from 3 to 7 large follicles. A proportion of the follicles in some of the lambs showed signs of ovulation or luteinisation. At both ages the result was greater than that seen after using 500 i.u. PMSG. Ovarian volumes were very large in those animals in which follicular development was excessive. All the lambs at 30 days of age showed elevated levels of oestrogens, reaching a mean peak of 656 $\pm$ 295 pg/ml on day 4 and falling again thereafter. However at 130 days of age the levels of oestrogens were no higher than those in control lambs.

From these preliminary results it was concluded that the response to PMSG-treatment was partly controlled by the dose used, an increase in the dose producing an increase in the number of large follicles. However there was also a tendency for the response to be greater at the younger ages, both with respect to follicular development and the secretion of oestrogens.

4.4.2 1974

The details regarding bodyweights at slaughter, ovarian

characteristics and uterine horn diameters for 7, 21, 35 and 49 day-old lambs are shown in Tables 4.2 to 4.5 respectively. Plasma levels of oestrogens are shown in Figs. 4.3 to 4.10 and the individual values for all animals are given in Appendix 3.

(a) Controls

The gross appearance of the reproductive tract at 7, 21, 35 and 49 days of age was similar in the Finnish and Blackface lambs. The reproductive tracts from the Blackface control lambs are depicted in Plates 4.1 to 4.4 showing the development that occurred between 7 and 49 days of age. At 7 days of age the ovaries were small, white and compact with no follicles visible on the surface (Plate 4.1a). However as can be seen from the cross-section of the ovary (Plate 4.1b) vesicular follicles were present in the ovaries at this age. At 21 days of age the ovaries had increased in size and small vesicular follicles were visible on the ovarian surface (Plate 4.2a) and on cross-section (Plate 4.2b). At 35 and 49 days of age the ovaries were covered in small vesicular follicles (Plates 4.3a and 4.4a) and at these ages the ovarian cortex was packed with many vesicular follicles (Plates 4.3b and 4.4b).

There was an increase in ovarian volume with advancing age in the control lambs (Tables 4.2 to 4.5), between 7-49 days in the Finnish lambs and between 7-35 days in the Blackface lambs. However, since only one lamb of each breed was included as a control animal at each age, no detailed comparison could be made between the breeds. The follicles on the ovarian surface were usually no greater than 2 mm in diameter although occasionally slightly larger

follicles were seen, as in the Finnish control lambs at 21 and 49 days of age, each of which had one 4 mm diameter follicle (Tables 4.3 and 4.5 respectively).

Uterine horn diameter increased with advancing age in the control lambs (Tables 4.2 to 4.5) between 7-49 days in the Finnish lambs and 7-35 days in the Blackfaces. As can be seen in the uterine cross-sections from Blackface control lambs (Plates 4.1c, 4.2c, 4.3c and 4.4c) the uterine glands had started to develop at 1 week of age and the glandular development increased with advancing age. The height of the epithelium in the caruncular and inter-caruncular regions of the uterus of the control lambs is shown in Table 4.6. Due to the small number of control lambs included at each age it was not possible to relate any differences in epithelial height to age.

The plasma levels of oestrogens in the control lambs are shown in Figs. 4.3 to 4.10 (and Appendix 3). As can be seen, the levels remained low in all the lambs never rising above 15 pg/ml.

(b) PMSG-treated

In both breeds the number of lambs responding to the administration of PMSG by the development of large follicles increased with age (Tables 4.2 to 4.5). One Finnish and one Blackface lamb were stimulated to produce large follicles at 7 days of age. At 21 days of age, three of four lambs in each breed responded, and all the lambs showed a response at 35 and 49 days of age. The response, in the lambs that were stimulated, ranged from 1-45 large follicles in the Blackfaces and 1-200 large follicles in the Finnish lambs. Due to this extreme variation and the small

number of lambs treated at different ages, there was no significant difference in response between the two breeds, whether the individual age groups were compared or all the stimulated lambs in each breed. However at all ages studied the greatest number of large follicles always occurred in a Finnish lamb.

With one exception, there were no signs of luteinisation or ovulation in the large follicles at all ages. The exception was the Finnish lamb, No. 27R, at 21 days of age, in which two follicles had ovulated. There was an increase in the mean ovarian volume with advancing age in the treated lambs of both breeds which reflected the increasing response to PMSG. However, as with the follicle results, there was no significant difference in the ovarian volumes between the breeds, due to the variation among individual animals.

At all ages studied during 1974 the lambs that responded to PMSG-treatment by the development of large follicles in their ovaries also showed an elevation in the plasma concentration of oestrogens compared to the levels in control lambs (Figs. 4.3 to 4.10, and Appendix 3). In these stimulated lambs (Finnish and Blackface) the mean level of oestrogens on day 1 was significantly greater than the mean level on day 0 ($P < 0.01$). The level continued to rise until day 3 or 4 but thereafter the concentration was variable, continuing to rise in some animals but falling in others. A comparison between the day 4 plasma levels of oestrogens in the Finnish and Blackface lambs showed no significant difference between the two breeds. This was true whether the individual age groups were compared or all the stimulated lambs. As with the

follicular response, however, the highest level of oestrogens at each age always occurred in a Finnish lamb.

The relationship between the number of large follicles in the ovaries and the plasma level of oestrogens on day 4 is shown in Fig. 4.11 for all the stimulated Finnish and Blackface lambs except those in which the follicle count was estimated. A significant correlation existed between these two parameters ($r = 0.92$; $P < 0.001$). The estimated counts tended to show a similar relationship with the level of oestrogens although there was greater variability.

The epithelial heights in the caruncular and intercaruncular regions of the uteri from the treated lambs are shown in Table 4.6. The histological sections from one Finnish lamb were not sufficiently complete for the measurements to be carried out. In both regions the epithelium was significantly higher in treated lambs than in the controls ($P < 0.02$ in each case).

Bodyweights of the lambs increased as they grew older to a similar extent in both breeds (See Tables 4.2 to 4.5). Bodyweight did not appear to influence the response to PMSG since in any particular age group the greatest result, in terms of follicular development, did not necessarily occur in the heaviest lambs.

From the results for 1974 it appeared that the response, in terms of number of large follicles or production of oestrogens, was not significantly different between the Finnish and Blackface lambs. This may have been due to the large variation in the response to 750 i.u. PMSG in conjunction with the small number of animals in each age group. It was noticeable, however, that the

greatest response at all ages always occurred in a Finnish lamb.

4.4.3 1975

(a) Controls

The details regarding bodyweights at slaughter, ovarian characteristics and uterine horn diameters for the Finnish Landrace and Scottish Blackface control lambs during 1975 are given in Tables 4.7 and 4.8 respectively. The mean levels of oestrogens in the control Finnish and Blackface lambs are shown in Figs. 4.12 and 4.13 respectively. The individual values for all animals are given in Appendix 4.

In the control lambs belonging to both breeds there were no large follicles visible on the surfaces of the ovaries. However, as described for the 35 day-old control lambs during 1974, the ovarian surfaces in all the Finnish and most of the Blackface lambs were covered with small vesicular follicles up to 2 mm in diameter. The ovaries of Blackface lamb No. 67 were small and white with no visible follicles on the surface. The mean ovarian weights and volumes in the control lambs were both significantly greater in the Finnish lambs than in the Blackfaces ($P < 0.05$ in each case).

The mean oocyte counts for the ovaries from Finnish and Blackface control lambs during 1975 are shown in Table 4.9. Some of the ovaries were processed badly, resulting in compression of the peripheral region of the sections. Therefore the counts from only three animals of each breed have been included in the results. There was no significant difference in the number of oocytes in

primordial or growing follicles between the two breeds. The number of oocytes in vesicular follicles was significantly greater in the Finnish lambs than in the Blackfaces ($P < 0.05$). The degree of follicular atresia was extremely variable among the lambs of both breeds. In a few animals there were no obvious degenerative changes in the vesciular follicles. In most lambs, however, some of the follicles were atretic and in a few lambs the majority of the vesicular follicles were in advanced atresia.

There was no significant difference between the mean uterine horn diameter for each breed. The mean levels of oestrogens in the control lambs are shown in Figs. 4.12 and 4.13 for the Finnish and Blackface lambs respectively. As can be seen the level remained low in both breeds; the mean concentration of oestrogens for all the control lambs, both Finnish and Blackface, was 6.9 ± 4.5 pg/ml.

(b) PMSG-treated

The details regarding bodyweights at slaughter, ovarian characteristics and uterine horn diameter for the Finnish and Blackface lambs treated with PMSG during 1975 are given in Tables 4.10 and 4.11 respectively. The plasma levels of oestrogens in the treated Finnish and Blackface lambs are shown in Figs. 4.12 and 4.13 respectively. The individual values for all animals are given in Appendix 4.

As in previous years there was considerable variation in the response of both Finnish and Blackface lambs to PMSG. Two of 13 (15%) Finnish lambs and 5 of 15 (33%) Blackface lambs failed to respond in terms of large follicles in their ovaries. This

difference between the breeds was not significant. Among the treated lambs that failed to develop large follicles in their ovaries, two lambs, Finnish No. 92 and Blackface No. 61, had ovarian weights and volumes much greater than found in the control lambs. The reproductive tract from the Finnish lamb No. 92 is depicted in Plate 4.5. It appeared that the ovaries of these two lambs had been stimulated by PMSG although no large follicles had developed. Although in most of the treated lambs that failed to develop large follicles the concentration of oestrogens remained low, similar to the control values, Blackface No. 61 showed an elevation in the concentration of oestrogens reaching a peak of 110 pg/ml on day 4.

In the lambs that did respond to PMSG by the development of large follicles, the response ranged from a single large follicle to the ovarian surfaces being covered in large follicles. As an illustration of each of these responses the reproductive tracts from two treated lambs are depicted in Plates 4.6 and 4.7. The mean result of 20.3 ± 14.3 large follicles in the Finnish lambs was not significantly different from the mean result of 10.3 ± 12.7 large follicles in the Blackface lambs (Tables 4.10 and 4.11). Although the range in the response was similar in the two breeds most of the Blackface lambs produced a small number of large follicles whereas the results in the Finnish lambs were more evenly distributed. To take into account this different distribution, the results for the two breeds were also compared by the Mann-Whitney statistical test. This also showed no significant difference between the Finnish and Blackface lambs in their

follicular response to PMSG.

In many of the lambs a proportion of the large follicles showed signs of luteinisation or ovulation as demonstrated in Plate 4.8. In both breeds the percentage of the total number of large follicles that showed signs of ovulation or luteinisation was calculated. The values of 35% for the Finnish lambs and 30% for the Blackface lambs were not significantly different.

As in 1973 and 1974 ovarian volumes in the PMSG-treated lambs reflected the number of large follicles in the ovaries. There was a significant correlation between ovarian volume and the number of large follicles ($r = 0.93$; $P < 0.001$) for all treated Finnish and Blackface lambs as shown in Fig. 4.14. Ovarian weight was also significantly correlated with the number of large follicles ($r = 0.91$; $P < 0.001$) for all treated Finnish and Blackface lambs as seen in Fig. 4.15. In the Finnish lambs both the mean ovarian weights and volumes were significantly greater in the treated lambs than in the controls ($P < 0.05$ in each case). In the treated Blackface lambs the mean ovarian weights and volumes were not significantly different from the control values.

The levels of oestrogens in the PMSG-treated lambs are shown in Figs. 4.12 and 4.13 for the Finnish and Blackface lambs respectively. In general, in the treated lambs that were stimulated to produce large follicles in their ovaries, the levels of oestrogens were elevated above those found in the control lambs. However in two Finnish lambs, Nos. 151S and 165, which had 2 and 3 large follicles respectively in their ovaries, the levels of

oestrogens remained low, similar to those found in control lambs. In the remainder of the stimulated lambs (Finnish and Blackface) the level of oestrogens was significantly elevated on day 1 compared to day 0 ($P < 0.01$). The level continued to increase to a peak on day 4 in all lambs. On day 5 the concentration had fallen again to a low level in all animals. The day 4 levels of oestrogens in the individual PMSG-treated lambs, that had large follicles in their ovaries, are shown in Table 4.12. There was no significant difference between the mean levels of 270 ± 263 pg/ml in the Finnish lambs and 233 ± 297 pg/ml in the Blackface lambs.

There was a highly significant correlation between the peak level of oestrogens and the number of large follicles in the PMSG-treated Finnish ($r = 0.94$; $P < 0.001$) and Blackface ($r = 0.95$; $P < 0.001$) lambs as seen in Fig. 4.16. The level of oestrogens per follicle has been calculated for the two breeds by dividing the plasma level of oestrogens on day 4 by the number of large follicles. The mean level of 23.3 ± 8.7 pg/ml/follicle for the Blackfaces was significantly greater than 10.0 ± 6.3 pg/ml/follicle for the Finnish lambs ($P < 0.001$).

The mean uterine horn diameters (See Tables 4.7, 4.8, 4.10 and 4.11) were significantly greater in treated compared to control lambs for both Finnish and Blackface breeds ($P < 0.02$ in each breed). PMSG-treatment resulted in an increase in the height of the uterine epithelium as demonstrated in Plate 4.9. Uterine epithelial measurements in treated and control lambs are shown in Table 4.13 for the caruncular and intercaruncular regions of the uterus. The histological sections from some treated lambs were not

sufficiently complete for the measurements to be carried out. In both regions the epithelium was significantly higher in the treated lambs than in the controls ($P < 0.001$ in each case).

There was no significant difference between the mean bodyweight in control and PMSG-treated lambs belonging to both breeds. Therefore the values have been pooled for further comparisons. The mean bodyweight for the Blackface lambs during 1975 was significantly lower than for the Blackface lambs at a corresponding age during 1974 ($P < 0.001$). This was probably due to the use of artificially-reared lambs during 1975. During 1973 and 1974 it was observed that the greatest response to PMSG did not always occur in the heaviest lamb. The results for 1975 verified this observation. Fig. 4.17 shows the relationship between bodyweight and the number of large follicles for all treated lambs of both breeds, and clearly there was no correlation ($r = 0.063$).

These results for 1975, using a larger number of lambs than in 1974 and a lower dose of PMSG, also demonstrated no significant difference in the response to PMSG between Finnish and Blackface lambs at 5 weeks of age. The percentage of lambs that developed large follicles, and the mean response in stimulated lambs, in terms of both follicular development and levels of oestrogens, were similar in the two breeds. A significant difference was recorded, however, in the oestrogen level per follicle, which was greater in the Blackface lambs.

4.5 DISCUSSION

Puberty in the sheep normally occurs at the beginning of the first or second breeding season after birth. The earliest age at which oestrous cycles and ovulation are initiated is therefore around 5 months of age. However the ovaries are by no means inactive during the period from birth to puberty. Vesicular follicles are present in the ovaries of sheep at birth (Mauleon, 1969; Land, 1970a). The subsequent growth and development of the ovaries has been studied in Merino lambs by Kennedy et al. (1974). An increase in the number of both growing and vesicular follicles had occurred by 4 weeks of age. Ovarian weights reflected the follicle counts and increased to a plateau at 4-8 weeks of age. In the present study the ovaries of neonatal Finnish Landrace and Scottish Blackface lambs were examined and their development followed the pattern described by Kennedy et al. (1974). At 7 days of age the ovaries were small with no visible follicles on their surface. A progressive increase in ovarian volume at 21 and 35 days of age was accompanied by an increase in follicular development. At 35 and 49 days of age the ovarian surface was covered in vesicular follicles generally no larger than 2 mm in diameter. In some of the lambs at 35 days many of these vesicular follicles were undergoing atresia. By 12 weeks of age Kennedy et al. (1974) observed a decrease in ovarian weight with many atretic scars in the ovaries and from this age until 33 weeks there was a reduction in the number of growing and vesicular follicles. It therefore appears that a wave of follicular

development occurs during the first few weeks of life in the sheep.

During the present studies the levels of total unconjugated oestrogens were estimated in samples of peripheral plasma from control lambs. At all ages, between 7 and 130 days, the levels were low throughout the sampling periods. The mean level of 6.9 ± 4.5 pg/ml in all control lambs (Finnish and Blackface) during 1975 was similar to the base levels of 5.1 ± 1.7 pg/ml and 5.3 ± 1.7 pg/ml recorded in mature Finnish and Blackface ewes (Chapter 3). The levels appeared to vary more in the lambs compared to the adults with individual values as high as 15 pg/ml. Due to the fact that the estimation of plasma oestrogens in the lambs was carried out on a smaller sample volume than for the adults the levels recorded in the lambs were at the lower end of the standard curve where the precision of the assay was not very good. These observations must therefore be viewed with caution.

Observations were also recorded on the uteri from control lambs during the present study. Uterine horn diameter increased between 7 and 35 days of age and uterine gland development also tended to increase over the same period. However, the uterine epithelial height in the caruncular and intercaruncular regions of the uterus showed no significant changes with age. The results conform to the pattern of uterine development in the neonatal lamb described by Kennedy et al. (1974). They observed a doubling of the uterine weight between birth and 4 weeks, although after this age it did not vary significantly until 33 weeks of age. At birth there were only a few endometrial folds projecting into the lumen and no glands were present, but these

developed progressively at later ages. They recorded the greatest height of uterine epithelium at birth, with a subsequent decline to the lowest height between 8 and 12 weeks of age. They attributed the uterine epithelial growth at birth to the presence of oestrogens in the blood of the foetus at term (Findlay and Cox, 1970). The gross development of the uterus between birth and 5 weeks of age may be related to the increasing follicular development during the same period.

During the studies described here PMSG and HCG were administered to neonatal lambs in an attempt to stimulate follicular development. Overall the results demonstrated a tremendous variation in the response to PMSG. This has been attributed by earlier workers to a variety of factors. Breed or strain differences have been found to influence the ovulation response following PMSG treatment (Bradford et al., 1971). In addition the stage of the oestrous cycle may influence the response (Wallace, 1954). Wallace (1954) suggested that different batches of PMSG may result in different mean ovulation rates. According to Nalbandov (1964) the ratio of FSH-like to LH-like activity in PMSG may vary. Furthermore McIntosh et al. (1975) have suggested that the clearance rate of PMSG from the blood may vary in relation to the sialic acid content of PMSG. It was not possible to use the same batch of PMSG throughout the three years of the present study since the manufacturers recommend a shelf-life of two years. However the same batch was used within any one year in an attempt to overcome the problem of variability between batches as far as possible.

Throughout these studies a constant dose of PMSG was administered to all the animals within any one group, irrespective of bodyweight. This is the customary way of using this hormone and would appear to be justified by the finding, during 1975, that within a group of lambs of the same age bodyweight had no significant effect on the response to PMSG, in terms of follicular development.

Preliminary studies during 1973 were designed to establish the effect of varying doses of PMSG in lambs of different ages. 250 i.u. stimulated the development of a single large follicle in one out of three lambs at 30 days of age. 500 i.u. consistently stimulated the growth of a small number of large follicles (range 1-7) in lambs at 30, 69, 94 and 130 days of age. The highest dose, 1000 i.u., was administered to lambs at 30 and 130 days of age and resulted in a greater and more variable response compared with 500 i.u. During 1974, 750 i.u. PMSG produced excessively stimulated ovaries in some animals. Since one intention of these studies was to compare the response to PMSG in Finnish and Blackface lambs it was important to produce as consistent a response as possible. Therefore the dose was reduced to 500 i.u. during 1975. Unfortunately the results obtained using 500 i.u. during 1975, with a range of 2-41 large follicles in the Finnish lambs and 1-40 large follicles in the Blackface lambs, were not as consistent as those obtained using the same dose during 1973. This may have been due partly to the unavoidable use of different batches of PMSG in different years but may also have resulted from the different group sizes and the different ages studied during

these two years.

For similar reasons it is difficult to compare these results with those of other workers. Moreover Mansour (1959) and Land and McGovern (1968), who have administered PMSG to neonatal lambs, were interested in producing large numbers of ova for transplant purposes. Nevertheless Land and McGovern (1968), using two doses, 350 i.u. and 750 i.u., of PMSG, also observed a greater response with the higher dose. Mansour (1959) used 1000 i.u. of PMSG and obtained excessive ovarian stimulation in some lambs similar to that recorded in a few lambs in this study using the higher doses.

During 1974 the response of Finnish Landrace and Scottish Blackface lambs was investigated at four different ages, 7, 21, 35 and 49 days. The results, showing an increase in the proportion of lambs stimulated to develop large follicles with advancing age, confirmed reports by earlier workers that the ovaries of very young lambs are refractory to exogenous gonadotrophins. Mansour obtained no response to 1000 i.u. PMSG in lambs of 7 days of age, with only a proportion responding at 28 days. Mauleon (1969) stated that the ovary of the lamb does not respond to the administration of PMSG and HCG before the 20 day of life. In contrast to their results 2 out of 8 lambs responded to 750 i.u. PMSG at 7 days of age in the present study. At 21 days 6 out of 8 lambs responded and at 35 days all lambs responded during 1974 when 750 i.u. PMSG were used. However only a proportion of 35 day-old lambs developed large follicles during 1975 when the dose was reduced to 500 i.u.

This absence of follicular development in new-born lambs treated with PMSG is in agreement with the results for several other species. Zarrow and Wilson (1961) failed to obtain ovulation following treatment with PMSG and HCG in mice prior to 13 days of age and in rats prior to 17 days of age. A response in terms of an increase in ovarian weight did not occur before 65-70 days of age in rabbits (Fox, Cavanaugh and Zarrow, 1964). However calves have been found to respond to PMSG at birth (Marden, 1953). It is possible that the ovaries of all mammals are refractory to exogenous gonadotrophins before a specific age which is characteristic of each species. The present results suggest that the age of first response, at least in the sheep, also varies considerably among individual animals.

In the past the failure of the neonatal ovary to respond to exogenous gonadotrophins has been attributed to the absence of follicles containing antra (Zarrow and Wilson, 1961). The onset of antrum formation occurs at 12-14 days of age in mice (Engle, 1931) and 11 days of age in rats (Hargitt, 1930). However in the sheep vesicular follicles were present in the ovaries of 7 day-old lambs during the present study and other workers have established the presence of vesicular follicles at birth (Mauleon, 1969; Land, 1970). Therefore in this species vesicular follicles are present in the ovaries before the appearance of ovarian sensitivity to PMSG.

Hertz and Hisaw (1934) showed that the insensitivity of the infantile ovary (<10weeks) in the rabbit was inherent within the ovary; 4 week old rabbit ovaries implanted into the kidney

of a juvenile rabbit (> 12-13 weeks) failed to respond to stimulation by pituitary extract, unlike the host ovary. Hisaw (1947) stated that the acquirement of sensitivity is correlated with the time at which the cells of the theca interna normally start differentiating into epithelioid tissue. Recognising the fact that oestrogens sensitise the ovary to PMSG injections (Williams, 1940), Mansour (1959) argues that refractoriness in postnatal lambs was due to a lack of secretion of oestrogens, resulting from the failure of the immature pituitary to secrete enough LH. This would support Hisaw's theory since oestrogens are thought to be secreted by the theca interna (See Chapter 1, section 1.3). As stated previously levels of oestrogens in control animals throughout the present study were too near the sensitivity limit for the assay to be able to state definitely that the ovaries of neonatal lambs are secreting oestrogens. During the first 3 weeks after birth levels of FSH and LH have been found to be similar to baseline concentrations found in the adult ewe on day 7 of the oestrous cycle (Foster et al., 1975). Clearly more information is required before this ovarian refractoriness in postnatal animals belonging to different species can be fully explained.

Following the development of ovarian sensitivity in the neonatal lamb the response, in terms of follicular development, appeared to be greatest in the younger lambs during the present study. During 1973 the response was greater at 30 days than at 130 days of age, following treatment with 500 or 1000 i.u. PMSG. During 1974, in lambs of 49 days of age and less, 750 i.u. PMSG

produced a range of 1-200 large follicles. A dose of 500 i.u. during 1975 produced a range of 1-41 large follicles at 35 days of age. It is difficult to compare these results with other studies, using different doses of PMSG and also different breeds of sheep. Nevertheless it does appear that the response to PMSG is greater and more variable during the first 8 weeks of life than at later ages. Mauleon (1969) observed a peak response at 30 days of age. Mansour (1959) obtained a greater response in young lambs, with the ovaries being covered in large follicles in some 4 and 8 week-old lambs. Other studies, involving the administration of PMSG to 9 week-old lambs (Land and McGovern, 1968) or adult ewes of various breeds (Bradford et al., 1971) did not result in the variation in follicular development seen in lambs of 7 weeks of age and less during the present study.

The pattern of response to PMSG seen in the neonatal lamb is similar to that recorded in other species. An increase in response with advancing age to a peak, followed by a subsequent decline in the response has been described for mice and rats (Zarrow and Wilson, 1961) and rabbits (Fox et al., 1964). Zarrow and Wilson (1961) considered the decline in response reflected an increased requirement for gonadotrophins. However from the present studies it would appear more likely that the greater response to PMSG during the first 8 weeks of life in the sheep reflects the situation in the control ovaries. The wave of follicular development resulting in a large number of small vesicular follicles probably accounts for the massive development of large follicles following the administration of PMSG. The variation in the degree

of follicular atresia observed in control lambs of 5 weeks of age during the present study may explain the wide range in the response to PMSG at this time.

During two years of the present study HCG was administered to the lambs following treatment with PMSG in order to study ovulation rates as well as follicular development. From the early work of Mansour (1959) in the lamb a dose of 500 i.u. was selected and administered 4 days after the PMSG, a regime which Mansour found satisfactory. During the present study the proportion of ovulated and luteinised follicles was 30-35%. Therefore the number of follicles that actually ovulated was not very great. Land and McGovern (1968) have shown that the time interval between administering PMSG and HCG can influence the proportion of follicles that ovulate; in 9 week-old lambs 20 out of 43 follicles (47%) formed corpora lutea when the interval was 2 days compared to only 9 out of 38 follicles (24%) when the interval was 3 days. This may account for the small number of follicles that actually ovulated during the present study.

The delayed administration of HCG may also account for the presence of luteinised follicles. Follicles were described as luteinised when they were no longer tense and shiny but appeared solid and discoloured. Internal haemorrhage had occurred in many of these follicles. Although they were termed luteinised, their histological structure was not determined. However they probably represented a response to HCG since they were not seen in the ovaries of lambs during 1974 that received PMSG alone. Hill and Parkes (1930) also reported haemorrhagic follicles in the ovaries

of PMSG-treated rabbits that failed to ovulate. The condition was associated with a well-luteinised granulosa round the periphery of the blood follicles. A similar finding has been reported in rabbits which are not allowed to mate during the breeding season (Hammond, 1925). The condition was due to follicles becoming mature, to the extent that congestion of the blood vessels of the theca had occurred, and then not receiving the correct stimulus for ovulation. Breakdown of the thecal blood vessels resulted in the formation of haemorrhagic follicles.

Following the use of PMSG alone during the present study neither ovulation or luteinisation were observed (with the exception of one lamb) at 7, 21, 35 and 49 days of age. This is consistent with the results of Mansour (1959) who found that treatment with PMSG alone did not produce luteunised follicles until 12 weeks of age and ovulation did not occur until 16 weeks of age. Furthermore Mansour (1959) found that even allowing 6 days after PMSG-treatment before examining the ovaries, spontaneous ovulation or luteinisation did not occur in the younger lambs. Therefore the absence of ovulation following PMSG-treatment during the present study was not due to the ovaries being examined at an earlier time than when PMSG and HCG were administered.

Reports of the use of PMSG in mature ewes indicate that ovulation of PMSG-induced follicles does occur spontaneously without the administration of a further exogenous stimulus (Cole and Miller, 1933). It has been suggested that secretion of oestrogens by the PMSG-induced follicles in the mature ewe has a positive feedback effect on the anterior pituitary stimulating the

release of an ovulatory surge of LH (McCracken et al., 1971). In the neonatal lambs in the present study elevated levels of oestrogens were detected following PMSG-stimulation. Therefore the stimulus for the release of LH was present. However Foster and Karsch (1975) failed to elicit a discharge of LH following the administration of oestradiol-17β at 3 weeks of age. At 7, 12 and 20 weeks LH surges of progressively increasing magnitude were elicited. Present studies (described in Chapter 5) have also demonstrated that exogenous oestradiol-17β will not consistently induce the release of LH in lambs of 42 days of age. Therefore the absence of spontaneous ovulation in neonatal lambs following PMSG-stimulation of the ovaries is probably due to the immaturity of the positive oestrogen feedback.

Throughout the present studies the level of oestrogens has been estimated in samples of peripheral plasma collected daily throughout an experimental period. A good correlation existed between the plasma level of oestrogens on day 4 and the number of follicles, 4 mm or more in diameter, in the ovaries. This suggested that the majority of the oestrogens were being secreted by the ovarian follicles rather than another ovarian compartment, such as the stromal tissue, or by the adrenal glands. This is consistent with current knowledge on the secretion of oestrogens in the sheep. There is evidence that the interstitial gland tissue may not secrete oestrogens (Roche et al., 1974) and although oestrone has been detected in adrenal venous blood of the ewe (Baird, 1968), the adrenal secretion of oestrogens in the neonatal lamb is not known.

The secretion of oestrogens by sheep follicles of various sizes *in vitro* has been studied by Moor (1973). High levels of oestrogens were produced by follicles greater than 4.5 mm in diameter whereas follicles smaller than this secreted low levels of oestrogens throughout the culture period. However in adult ewes, stimulated by PMSG, follicles 2 mm or more in diameter have been shown to secrete significant amounts of oestrogens (Moor, Hay, McIntosh and Caldwell, 1973). This probably explains the elevated levels of oestrogens in a Blackface lamb (No. 61), during 1975 of this study, which had no large follicles in the ovaries. Ovarian size in this lamb was considerably greater than in control lambs of the same age, due to a massive development of small vesicular follicles. These follicles were responsible probably for the oestrogens secreted in this animal. Another anomaly in two Finnish lambs during 1975 (Nos. 151S and 165) was the presence of two or three large follicles in the ovaries associated with consistently low levels of oestrogens similar to those found in control lambs. Due to the small plasma sample size used for the estimation of oestrogens in the neonatal lambs the peak levels in these two animals may have been below the detection limit for the radioimmunoassay.

In the PMSG-treated lambs in this study there was a tendency for the production of oestrogens to be dependent on the age of the animal. During 1973 in lambs of 130 days of age the levels of oestrogens were not elevated above those in the control lambs, following treatment with 500 or 1000 i.u. PMSG, despite the presence of many large follicles in the ovaries of some of the

lambs. At 69 and 94 days of age only slightly elevated levels of oestrogens were detected following the administration of 500 i.u. PMSG. However in lambs of 49 days of age and younger the presence of large follicles in the ovaries was associated with elevated levels of oestrogens following 500, 750 or 1000 i.u. PMSG (and during all three years of the study). Furthermore the value of 23.3 $\pm$ 8.7 pg/ml/follicle calculated for the Blackface lambs during 1975 was greater than the mean peak value of 9.1 $\pm$ 5.4 pg/ml in mature Blackface ewes during prooestrus, with around one preovulatory follicle in their ovaries. Although the value of 10.3 $\pm$ 6.3 pg/ml/follicle calculated for the Finnish lambs was similar to the mean peak value of 11.4 $\pm$ 4.1 pg/ml in Finnish ewes during prooestrus, there would have been around 2 preovulatory follicles in the Finnish ewes at this time.

It is possible that the many small vesicular follicles present in the ovaries of the younger lambs may have contributed to the high levels of oestrogens following PMSG-treatment. Moor *et al.* (1973) have shown that after stimulation by PMSG *in vivo* small vesicular follicles (2-3 mm diameter) secreted significant amounts of oestrogens in culture. However another explanation may be that the secretion of oestrogens by preovulatory follicles in the cycling ewe is terminated naturally at an earlier stage than it was terminated artificially in the experimental lambs. This could account for the higher peak levels of oestrogens in the lambs.

In the PMSG-stimulated lambs an elevation in the concentration of oestrogens had occurred by 24 hours after treatment (*i.e.* when the next sample of blood was collected). Moor *et al.*

(1973) found that after the administration of PMSG *in vivo* some follicles, explanted after only 5 minutes, secreted significantly greater amounts of oestrogens in culture than follicles from control sheep. In the present study the levels continued to rise steadily for 3-4 days. After this time, in lambs that received only PMSG, the levels became irregular, falling in some animals and continuing to rise in others. In contrast, in the lambs that received HCG, the level of oestrogens fell immediately to a low level. Moor (1974) has shown from *in vitro* and *in vivo* studies that oestrogen secretion by large follicles can be rapidly terminated by LH. It is highly likely therefore that HCG was responsible for the immediate fall in the levels of oestrogens in the present studies.

In intact adult sheep treated with PMSG the secretion of oestrogens is thought to be responsible for stimulating the rise in LH that occurs within 41-46 hours of treatment (Cumming *et al.*, 1971a). Moor *et al.* (1973) found that the level of oestrogens in the ovarian vein blood of adult ewes, following treatment with PMSG, was still elevated at 60 hours after treatment but had declined by 84 hours. This implied that in PMSG-treated ewes the secretion of oestrogens may be terminated by the endogenous release of LH. In addition Moor (1974) has suggested that in the normal cycling ewe endogenous LH is responsible for terminating the secretion of oestrogens by the preovulatory follicles.

During 1974 and 1975 of the present studies a significant correlation was found to exist between the plasma level of oestrogens on day 4 and the number of large follicles in the ovaries. It

must be remembered that during 1974 the follicle count was recorded on day 4 whereas during 1975 it was recorded on day 6. This did not appear to influence the correlation. Other workers, using different species, have also found a good correlation between the rate of oestradiol-17β production and the number of ovulations in superovulated cows (Saumande and Pelletier, 1975) and between the rate of urinary oestrone excretion and the degree of ovarian stimulation in infertile women treated with gonadotrophins (Hancock, Scott, Stitch, Levell, Oakey and Ellis, 1970). A positive relationship has also been demonstrated between mean levels of oestrogens and the dose of PMSG used to superovulate cows (Henricks, Hill, Dickey and Lammond, 1973) and pigs (Guthrie, Henricks and Handlin, 1974). The ability to relate accurately the level of oestrogens in the peripheral circulation to the number of ovulating follicles would be valuable in superovulation studies, whether these were aimed at the production of multiple births or the production of ova for transplant experiments. The present studies suggest that it may be possible, to some extent, to determine the degree of follicular development in sheep by estimating the plasma level of oestrogens.

It has also been demonstrated from the present results that the breed of sheep may influence the relationship between the number of large follicles and the plasma level of oestrogens. During 1975 the mean level of oestrogens/follicle was greater in the Blackface lambs than in the Finnish lambs. There are two possible explanations for this finding. Firstly the ovaries in the two breeds may not have matured to a similar degree at 5 weeks

of age resulting in differing abilities to produce oestrogens at this age. Alternatively ovarian follicles may secrete greater amounts of oestrogens in Blackface sheep than in Finnish sheep, irrespective of age. The results for mature cycling ewes presented in Chapter 3 showed that the peak level of oestrogens during prooestrus was similar in the two breeds, although there would have been a larger number of pre-ovulatory follicles in the ovaries of the Finnish ewes at that time. Although these results could have been due to the pre-ovulatory follicles in the Finnish ewes developing slightly out of phase, taken with the results for the neonatal lambs they imply that ovarian follicles in the two breeds do secrete different quantities of oestrogens.

Following the treatment of lambs with exogenous gonadotrophins in the present study an alteration in uterine structure was recorded. The uterine horn diameter was significantly greater in the treated lambs than in the controls. Furthermore there was a significant increase in uterine epithelial height. In the mature ewe the maximum development of uterine epithelium together with the greatest proliferation and secretory activity of the uterine glands occurs during prooestrus (Restall, 1966). This coincides with the highest circulatory level of oestrogens recorded during the cycle (Scaramuzzi et al., 1970). Kennedy et al., (1974) related the increased uterine epithelial height at birth to the presence of oestradiol-17α and oestradiol-17β in the plasma of the sheep foetus at term (Findlay and Cox, 1970). Liefer et al. (1972) have shown that the uterus of the neonatal lamb requires the presence of the ovaries for normal growth. The same authors

obtained a significant increase in uterine weight following the administration of oestradiol-17β to both intact and ovariectomised lambs, demonstrating that the uteri were capable of responding to exogenous oestrogens. The uterine development seen in PMSG-treated lambs in the present study demonstrates the biological activity of endogenously secreted oestrogens. The responsiveness of the uterus in the neonatal lamb is in contrast to the apparent insensitivity at similar ages of the hypothalamic centres responsible for the feedback control of the pituitary.

The intention, throughout the present studies was to compare ovarian activity in Finnish Landrace and Scottish Blackface sheep. In normal 5 week-old lambs the number of oocytes in vesicular follicles was significantly greater in the Finnish lambs compared to the Blackfaces. This probably accounted for the significantly greater ovarian weight and volume in the Finnish lambs at this age. The number of oocytes in primordial and growing follicles was not different in the two breeds. Similarly Land (1970a) recorded a greater proportion of oocytes in follicles with an antrum in Finnish x Blackface lambs compared to Blackfaces at birth. He suggested that the basal gonadotrophin levels, or the ovarian sensitivity to gonadotrophins must be greater in the more fecund breed of lamb.

Ovarian sensitivity to treatment with exogenous gonadotrophins was compared in Finnish and Blackface lambs during the present study. The results were assessed in terms of the proportion of lambs responding, the number of large stimulated follicles, the proportion of these follicles that showed signs of

ovulation or luteinisation and the plasma level of oestrogens. In all aspects of the response, and during the two years of the study when the two breeds were compared, there was no significant difference between the Finnish and Blackface lambs.

Since the normal development of vesicular follicles has been shown to be greater in the Finnish lambs it was surprising that the ovarian response to a further exogenous gonadotrophic stimulus was similar in the two breeds. It suggests that the situation in the 5 week-old lamb must be different from that in the adult in which a significant difference in the response to PMSG has been reported between breeds and strains of sheep of differing fecundity (Bindon et al., 1971; Bradford et al., 1971; Trounson and Moore, 1972).

As discussed previously in this Chapter, these studies were conducted at an age when the positive and negative oestrogen feedbacks, controlling the pituitary secretion of gonadotrophins, have not fully matured. The oestrogens stimulated in the lambs are therefore unlikely to have influenced the release of gonadotrophins by the animal's own pituitary. In that case the results obtained in the lambs, following treatment with PMSG, represented solely the response to this exogenous stimulus. Since the stimulated development of large follicles was similar in the Finnish and Blackface lambs it can be concluded that ovarian sensitivity was similar in the two breeds during the neonatal period.

CHAPTER FIVE

THE EFFECT OF METHALLIBURE ON NORMAL AND INDUCED FOLLICULAR DEVELOPMENT IN NEONATAL LAMBS

5.1 INTRODUCTION

The relationship between the pituitary secretion of LH and ovarian steroid secretion is well documented for the adult ewe (Geschwind, 1972). However the early development of this relationship in the foetal and neonatal lamb is not fully known.

In the foetal lamb both FSH and LH have been detected in the pituitary. An increase in LH content with gestational age has been reported (Foster, Roach, Karsch, Norton, Cook and Nalbandov, 1972a; Mauleon and de Reviers, 1969) reaching higher levels in male than in female lambs (Foster et al., 1972a). Pituitary FSH content did not vary at different ages but was greater in female than in male foetal lambs (Mauleon and de Reviers, 1969).

The levels of LH have been estimated in the circulation of the foetal lamb (Foster et al., 1972a; Alexander et al., 1973) but there is a lack of information on the blood levels of FSH in the foetus. Serum LH levels reached a maximum during mid-gestation (Foster et al., 1972a). A study of the placental transfer of LH in the sheep showed that LH in the circulation of the foetal lamb was probably of foetal pituitary origin (Foster, Karsch and Nalbandov, 1972b). Foster et al. (1972a) have suggested that the decreasing levels of serum LH during the final third of gestation are the result of rising levels of placental steroids exerting a negative feedback on the foetal pituitary.

At birth a significant decrease in pituitary LH levels has been demonstrated by Foster et al. (1972a) which may have been due to the sudden removal of the maternal influence on the lamb.

However, the same workers could not detect an accompanying rise in serum LH at this time. Both serum and pituitary LH concentrations have been shown to rise gradually during the first 18 days of life in the female lamb (Foster et al., 1972a)and Land, Thimonier and Pelletier (1970) have shown that LH levels in the serum continue to rise between 13 and 77 days of age. In comparison, the concentrations of pituitary and serum LH in males remained steady during the first 18 days of life. There is no information concerning the pituitary and serum concentrations of FSH in the neonatal lamb.

There is evidence to suggest that hypothalamic control of synthesis and release of gonadotrophins by the pituitary is at least partially functional in the foetal and neonatal lamb. Foster, Jackson, Cook and Nalbandov (1972d) detected luteinising hormone releasing factor (LRF) activity in the hypothalamus of the neonatal lamb although they failed to detect it prenatally. However, the pituitary of both the late foetal and neonatal lamb was capable of responding to the administration of LRF, by the release of LH (Foster, Cruz, Jackson, Cook and Nalbandov, 1972c). In the neonatal lamb the response was lowest during the immediate post-natal period due, it was suggested, to the low pituitary stores of LH at this time.

Although the hypothalamic-hypophyseal axis may be functional in the new-born female lamb the relationship between this axis and the gonads is not fully developed. The fact that serum LH levels increase after birth suggests that in the female lamb the negative oestrogen feedback is not established at this

age (Foster et al., 1972a). In addition Foster et al. (1975) have reported an episodic release pattern for LH in intact female lambs after 4-5 weeks of age that was similar to that in the long-term ovariectomised adult. This is in comparison to the male lamb in which serum LH levels remained low after birth (Foster et al., 1972a). Castration at birth (Foster et al., 1972e) caused serum LH levels to rise in male lambs, further evidence that the testes are capable of suppressing circulating gonadotrophins at this age. Ovariectomy of the new-born female lamb had no effect on serum LH levels during the first two weeks after birth (Foster et al., 1972e).

The lack of a fully competent negative control system for LH is not due to the inability of the hypothalamo-hypophyseal unit to recognise circulating ovarian steroids since serum LH has been suppressed to undetectable levels by exogenous oestradiol-17β (Liefer et al., 1972). This implies that the ovaries of the neonatal lamb do not produce sufficient quantities of an inhibitory steroid to suppress the release of LH. However, the greater uterine weight at 6 weeks after birth in intact lambs compared to neonatally ovariectomised females (Liefer et al., 1972) suggests that the ovary becomes competent to produce oestrogens early in postnatal development. Foster et al. (1975) have recently shown that ovariectomy at 2 weeks of age produced a delayed rise in both FSH and LH levels beginning 4-5 weeks later. Their results indicated that by 9 weeks of age the ovary of the lamb had influenced the secretion of FSH and LH.

The positive feedback effect of oestrogens, which facilitates the secretion of LH, is now well documented (Everett,

1964). It is generally accepted that in the mature cycling ewe the increased secretion of oestrogen, during prooestrus, stimulates the preovulatory release of LH (Goding et al., 1969; Scaramuzzi et al., 1970). The administration of oestradiol-17β to anoestrous ewes was followed in all animals by increased LH secretion (Goding et al., 1969; Symons et al., 1973). It has been suggested that although the anterior hypothalamus is necessary for this positive feedback, the anterior pituitary may be the main site of action of oestrogen (Döcke and Dörner, 1965).

There is evidence to suggest that the positive oestrogen feedback mechanism is not fully competent in the neonatal lamb. Land et al. (1970) administered oestradiol benzoate to female lambs of various ages to provoke the release of LH. Only 1 of 6 lambs responded at 13 days of age, whereas at 38 days of age 5 of 6 lambs showed a response. Similarly Foster and Karsch (1975) obtained no release of LH following treatment with oestradiol-17β at 3 weeks of age, whereas progressively increasing surges of LH were stimulated from 7 weeks of age onwards.

The role of gonadotrophins in the normal development of the ovary in the foetal and neonatal lamb is not clear. Hypophysectomy of foetal lambs in utero (Liggins and Kennedy, 1968) appeared to show that ovarian growth would proceed independently of pituitary secretions. Nevertheless, folliculogenesis is initiated during foetal life and by the end of gestation there are many growing and vesicular follicles present in the ovary (Mauleon, 1969). The continual secretion of both FSH and LH are thought to be necessary for the normal development of growing and vesicular

follicles in the mammalian species that have been studied (Schwartz, 1974).

Throughout the present studies various aspects of ovarian activity have been examined in Finnish Landrace and Scottish Blackface sheep. In Chapter 4 the response to PMSG was compared in neonatal lambs belonging to the two breeds. During any study designed to compare ovarian sensitivity to exogenous gonadotrophins, the results may be confused by interference from endogenous gonadotrophins. In an attempt to overcome this problem the use of the pituitary blocking agent, 1-α-methylallylthiocarbamoyl-2-methylthiocarbamoylhydrazine (I.C.I. 33,828 or methallibure), has been investigated in the neonatal lamb.

The pituitary inhibiting action of methallibure was initially observed in rats (Paget, Walpole and Richardson, 1961). The compound was administered orally to male rats, producing atrophy and loss of secretory activity in the accessory sex glands. Treatment for a longer period also produced damage to the spermatogenic epithelium. Loss of fertility has been observed in treated male rats (Walpole, 1965). Methallibure has also been shown to suppress sperm production and libido in male rabbits (Skinner and Adams, 1969), pigs (Call, Barker and Cummings, 1969) and dogs (Call and Barker, 1967).

In female rats, orally dosed with methallibure, cyclic activity, as assessed by vaginal smears, ceased within 2-3 days (Paget et al., 1961). There was a loss in ovarian weight and histological examination showed a decrease in the number of maturing follicles.

The ability of methallibure to inhibit pituitary function in female pigs has been utilised for practical oestrous synchronisation in this species (Polge, 1965). The compound was highly effective in suppressing oestrus and ovulation in all animals, provided oral administration was continued for at least 20 days. Ovaries of treated animals contained only a few small follicles and the corpora lutea were pale and regressing; the uteri were small, resembling prepubertal uteri (Polge, 1965). After withdrawal of treatment follicular development was initiated quite quickly, the average length of time from withdrawal to the next oestrus being 6 days.

The addition of methallibure to the diet of cycling ewes was not very successful in suppressing oestrus (Gerrits, Kraeling and Sidwell, 1965b) but the results suggested that higher levels of methallibure than were used may be necessary to synchronise oestrus in the ewe.

From the initial work on the effects of methallibure, it was concluded that the compound produced reversible inhibition of pituitary gonadotrophic function (Paget et al., 1961). The finding that pituitary glands from treated rats contained less total gonadotrophin and less FSH than the glands from control rats (Brown, 1963) suggested that it acted by reducing the formation of pituitary gonadotrophins. Another study, using rats, showed that both pituitary and serum LH levels were lowered (Labhsetwar and Walpole, 1972) implying that both synthesis and release of pituitary LH were inhibited. In pigs methallibure has been shown to cause an increase in pituitary FSH and LH activity (Garbers and First,

1969a; Stormshak, Leverage, Kelley, Gerrits and Howland, 1970) suggesting that in this species synthesis of gonadotrophins was not inhibited but their release had been blocked. Garbers and First (1969a) explained this difference between the two species as being due to the different treatment regimes that were used.

Apparently methallibure does not have a direct effect on the gonads. Paget et al. (1961) demonstrated that methallibure did not affect the gonadal response to exogenous hormones in rats of either sex and Polge and Day (1969) have shown that PMSG is capable of stimulating ovulation in gilts under methallibure treatment. Although Brown (1963) obtained a modified response to exogenous gonadotrophins in methallibure-treated rats, he considered this may not have been due to a specific effect at the gonadal level but either to suppression of endogenous gonadotrophins or to the effect of methallibure on the general condition of the test animals.

Methallibure has been shown also to inhibit other aspects of pituitary function. From a study of the effects on thyroid function in rats and mice (Tulloch, Crooks and Brown, 1963) it was concluded that methallibure had inhibited pituitary thyrotrophic activity as well as interfering directly with the trapping and protein-binding of iodine by the thyroid gland. The simultaneous administration of methallibure with growth hormone has been shown to suppress the action of the hormone in inducing nitrogen retention and body growth (Ramaiah and Gangadhara, 1969). Inhibition of lactation in methallibure-treated sows (Gerrits, Johnson and Kraeling, 1965a; Garbers and First, 1968) and rats

(Benson and Zagni, 1965) could be prevented by the administration of oxytocin, implying that the effect of methallibure was at the pituitary or hypothalamic level, although possible direct effects on the mammary gland were not entirely ruled out.

It has been suggested that methallibure may act via the hypothalamus to alter pituitary function. By surgically implanting small quantities of methallibure intracranially in guinea pigs Malven (1971) obtained greater suppression of ovulation with implants within or near the hypothalamus than in the anterior pituitary. However Stormshak et al. (1970) found that methallibure treatment of gilts did not affect the LRF activity of the hypothalamus.

The present studies were designed to investigate the effect of methallibure treatment on normal ovarian development and function in the neonatal lamb. The mode of action of methallibure was studied by measuring the release of LH following stimulation by luteinising hormone-releasing hormone (LH-RH) or oestradiol-17β. Ovarian sensitivity to PMSG-treatment was compared in Finnish Landrace and Scottish Blackface lambs that had received methallibure from birth.

5.2 MATERIALS

5.2.1 Animals

These studies were performed on Finnish Landrace and Scottish Blackface lambs born during the spring of 1975. The lambs were obtained from the same sources as those described in Chapter 4 (Section 4.2.1) for 1975.

5.2.2 Drugs

(a) Methallibure

Methallibure was generously provided by Dr. A. L. Walpole of Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Cheshire. The structural formula of methallibure is -

$$CH_2 = CH.\underset{\displaystyle CH_3}{\underset{|}{CH}}.NH.CS.NH.NH.CS.NH.CH_3$$

The pure methallibure powder was stored at $4^{o}C$ and a fresh suspension prepared daily for administration to the lambs. 800 mg of methallibure were added to 20 ml of a solution of 0.5% Tween 80 (Sigma) in normal saline, giving a concentration of 40 mg/ml. This was mixed thoroughly on a rotary mixer to produce an even suspension.

(b) Chorulon and Folligon

These were used as described in Chapter 4 (Section 4.2.2).

(c) Oestradiol-17β

The stock solution of 200 µg/ml oestradiol-17β in ethyl alcohol, prepared for use in the radioimmunoassay (Chapter 2,

Section 2.2) was used to provide oestradiol-17β for administration to lambs. One ml was evaporated to dryness in a glass vial to which 8 ml of ethyl oleate (B.D.H. Chemicals Ltd.) was then added. This gave a concentration of 25 µg/ml which was stored at 4°C until used.

(d) Luteinising hormone-releasing hormone (LH-RH)

LH-RH was a generous donation from Dr. J. M. J. Best of Hoechst U.K. Ltd., Hoechst House, Salisbury Road, Hounslow, TW4 6JH. It was supplied as a sterile solution in buffer at a concentration of 200 µg/ml. This was stored at 4°C until required. 0.5 ml was withdrawn and made up to 10 ml with saline to give a concentration of 10 µg/ml. This was stored at 4°C until administered to the lambs.

5.3. METHODS

5.3.1 Experimental procedure

(a) Methallibure administration

Methallibure treatment began 1 day after birth and continued until the lambs were killed. The lambs were weighed at the start of treatment and thereafter once a week, for the purpose of establishing the dose of methallibure. A final bodyweight was recorded before the lambs were killed. Methallibure was administered daily to the lambs by a single subcutaneous injection at a dose rate of 10 mg/kg. The daily dose for an individual animal was calculated on the basis of the most recently recorded bodyweight. In some lambs small abcesses developed at the injection sites and to avoid these the injection site was varied.

(b) PMSG administration

At 35 days of age, 7 methallibure-treated Finnish Landrace lambs and 5 methallibure-treated Scottish Blackface lambs received 500 i.u. PMSG by subcutaneous injection on day 0, followed on day 4 by 500 i.u. HCG administered by intravenous injection. Six Finnish and 5 Blackface methallibure-treated lambs received injections of sterile water in place of gonadotrophins. 10 ml samples of heparinised blood were collected daily between 9-10 a.m. from the jugular vein of each lamb from day 0 until slaughter. Plasma was separated from the blood samples and stored as described in Chapter 2 (Section 2.3.1) until the concentration of oestrogens could be estimated. The lambs were killed on day 6

by an intravenous injection of pentobarbitone sodium.

The normal lambs, used during 1975 and described in Chapter 4, are compared in the present Chapter with the methallibure-treated lambs. The mean results for the normal lambs, both control and PMSG-stimulated, are given alongside the mean results for the methallibure-treated lambs in the Results section of this Chapter.

(c) Oestradiol-17β administration

At an average age of 43 days (range 42-44) LH release following the administration of oestradiol-17β was studied in methallibure-treated and normal lambs. These were animals that had not been used for any previous experiments. The number of animals involved was 3 methallibure-treated Finnish lambs and 2 methallibure-treated Blackface lambs, and the same number of normal lambs of both breeds. 25 μg of oestradiol-17β in 1.0 ml of ethyl oleate were administered by intramuscular injection into the hind limb. 10 ml samples of heparinised blood were collected from the jugular veins of treated lambs, immediately prior to the injection of oestradiol-17β and every 2 hours until 18 hours after treatment. A further sample was collected at 26 hours after treatment. Plasma was separated from the samples as described in Chapter 2 (Section 2.3.1) and stored at -15°C until the samples were estimated for LH content.

(d) LH-RH administration

At an average age of 45 days (range 43-46) LH release following the administration of LH-RH was studied in methallibure-treated and normal lambs. These were animals that

had not been used for any previous experiments. The number of animals involved was 3 methallibure-treated Finnish lambs and 3 methallibure-treated Blackface lambs, and the same number of normal lambs of both breeds. A pre-treatment sample of heparinised blood was removed from the jugular vein and 5 µg LH-RH in 0.5 ml saline was immediately injected back into the vein via the same needle. Further samples of heparinised blood were collected at 20, 40, 60 and 80 minutes after treatment. Plasma was separated from the samples as described in Chapter 2 (Section 2.3.1) and stored at -15°C until the samples were estimated for LH content.

5.3.2 Examination of the reproductive tract

Following the slaughter of the methallibure-treated lambs that were described in Section 5.3.1.b, the reproductive tracts were removed and the external morphology of the ovaries and uterus examined, as described for normal lambs in Chapter 4 (Section 4.3.3). Serial histological sections were prepared from the ovaries of the Finnish and Blackface lambs that were treated with methallibure alone and oocyte counts carried out as described in Chapter 4 (Section 4.3.4). Histological sections were prepared from the uteri of methallibure-treated lambs, including those that received PMSG, and measurements of the uterine epithelium carried out as described in Chapter 4 (Section 4.3.4).

5.3.3 Examination of the pituitary glands

The pituitary glands were removed, at slaughter, from the

methallibure-treated lambs, that were described in Section 5.3.1.b, and also from the normal lambs described in Chapter 4 for 1975. Access to the pituitary gland was achieved by dissection dorsally through the cranial cavity. After stripping the skin from the dorsal surface of the skull, a pair of bone cutters was used to remove the top of the skull. This allowed the brain to be lifted carefully from the cranial cavity, sectioning the pituitary stalk, cranial nerves and the brain stem as they became accessible. The pituitary gland was then visible in the sella turcica and, after snipping through the overlying dura mater, it was lifted free. All adhering tissue was dissected away and the glands were blotted free of surface blood before being weighed to an accuracy of 10^{-3} g.

5.3.4 Estimation of plasma oestrogens

Total unconjugated oestrogens were estimated in the samples of plasma collected from the lambs using the radioimmunoassay described in Chapter 2.

5.3.5 LH estimation

LH was determined by a double antibody radioimmunoassay similar to that described by Carr and Land (1975). A rabbit (anti-ovine LH) antiserum, which showed minimal cross-reaction with other pituitary hormones, was employed. The reference preparation, NIH-LH-S18, was used as standard. The radioactive tracer was CNRS-LH-MB labelled with ^{125}I. A specific goat anti-rabbit Immunoglobulin-G was used as the precipitating antiserum. The radioimmunoassay was carried out in the laboratory of Mr. W. R. Carr

at the Animal Breeding Research Organisation, The Kings Buildings, West Mains Road, Edinburgh EH9 3JQ.

5.4 RESULTS

5.4.1 General condition of the lambs

The individual bodyweights of the methallibure-treated lambs, immediately before they were killed, are shown in Tables 5.1 and 5.2 for the Finnish Landrace and Scottish Blackface lambs respectively. The mean bodyweight of 6.7 $\pm$ 1.2 kg for the Finnish lambs that did not receive PMSG was not significantly different from the mean bodyweight of 6.7 $\pm$ 1.1 kg for those that received PMSG. Similarly the mean bodyweight of 5.1 $\pm$ 1.9 for Blackface lambs that did not receive PMSG was not significantly different from the mean bodyweight of 6.0 $\pm$ 0.8 kg for those that did. Therefore, as in the normal lambs (See Chapter 4, Section 4.4.3) PMSG had no significant effect on bodyweight. For this reason the values were pooled for further comparisons.

There was no significant difference between the mean bodyweights of 6.7 $\pm$ 1.1 kg for the methallibure-treated Finnish lambs and 5.5 $\pm$ 1.4 kg for the methallibure-treated Blackface lambs. The effect of methallibure-treatment on bodyweight is shown in Table 5.3 which gives the mean bodyweights of methallibure-treated lambs alongside those of normal Finnish and Blackface lambs for 1975 (See Chapter 4, Section 4.4.3). Methallibure-treatment caused a significant reduction in bodyweight in both breeds ($P < 0.02$ in each breed).

The Blackface lambs used during 1975 were all artificially reared. Treatment with methallibure resulted in a depression of appetite in most animals, although the degree of inappetance ranged

from a lack of enthusiasm at feeding-time to an actual reduction in the amount of milk taken. A complete refusal to feed voluntarily resulted in the death of two lambs after about 3 weeks of treatment. As can be seen in Table 5.2 others, which survived, had very low bodyweights when they were eventually killed.

Another serious side-effect observed in the methallibure-treated lambs of both breeds was an apparent loss of control over their voluntary muscles. This was first seen after about 2 weeks of treatment and more animals became affected as treatment continued. Seemingly normal lambs would suddenly become limp and collapse to the ground. When the lambs were still young they rapidly returned to normal, especially when stimulated by noise or touch. However, as the treatment continued and the lambs grew older, there was a tendency for longer periods of collapse to occur, when stimulation did not cause a recovery. Furthermore, some of the lambs at older ages were more chronically affected and appeared perpetually sleepy with partial loss of muscle control. If these animals could be persuaded to stand they showed buckling of the limbs, a relaxed abdomen and drooping of the head and neck. By the time the lambs were killed, most were affected by one or other form of this side-effect. However a few, belonging to both breeds, were apparently unaffected.

Some of the treated lambs were examined clinically and, with the exception of the above phenomenon, were otherwise normal. Clinical biochemistry was carried out on samples of blood from treated and normal lambs from both breeds. The results of urea, sodium, potassium, chloride, calcium, magnesium, phosphorus,

bilirubin, alkaline phosphatase, alanine transferase, aspartate transferase, albumin and globulin estimations were all within the normal range for sheep.

5.4.2 Ovarian characteristics

The ovarian characteristics of the individual lambs treated with methallibure are shown in Tables 5.1 and 5.2 for the Finnish and Blackface lambs respectively. The ovaries from the methallibure-treated lambs were similar in appearance to those from control lambs of the same age, described in Chapter 4 (Section 4.4.3). With one exception their ovaries were covered in small vesicular follicles up to 2 mm in diameter. The exception was Blackface lamb No. 72 whose ovaries were small with no visible follicles on the surface. Furthermore the ovaries in some of the methallibure-treated lambs were larger than the ovaries from the control lambs. However, as can be seen in Table 5.4, methallibure-treatment did not significantly affect the mean ovarian volume or ovarian weight.

The administration of PMSG to methallibure-treated lambs produced a response similar to that in normal lambs treated with PMSG (described in Chapter 4, Section 4.4.3). Some animals, 2 of 7 Finnish lambs (29%) and 3 of 5 Blackfaces (60%), were not stimulated to develop large follicles. In the animals that were stimulated the response ranged from 1 to 130 large follicles in the Finnish lambs and 9 to 60 large follicles in the Blackface lambs. Although Blackface lamb No. 76 did not develop large follicles, the ovaries were larger than in control lambs

suggesting that they may have been stimulated by PMSG. In some of the stimulated lambs a proportion of the large follicles showed signs of luteinisation or ovulation.

The ovarian weights and volumes of individual stimulated lambs reflected the presence of large follicles. However due to the large variation in the response to PMSG, there was no significant difference, within both breeds, between the mean ovarian weight and volume in lambs that were treated with methallibure and PMSG, and those that received only methallibure.

5.4.3 Oestrogen levels

The plasma levels of oestrogens in individual methallibure-treated lambs that also received PMSG, and the mean levels in lambs that received only methallibure are shown in Figs. 5.1 and 5.2 for the Finnish and Blackface lambs respectively. The individual values for all experimental animals are given in Appendix 5.

The overall mean plasma level of 7.4 ± 5.9 pg/ml for all Finnish and Blackface lambs that were treated with methallibure alone, was not significantly different from the mean value of 6.9 ± 4.5 pg/ml for the control lambs of both breeds (Chapter 4, Section 4.4.3).

In the lambs that received PMSG the pattern of secretion of oestrogens varied according to the degree of ovarian stimulation. In the lambs that had no large follicles in their ovaries the levels remained low during the sampling period. In the lambs that were stimulated to develop large follicles the levels of oestrogens were as variable as those in the normal lambs that received PMSG

during 1975 (Chapter 4, Section 4.4.3). The levels rose to a peak on day 4, when HCG was given, and had fallen to a low level again the following day. The relationship between the day 4 level of oestrogens and the number of large follicles in methallibure-treated lambs was similar to that recorded for the normal lambs.

5.4.4 Uterine measurements

The uterine horn diameters for the individual lambs are shown in Tables 5.1 and 5.2 for the Finnish and Blackface lambs respectively. The mean uterine horn diameter in methallibure-treated Finnish lambs that received PMSG, was significantly greater than in the Finnish lambs that received only methallibure ($P < 0.02$). In the Blackface lambs PMSG-treatment had no significant effect on uterine horn diameter.

The mean uterine horn diameter in methallibure-treated lambs is compared to the results for the control lambs (See Chapter 4, Section 4.4.3) in Table 5.5. This shows that methallibure-treatment had no significant effect on the diameter of the uterine horn.

The uterine epithelial measurements for the methallibure-treated Finnish and Blackface lambs are shown in Table 5.6 for the caruncular and intercaruncular regions of the uterus. The histological sections from some Finnish lambs were not sufficiently complete for the measurements to be carried out, and these results could not be included. For comparison, the values for the control lambs (See Chapter 4, Section 4.4.3) are also shown. In both breeds methallibure-treatment had no significant effect on the

uterine epithelial height, the mean values being similar to those for the control lambs. The administration of PMSG to methallibure-treated lambs resulted in a significant increase in the uterine epithelial height in the caruncular ($P < 0.05$) and intercaruncular regions ($P < 0.02$).

5.4.5 Pituitary measurements

The pituitary weights for individual lambs of both breeds are shown in Table 5.7. The weights of the pituitary glands from methallibure-treated lambs are shown, as well as those from the normal lambs used during 1975 (described in Chapter 4). Not all of the pituitary glands were removed intact and the weights of complete glands only have been included in the results.

Treatment with PMSG had no significant effect on pituitary weights in normal and methallibure-treated lambs of both breeds. Therefore the results have been pooled for further analysis. The mean pituitary weight in the methallibure-treated Finnish lambs was significantly less than in the normal Finnish lambs ($P < 0.01$). In the Blackfaces there was a tendency for the pituitary weight to be greater in the methallibure-treated lambs than in the normal animals, although the difference was not significant.

Pituitary weight was also related to bodyweight for each individual lamb. These relative pituitary weights are shown in Table 5.8. The mean relative pituitary weight was significantly lower in methallibure-treated Finnish lambs than in normal animals ($P < 0.05$). In the Blackfaces the mean relative pituitary weight was significantly greater in the methallibure-treated than in

normal animals ($P < 0.001$).

5.4.6 Oocyte counts

The mean oocyte counts for the ovaries from methallibure-treated lambs, Finnish and Blackface, are shown in Table 5.9. There was no significant difference, between the two breeds, in the mean number of oocytes in primordial, growing or vesicular follicles. The results for the methallibure-treated lambs were compared with those for normal lambs of the same age described in Chapter 4 (Section 4.4.3). When the breeds were considered individually, there was no significant difference, in the number of oocytes in all classes of follicles, between methallibure-treated and normal lambs. However, when the results for the two breeds were combined, to overcome the disadvantage of small group sizes, the mean number of oocytes in primordial follicles was significantly greater in the methallibure-treated lambs ($P < 0.05$).

5.4.7 LH levels

The plasma LH concentrations in 45 day-old Finnish and Blackface lambs treated with 5 µg LH-RH are shown in Figs. 5.3 and 5.4 respectively. In 5 of 6 normal lambs the highest LH concentration was recorded in the 20 minute blood sample. There was no significant difference between the mean concentrations of 39.3 ± 12.6 and 36.3 ± 40.7 ng/ml in the Finnish and Blackface lambs respectively at this time.

In the methallibure-treated lambs that received LH-RH, the highest LH concentration occurred most frequently in the

40 minute sample. The mean LH concentrations at this time, 6.3 ± 4.9 and 6.8 ± 3.1 ng/ml for the Finnish and Blackface lambs respectively, were not significantly different. The mean LH concentration of 6.6 ± 3.7 ng/ml in the 40 minute samples from these methallibure-treated lambs was significantly less than the mean peak concentration of 37.8 ± 27.0 ng/ml for the normal lambs of both breeds ($P < 0.01$).

The plasma LH concentrations in 43 day-old Finnish and Blackface lambs treated with 25 µg oestradiol-17β are shown in Figs. 5.5 and 5.6 respectively. Two normal lambs, one Finnish and one Blackface, showed a response to oestradiol. Peak levels of 30 and 26 ng/ml, at 18 hours and 14 hours respectively, were recorded in these two lambs. In the remainder of the lambs, both normal and methallibure-treated, the concentration remained below 5 ng/ml at all times.

5.5 DISCUSSION

The prolonged treatment of neonatal lambs with methallibure during the present study resulted in several severe side-effects. A significant reduction in bodyweight was recorded in both Finnish and Blackface lambs by the time they were killed at the end of the treatment period. Since the Blackface lambs were all artificially reared during 1975, it was possible to compare the appetites of treated lambs with those of their normal counterparts. There was a depression of appetite in the treated group, although the degree of inappetance varied among the lambs. Owing to the fact that the Finnish lambs were all reared on their mothers, it was not possible to compare the appetites of methallibure-treated and normal lambs. However it seems likely that the lower bodyweights in the treated lambs were due to a loss of appetite in this breed also.

Other workers have observed a reduction in bodyweight in treated rats (Benson and Zagni, 1965; Labhsetwar and Walpole, 1972), mice (Brown, 1963) rabbits (Skinner and Adams, 1969) and gilts (Stratman and First, 1965). Methallibure has been shown to suppress the action of endogenous growth hormone in rats, and also to inhibit the anabolic action of exogenous growth hormone (Ramaiah and Gangadhara, 1969). This suppression of the action of growth hormone may be partly responsible for the lower bodyweights in methallibure-treated animals. During the present study depression of appetite occurred in the treated lambs and this has been reported also in treated sows (Gerrits and Johnson, 1965).

Growth hormone has been shown to have no effect on appetite in sheep (Baile and Martin, 1971). The reduction in bodyweight in methallibure-treated animals may result therefore from two actions of methallibure, firstly suppressing the action of growth hormone and secondly depressing appetite.

Another serious side-effect, observed in the lambs treated with methallibure in the present study, was the drowsiness that occurred in most animals, with a complete loss of control over voluntary muscles on some occasions. Lethargy has also been reported in boars (Call et al., 1969) and dogs (Call and Barker, 1967) following treatment with methallibure. Brown (1963) also considered that his treated mice and rats were less lively than control animals. Humans who were treated with methallibure (Walpole, 1965) complained of lethargy and somnolence. It has been observed that these symptoms of lethargy pass off when the compound is withdrawn (Walpole, 1965; Call et al., 1969). The acute attacks of muscular collapse seen in some of the lambs in the present study have not been reported previously. They may have represented a more serious development of the lethargy effect produced by methallibure, although in that case it was surprising that the acute form was seen earlier than the drowsiness. No information exists on whether methallibure produced these effects by a peripheral action on the muscles themselves or an action at the level of the central nervous system.

Various other side-effects have been described in methallibure-treated animals, which may or may not be mediated through the central nervous system - for example the vomiting and

nausea that occurred in humans (Walpole, 1965) and increased thirst in pigs (Call et al., 1969; Polge, 1965). Other side-effects have included respiratory distress in boars (Call et al., 1969), a red body rash in sows (Polge, 1965) and teratogenic effects in the piglets born to sows that received methallibure during pregnancy (King, 1969).

Evidence of toxicity was described in mice and rats treated with methallibure, with death in some cases (Brown, 1963). Two of the lambs being treated with methallibure in the present study died after about 3 weeks of treatment, although this was attributed to anorexia. Clinical examination, including certain biochemical investigations, revealed no abnormalities in the normal or methallibure-treated animals.

To examine the effect of methallibure-treatment on the development of the reproductive tract in neonatal Finnish and Blackface lambs, the ovaries and uteri were compared with those in normal lambs. The gross appearance of ovaries from the treated lambs was similar to those in the controls, with small vesicular follicles covering the ovarian surface. The bodyweights of 2 of 5 treated Blackface lambs were very low by the time of slaughter and this may have affected normal growth of the reproductive tract in these 2 animals. In one of these animals the ovaries were small with no vesicular follicles on the surface. However, the ovaries in one Blackface control lamb at the same age had a similar appearance. Both mean ovarian volume and weight were not significantly different between treated and control animals of both breeds. However, the mean number of oocytes in primordial follicles was

significantly greater in the ovaries of the methallibure-treated lambs. A significantly greater number of primordial follicles has also been observed in the ovaries of rats that have been hypophysectomised (Jones and Krohn, 1961). Schwartz (1974) has suggested that gonadotrophins may be required to stimulate the movement of follicles from the "non-proliferating" to the "proliferating" pool. However, in the ovaries of lambs in this study, the numbers of oocytes in growing and vesicular follicles were not significantly altered by methallibure-treatment.

Other workers have described changes in ovarian morphology following the administration of methallibure to various mammalian species. In female rats (Paget et al., 1961) there was a reduction in ovarian weight, and histological examination showed a decrease in the number of maturing follicles. Brown (1963) also observed a reduction in the weight of the ovaries in a proportion of rats and mice treated with methallibure. In sows Polge (1965) found only a few small follicles and regressing corpora lutea in the ovaries of treated animals, and Garbers and First (1969a) also found a reduction in the number of follicles, larger than 5 mm, in the ovaries of treated gilts. A suppression of ovulation has also been recorded in treated animals (Polge, 1965; Garbers and First, 1969b).

Methallibure is also effective in suppressing oestrus in treated animals. In female rats, cyclic activity, as determined by vaginal smears, ceased within 2-3 days of the start of treatment and was depressed for as long as dosing continued (Paget et al., 1961). In pigs, provided the dose of methallibure

was adequate, the suppression of oestrus was achieved, resulting in the compound being used for the synchronisation of oestrus in this species (Polge, 1965).

Some workers have reported alterations in the uteri of animals treated with methallibure. In sows Polge (1965) found that the uteri were small, resembling those in prepubertal animals, and similarly, in rats and mice, Brown (1963) observed a reduction in uterine weight. This provided further evidence that ovarian steroid secretion had been depressed in these animals. In the present study both the uterine horn diameter and the height of the uterine epithelium were similar in methallibure-treated and normal lambs. Leifer et al. (1972) have shown that the uteri of intact lambs at 44 days of age were heavier than those from lambs that had been ovariectomised 5 days after birth. This implied that the ovaries were secreting sufficient oestrogens to influence the growth of the uteri by 44 days of age. The similarity in uterine characteristics between methallibure-treated and normal lambs at 6 weeks of age in the present study therefore suggests that methallibure had not suppressed the secretion of oestrogens by the ovaries.

There have been few previous reports of the use of methallibure in sheep. Gerrits et al. (1965b) achieved suppression of oestrus in 5 out of 12 cycling ewes that were being group-fed methallibure at the rate of 0.84 to 1.25 mg/kg of bodyweight/day. From the weight gains of individual ewes during the treatment period it was suggested that ewes with altered cycles may have received more than the calculated average dose.

The authors concluded that a higher dose of methallibure may be necessary to synchronise oestrus effectively in the ewe. In comparison the successful synchronisation of oestrus in sows (Polge, 1965) has been achieved by the oral administration of methallibure at the rate of 100 mg per pig per day. This represented a dose rate of 0.60 to 1.19 mg/kg of bodyweight/day, which was similar to that described above for ewes. However, Garbers and First (1969c), working with gilts, have found that methallibure blocked ovulation, oestrus and follicular growth at different dose levels, with ovulation being blocked at the lowest dose. It seems possible therefore that in ewes, also, the degree of suppression of oestrous cycles may depend on the dose level of methallibure.

When administered as a single intravenous injection to pubescent male lambs (Skinner and Adams, 1969) methallibure actually improved sexual function. This was in apparent contrast to previous findings for the rat (Walpole, 1965), dog (Call and Barker, 1967) and boars (Call et al., 1969) in which oral administration produced loss of libido and in some cases had an adverse effect on spermatogenesis. The different method of treatment in the male lambs, a single intravenous injection, compared to constant oral dosing in the other species, may have explained the different result.

It appears therefore that the route of administration, the dose level and the duration of treatment with methallibure may influence the degree of depression of gonadal function. In the present study methallibure was administered by subcutaneous

injection, following the advice of Dr. Walpole that oral administration was toxic in ruminants. Subcutaneous administration has proved successful in suppressing reproductive function in rats and mice (Brown, 1963) although other workers, using approximately a quarter of the dose, achieved ovarian suppression in rats, but not mice. This was further evidence that the dose level of methallibure is important. In the present study methallibure was administered at the rate of 10 mg/kg of bodyweight/day for approximately 6 weeks. In view of the dose rates described above for adult ewes and sows, this would appear to be an adequate dose, even taking into consideration the different route of administration.

Information on the mode of action of methallibure in suppressing gonadal function indicates that it acts centrally, altering the normal pattern of gonadotrophin secretion by the pituitary. In the present study the pituitary glands were removed from experimental lambs at slaughter. Treatment with methallibure produced a significant reduction in mean pituitary weight in the Finnish lambs, whereas in the Blackfaces pituitary weight was not significantly different in the treated lambs. To allow for any general growth-suppressant action of methallibure, pituitary weight was also related to the bodyweight of each individual animal. This accentuated the reduction in pituitary weight in the treated Finnish lambs and further showed a significant increase in pituitary weight in the treated Blackfaces. This difference between the two breeds is difficult to explain. It could result from a variation in the accumulation of hormones in

the pituitary glands between the two breeds. The results of LH-RH stimulation (discussed later) suggested a similar effect on pituitary LH function in the Finnish and Blackface lambs, although this does not exclude a variation in the effect on other pituitary hormones. Other workers have reported no alteration in anterior pituitary weight in sows and gilts which had received methallibure (Garbers and First, 1969a; Stormshak et al., 1970). However, Garbers and First (1969a) did record a significant increase in posterior pituitary weight in sows treated with methallibure for 10 days, beginning on day 3 of the cycle.

There have been several reports of changes in the pituitary content of FSH and LH. The pituitary glands from treated rats were found to contain less total gonadotrophin and less FSH than the glands from control rats (Brown, 1963). Another study, using rats, showed that both pituitary and serum LH levels were lowered (Labhsetwar and Walpole, 1972). These results suggested that, in rats, methallibure acted by reducing the formation of pituitary gonadotrophins as well as suppressing their release. In pigs methallibure has been shown to cause an increase in pituitary FSH and LH activity (Garbers and First, 1969b; Stormshak et al., 1970) suggesting that in this species synthesis of gonadotrophins was not inhibited but their release had been blocked.

Malven (1971) has carried out experiments to determine the site at which methallibure may act to inhibit gonadotrophin secretion. By surgically implanting small quantities of methallibure intracranially in guinea pigs he obtained greater

suppression of ovulation with implants within or near the hypothalamus than in the anterior pituitary. However, Stormshak et al. (1970) found that methallibure-treatment of gilts failed to affect the LRF activity of the stalk median eminence. To investigate further the site of action of methallibure in neonatal lambs in the present study, the release of LH following treatment with oestradiol-17β or LH-RH was compared to the response in normal lambs at approximately 6 weeks of age.

The administration of oestradiol-17β to lambs at 6 weeks of age stimulated an increase in the plasma LH concentration in 1 of 3 and 1 of 2 normal Finnish and Blackface lambs respectively.

Other workers have also administered oestradiol to neonatal lambs of varied ages. Land et al. (1970) obtained a response in terms of a rise in plasma LH concentration, in 1 of 6 lambs at 13 days of age. At later ages, 38 and 77 days, 5 of 6, and 6 of 8 lambs respectively responded. At 11 weeks of age Thimonier et al. (1972) obtained a response in only 17 of 22 lambs of various breeds. From these reports, and the absence of a response in some of the lambs in the present study, it appears that the positive feedback mechanism, by which oestrogen induces a release of LH, is not fully competent in the neonatal lamb. None of the five methallibure-treated lambs, in the present study, showed an increase in plasma LH concentration following the administration of oestradiol-17β. Owing to the small number of lambs within the groups, it is not possible to say whether this was due to an effect of methallibure, or to the immaturity of the positive oestrogen feedback mechanism.

The ability of LH-RH to induce the release of LH from the pituitary of the sheep is well established (Arimura, Debeljuk, Matsuo and Schally, 1972; Crighton, Foster, Haresign and Scott, 1975). The administration of LH-RH to groups of Finnish and Blackface lambs at 6 weeks of age in the present study resulted in a significant increase in plasma LH levels. Foster et al. (1972c) have previously demonstrated that the pituitary of the lamb during the late foetal and neonatal stages of development is able to respond to purified hypothalamic extract. It was shown that the magnitude of the response by 3 and 11 day-old lambs was less than that of 126 to 138 day-old foetal lambs. The authors considered this was due to the drop in pituitary LH levels recorded at birth in the lamb (Foster et al., 1972a) which may have resulted in a decrease in the stores of LH available for release during the post-natal period. By day 68 of neonatal life, the response to the releasing hormone did not differ from that in foetal lambs. At this time the authors obtained a mean increase in serum LH of 6.8 ± 3.23 ng/ml. This was less than the mean peak level of 37.8 ± 27.0 ng/ml found in the normal lambs in the present study. The difference may have been due to the use of a hypothalamic extract in the study of Foster et al. (1972c) compared to the synthetic LH-RH used in the present study.

A smaller and delayed release of LH occurred in methallibure-treated lambs in the present study, following the administration of LH-RH. This may have been due to a greater previous release of LH, a reduced sensitivity to LH-RH or a diminished synthesis of LH in the treated lambs. Foster et al.

(1972c) carried out repeated injections of purified hypothalamic extract at 1 hour intervals for 3 successive hours in female foetal lambs. The amount of LH released in response to the second and third injections tended to be greater than that after the first challenge. Therefore it seems unlikely that the reduced response to LH-RH in methallibure-treated lambs during the present study was due to a greater release of LH prior to LH-RH administration, especially since this would have been expected to result in greater follicular development in the treated lambs which was not the case. A variation in responsiveness to LH-RH has been recorded in ewes at different times during the oestrous cycle (Reeves, Arimura and Schally, 1971) which the authors suggested was due to changes in the level of oestrogens sensitising the pituitary. A reduction in pituitary LH stores has been recorded in rats treated with methallibure (Labhsetwar and Walpole, 1972). Therefore it would appear most likely that the reduced release of LH in response to an injection of LH-RH in treated lambs in the present study was due to a reduced synthesis of LH.

LH-RH and oestradiol were administered to the lambs in an attempt to determine the site of action of methallibure. A normal response to LH-RH, but a reduced response to oestradiol-17β, in the methallibure-treated lambs compared to the controls, would have suggested that methallibure was acting via the hypothalamus, whereas a reduced response to both hormones in the methallibure-treated lambs would have implied that methallibure was acting on the pituitary. Although the response of the lambs to oestradiol-17β was inconclusive, the reduced response to LH-RH suggests that

pituitary function had been altered by methallibure-treatment. Any effect of methallibure on the hypothalamus remains uncertain.

It can be concluded that methallibure-treatment of the neonatal lamb had some effect on the secretion of LH by the pituitary. The greater number of oocytes in primordial follicles in the treated lambs than in controls may have reflected the reduced release of LH in the treated lambs. However the number of oocytes in growing and vesicular follicles was not significantly different, and ovarian volume and weight were not affected by treatment. Furthermore uterine development, as an indication of ovarian function, was also similar in the treated and control groups. This suggests that gonadotrophin secretion was not entirely suppressed by methallibure-treatment, which is supported by the finding that the LH release in response to LH-RH administration was reduced, but not suppressed altogether.

Methallibure was used during the present studies in an attempt to suppress the endogenous secretion of gonadotrophins prior to a comparison of ovarian sensitivity in Finnish and Blackface lambs. At 35 days of age PMSG and HCG were administered to groups of lambs, belonging to both breeds, that had been receiving methallibure from birth. The range in follicular development was as variable as that recorded in the normal lambs with some animals failing to respond and others being stimulated to develop large numbers of follicles. The production of oestrogens followed a similar pattern to that in the normal lambs and the effect on the uterus was also similar.

It has been shown by previous workers that methallibure

did not affect the gonadal response to exogenous hormones in rats (Paget et al., 1961), and PMSG stimulated ovulation in gilts under methallibure-treatment (Polge and Day, 1969). The normal response to PMSG in the present studies has confirmed that methallibure does not have a direct effect on the ovaries, but exerts an effect at the level of the brain or pituitary. However, if follicular development had been totally suppressed by the action of methallibure, to the extent that only primordial follicles were present in the ovaries, an altered response to PMSG would be expected. The development of growing and vesicular follicles was not affected by treatment, which probably accounts for the apparently normal response to PMSG.

Although the response to PMSG in methallibure-treated lambs in the present study was similar to that in control lambs, the small number of lambs studied and the variation in the response meant that the results between the two breeds could not be compared statistically. Therefore it was not possible to reach any conclusions regarding ovarian sensitivity in the Finnish and Blackface lambs under the influence of methallibure.

It can be concluded from these studies that, although methallibure has been shown to suppress ovarian development and function in other species, it has a minimal effect in the neonatal lamb. As discussed previously, the dose level of methallibure may influence the degree of gonadal suppression. Unfortunately, an increase in the dose administered to the neonatal lamb, whilst possibly suppressing further the release of LH, may also enhance the serious side-effects, probably resulting in the death of many

animals. Therefore methallibure has been shown to be of little use in suppressing endogenous circulating gonadotrophins in the neonatal lamb, prior to an investigation of ovarian sensitivity in response to exogenous gonadotrophins. However, the use of this compound for a similar purpose in other species should not be ruled out.

GENERAL DISCUSSION

GENERAL DISCUSSION

The present studies were designed to contribute further information on certain aspects of follicular development and function in sheep. The investigations were carried out in two breeds of sheep, Finnish Landrace and Scottish Blackface, and small flocks of these two breeds were established to provide experimental animals. Over a period of 3 years the Finnish flock produced a mean litter size of 2.37 lambs; the Blackface flock produced a mean litter size of 1.29 lambs over a period of 2 years. These results agreed with the reports of other workers (Donald and Read, 1967) that the Finnish breed was far more prolific than Scottish Blackfaces and other British breeds. In addition to the greater fecundity of Finnish Landrace sheep, significant differences in litter size have been recorded among many other strains and breeds of sheep (Bradford, 1972). In view of the importance of litter size in contributing towards the overall reproductive performance of sheep, there have been many investigations into the factors responsible for variations in litter size.

Wheeler and Land (1973) have reported ovulation rates for Finnish and Blackface ewes that were of a similar order of magnitude as their litter sizes. Packham and Triffitt (1966) compared the number of corpora lutea observed at laparotomy, with previous and subsequent lambing records, in strains of Merino sheep selected for and against twinning. They concluded that differences in prolificacy, resulting from the selection process, were due to genetic differences in the number of eggs shed at ovulation. This

confirmed earlier observations by Marshall (1904) that the number of corpora lutea present in sheep at slaughter corresponded with the normal lambing performance of the breed. Although differences in ovulation rate have been shown to account for differences in prolificacy, there is little information on the relationship between follicular development during the oestrous cycle with the number of follicles that rupture at ovulation. Following the removal of one ovary on days 2, 8 or 14 of the oestrous cycle, Land (1973a) observed no reduction in ovulation rate at the subsequent oestrus, suggesting that ovarian compensation had occurred by that time. It was concluded from these studies that ovulation rate in the sheep was not determined finally until after day 14 of the cycle.

The relationship, between circulating levels of gonadotrophins and normal follicular development during the oestrous cycle of the sheep, remains unclear. The levels of LH and FSH remain low throughout most of the cycle, when waves of follicular development are occurring, with peaks of both hormones occurring before ovulation (Cunningham, Symons and Saba, 1975). The surge of LH at this time is thought to be responsible for the ovulation of mature follicles (Cumming et al., 1971b). Variations in ovulation rate that have been recorded among different breeds and strains of sheep may be the result of differing levels of gonadotrophins in the circulation or differences in ovarian sensitivity to these gonadotrophins.

Circulating levels of LH during the oestrous cycle have been related to the number of follicles rupturing at ovulation. Land et al. (1973) reported that plasma LH concentration was

significantly greater in Romanov ewes than in other less fecund breeds, on days 1 and 8 of the cycle. The day 1 differences may have arisen from the delayed ovulatory discharge of LH in the Romanovs. However, the day 8 differences were in accordance with data on pituitary LH activity and unpublished information on LH levels in the urine, reported by Land et al. (1972a), that suggested a greater release of LH from the pituitary during mid-cycle in Finnish x Blackface ewes than in Merino x Blackface ewes. Pituitary LH activity was similar in the two breeds around oestrus. Similarly Land et al. (1973) showed that the ovulatory discharge of LH was not related to ovulation rate. It is difficult to correlate these findings with those of Land (1973a), except in terms of a differing sensitivity to gonadotrophins at the time when he suggested ovulation rate was determined. Land et al. (1972a) suggested that, although the ovulation rate may not be determined finally until after day 14, the normal ovulation rate may be determined somewhat earlier.

During the present studies a further aspect of normal endocrinological function was studied in relation to prolificacy. Peripheral plasma levels of oestrogens were estimated around the time of oestrus in Finnish and Blackface ewes. The development of a sensitive radioimmunoassay method meant that the level of oestrogens could be estimated in samples of peripheral plasma collected for a period of several days, either side of oestrus. The prooestrous peaks of oestrogens were compared between the two breeds. Although the rate of increase in the level of oestrogens was similar and the peak levels in each breed were not significantly

different, significantly elevated levels of oestrogens occurred over a period of two days in the Finnish ewes and only one day in the Blackfaces. The longer duration of the oestrogenic stimulus in the Finnish ewes may be partly responsible for the longer duration of oestrus recorded for this breed (Donald and Read, 1967), although greater sensitivity to oestrogens has also been recorded in Finnish ewes than in Blackfaces (Land et al., 1972b).

Oestrogens secreted by mature follicles are also thought to be responsible for stimulating the preovulatory surge of LH (Goding et al., 1969). The longer period of secretion of oestrogens in the Finnish ewes may be required for the release of LH in this breed if, as has been suggested by Land et al. (1973), the hypothalamus is less sensitive to the positive feedback of oestrogens in more prolific breeds.

It is not known whether the multiple follicles in Finnish ewes rupture simultaneously or not. Neither have the timing of ovulation and the surge of LH release been related to the longer duration of oestrus recorded for the Finnish breed. Until these facts are known, the function of a delayed LH release in the more prolific breeds cannot be determined.

As a means of determining the physiological factors responsible for genetic variations in ovulation rate, the ovarian response to exogenous gonadotrophins has been studied in breeds and strains of ewes of differing fecundity. Bindon et al. (1971) and Trounson and Moore (1972) have administered PMSG during the oestrous cycle to groups of Merino ewes with a high and low incidence of multiple births. The response, both in terms of

ovarian follicular development and the number of ovulations, was significantly greater in the group of ewes that was naturally more prolific. Bradford et al. (1971) have investigated the response to PMSG in several breeds of sheep. Ewes of the Finnish Landrace breed, with the highest natural fecundity, produced significantly more fertilised eggs than ewes of the other breeds. However, all these studies were conducted in ewes with intact pituitary glands. The results may have been the result of synergism between administered PMSG and endogenous gonadotrophins. Therefore it was not established whether variations in ovarian sensitivity to gonadotrophins contribute to the different ovulation rates in sheep.

In an attempt to clarify the physiological factors responsible for differences in ovulation rates in mature cycling ewes, there have been many investigations in prepubertal animals belonging to breeds or strains of sheep of differing fecundity.

During the present studies, normal ovarian development was examined in neonatal Finnish and Blackface lambs. An increase in ovarian volume was recorded between 7 and 35 days of age, accompanied by increasing follicular development. At 7 days of age there were no follicles visible on the ovarian surface; by 35 days of age the ovaries were covered by many small vesicular follicles. The results of these studies, in Finnish and Blackface lambs, fitted the pattern of follicular growth described by Kennedy et al. (1974) in Merino lambs. They recorded an increase in ovarian weight between birth and 4 weeks and between 4 and 8 weeks of age. An increase in the number of growing and vesicular follicles occurred between birth and 4 weeks. The age at which this follicular growth reaches

a peak has not been determined accurately. Degenerative changes were evident in the vesicular follicles in many 5 week-old lambs during the present study. By 12 weeks of age the number of growing and vesicular follicles has declined again (Kennedy et al., 1974). Although the number of Finnish and Blackface lambs studied at individual ages during the present study was too small to allow detailed comparisons, the rate of ovarian development appeared to be similar in the two breeds. In view of the comparable findings reported by Kennedy et al. (1974) for Merino lambs, the wave of follicular development during the neonatal period in the sheep may well be common to all breeds.

The factors responsible for the increase in follicular development, after birth, are not clear. It may be a continuation of folliculogenesis initiated during foetal life or, alternatively, it may be triggered by a particular stimulus, such as hormonal changes at parturition. For instance, Foster et al. (1972a) detected a sudden decrease in pituitary LH at birth, although they did not detect a corresponding increase in serum LH. In addition, the factors responsible for terminating the wave of follicular development in the neonatal lamb are not known. The decline in the number of growing and vesicular follicles coincides approximately with the maturation of the negative feedback mechanism in this species. Foster et al. (1975) have shown that the ovarian inhibition of LH release may have begun to develop by 9 weeks of age. If the growth of follicles in the neonatal ovary is accompanied by an increase in the basal secretion of steroids, this could be responsible for the development of a negative feedback. Since this

would result in a reduction in circulating levels of gonadotrophins, it could account for the decline in the number of follicles in the ovaries of older lambs. During the present study, levels of oestrogens in control lambs remained basal at all ages. However, this may have been due to the limitations of sensitivity for the radioimmunoassay used in this study. With the methods for assaying oestrogens available at the present time, it would appear to be necessary to sample ovarian venous plasma in order to determine, firstly, whether oestrogens are being secreted during the neonatal period and, secondly, whether there are any qualitative or quantitative changes in the pattern of secretion with age.

Although gross ovarian development appeared similar in Finnish and Blackface lambs during the present study, differences in ovarian morphology between the two breeds are present during the neonatal period. Land (1970a) reported more oocytes in the ovaries of Blackface lambs at birth, although the proportion of oocytes in vesicular follicles was greater in Finnish x Blackface lambs. In the 5 week-old lambs during the present study there was no significant difference in the number of oocytes in primordial follicles, although there was a larger number of oocytes in vesicular follicles in the Finnish ovaries than in the Blackfaces. Trounson et al. (1974) have reported that the number of primordial follicles was greater at birth in the ovaries of Merinos, selected against multiple births, than in lambs selected for multiple births. By 5 months of age there was no significant difference between the two groups. Just as in the adult, the greater development of vesicular follicles in neonatal lambs, belonging to more

fecund breeds, may be the result of higher circulating levels of gonadotrophins, or greater ovarian sensitivity to gonadotrophins.

There have been several reports of blood and pituitary concentrations of gonadotrophins in female lambs, in relation to the fecundity of their breed-type. At 30 days of age, Bindon (1973) detected significantly higher plasma LH levels in a more prolific strain of Merinos. At 5 months, Trounson et al. (1974) found that, in addition to plasma LH levels being higher in Merino lambs selected for multiple births, there was a similar, although not significant, trend for pituitary FSH and LH levels to be greater. This suggested that both the synthesis and release of gonadotrophins had been affected by selection. Bindon and Turner (1974) have suggested that a transient episodic pattern of LH release may occur earlier in life in lambs belonging to a more fecund strain of Merinos.

From these observations it is well-established that plasma levels of LH are higher in more prolific individuals during the neonatal period. This could account for the greater development of vesicular follicles in Finnish lambs than in Blackface lambs at 35 days of age during the present study. It may well be that differences in circulating gonadotrophin levels also exist during the foetal period. This could explain the greater proportion of vesicular follicles in the ovaries of Finnish lambs at birth (Land, 1970a). However, the reported differences in gonadotrophin levels do not discount the possibility that variations in ovarian sensitivity may contribute to the greater follicular development in the Finnish lambs.

During the present studies, ovarian sensitivity was compared in Finnish and Blackface lambs, by the administration of exogenous gonadotrophins. A proportion of the lambs, at 7, 21 and 35 days of age, failed to respond to PMSG, confirming reports by previous workers that the ovaries of the new-born lamb are refractory to stimulation by exogenous gonadotrophins. The proportion of animals, that failed to respond at these three ages, was similar for the Finnish and Blackface groups. In the animals that did respond to treatment, the mean development of large follicles, and the proportion of these follicles that showed ovulation or luteinisation in response to HCG-administration, were not significantly different between the two breeds. It was established, during the present study, that the induced follicular development in the neonatal lambs was associated with significantly elevated levels of oestrogens. As with the follicle results, the mean peak levels of oestrogens in the Finnish and Blackface lambs were not significantly different. Therefore, it was concluded from these studies that ovarian sensitivity to exogenous gonadotrophins was similar in the two breeds, at least between 7 and 49 days of age.

As mentioned previously in this discussion, the difficulty usually encountered in studies designed to investigate ovarian sensitivity in response to exogenous gonadotrophins is that the degree of participation of endogenous hormones cannot be determined. During the age period covered by the present studies (7-49 days) the positive and negative feedback control of endogenous gonadotrophin secretion, by the ovaries, may not have developed in

the female lamb (Foster and Karsch, 1975; Foster et al., 1975). Therefore, oestrogens secreted by the PMSG-stimulated ovaries during the present study were unlikely to have influenced the release of endogenous gonadotrophins. In which case the results of PMSG-treatment in the Finnish and Blackface lambs probably represented the response to this exogenous stimulus alone.

Nevertheless the fact remains that the basal circulating levels of LH in neonatal lambs have been shown to be greater in more fecund breeds. In an attempt to eliminate the influence of endogenous gonadotrophins in the Finnish and Blackface lambs completely during the present study, methallibure was used. This compound has been shown to suppress follicular development in other species (Paget et al., 1961; Polge, 1965), probably by suppressing the synthesis or release of pituitary gonadotrophins.

Following the administration of methallibure continuously from birth, the ovaries of 35 day-old lambs contained a significantly greater number of oocytes in primordial follicles compared to normal lambs at the same age. It has been suggested that gonadotrophins may be necessary for the movement of follicles from the "non-proliferating" to the "proliferating pool" (Schwartz, 1974). From the reduction in the release of LH, in response to LH-RH, in the methallibure-treated lambs, it can be postulated that the endogenous release of LH may have been suppressed in these animals. This could account for the greater number of oocytes in primordial follicles in the ovaries of the treated lambs. However, the number of oocytes in growing and vesicular follicles was not significantly different between the treated and normal groups of

lambs, which probably explains why the response to PMSG in the treated animals was similar to the response in normal lambs.

It was concluded that, at the dosage used, methallibure was not entirely successful in suppressing the release of gonadotrophins in the neonatal lamb. As a result, it was not possible to compare ovarian sensitivity to PMSG in Finnish and Blackface lambs, in the complete absence of endogenous gonadotrophins. The serious side-effects observed in the treated lambs were an additional complicating factor. Other techniques exist for the removal of circulating endogenous gonadotrophins. They include surgical hypophysectomy and the use of antigonadotrophin sera. However, both methods are not without difficulties.

From a consideration of the results from the present studies, as well as the findings of other workers, various conclusions can be reached on the control of ovulation rate in the sheep. During the present studies, it was shown that ovarian sensitivity was similar in Finnish and Blackface lambs, between 7 and 49 days of age. It is unlikely that differences in ovarian sensitivity develop between the neonatal period and puberty. Therefore ovarian sensitivity to gonadotrophins probably does not contribute to variations in ovulation rate. In view of the many reports of higher levels of LH in the blood, in more prolific breeds and strains of sheep, both in the prepubertal lamb and during the oestrous cycle, it would appear likely that ovulation rate is governed by the degree of gonadotrophic stimulation. Furthermore, Land and Carr (1975) have postulated that a lower sensitivity of the hypothalamo-hypophyseal axis to feedback from

the gonads may be responsible for the higher levels of gonadotrophins in more fecund breeds. The finding during the present study, that the stimulated plasma level of oestrogens/follicle was significantly greater in Blackface than in Finnish lambs, may fit this theory.

Due to the important contribution made by litter size to overall reproductive performance, there have been many attempts to increase litter size in sheep. This could be achieved by the introduction of more prolific breeds into a flock. However, more prolific sheep may have undesirable characteristics, such as poor growth or conformation. Another way is to improve the lambing performance of existing sheep. In addition to improving environmental factors, such as management and feeding, many attempts have been made to increase the natural ovulation rate by the use of hormonal treatments. However, despite all the research which has been carried out, practical and reliable techniques for modifying reproductive performance have not been developed. Another line of approach is the selection, within the existing flock, of more prolific individuals.

In 1953, Reeve and Robertson concluded from a review of the literature that, although there may be breed differences in the incidence of multiple births, the small genetic variation and marked influence of non-genetical factors meant that selection for increased incidence was not likely to be worthwhile. However, at around the same time, Hammond (1952) drew attention to the progress that had already been made by breeding from twin ewes. Since then several workers (Turner, Hayman, Triffitt and Prunster, 1962; Young,

Turner and Dolling, 1963; Turner, 1969) have claimed that satisfactory, although slow, progress can be achieved by selecting for multiple births over several generations. Land (1974) categorised the four main factors responsible for the slow response as, firstly, the low heritability, secondly the trait can be measured only in females, thirdly, even in females the trait can often not be measured until they are two to three years of age, and, fourthly, the low reproductive rate itself limits the selection pressure which can be applied.

The advantages to be gained from the development of a simple and convenient method for measuring genetic variation in ovulation rate, before the normal reproductive age, are obvious. In the present studies the response to the same dose of PMSG was compared between Finnish and Blackface lambs. Although the chief intention was to investigate ovarian sensitivity in the two breeds, the technique may have had a practical application if the response had been related to prolificacy. To overcome the problems associated with assessing follicular development, peripheral plasma levels of oestrogens were estimated. A highly significant correlation existed between the peak level of oestrogens and the number of large follicles in the ovaries. This suggests that it may be possible to monitor follicular development by measuring the plasma concentration of oestrogens.

However, the mean response to PMSG, in terms of follicular development and the levels of oestrogens, was not significantly different between the two breeds. The reason for this finding, as suggested previously in this discussion, may be the immaturity of

feedbacks between the ovaries and the hypothalamo-hypophyseal axis at 49 days of age and earlier. In which case, a difference in response to PMSG, between the two breeds, may develop at slightly older ages. It was not the intention, during the present studies, to observe any long-term effects of PMSG-treatment on the ovaries. Therefore it is not known whether the treatment at this age might result in permanent scarring of the ovaries, especially in those lambs in which there was massive development of large follicles.

Although ovarian activity has the advantage of being measurable in young lambs, it suffers from the disadvantage of being limited to the female side. Several investigations have shown that plasma LH levels may be related to the prolificacy of the breed-type, with the advantage that the trait is measurable in young lambs of both sexes (Bindon, 1973; Trounson et al., 1974; Carr and Land, 1975). However, the fluctuation of LH levels throughout the day has been shown to be too great for single samples to be useful as a selection criterion. Land (1973b) suggested that testis growth in the lamb may be used as a simple measure of circulating gonadotrophins and Carr and Land (1975) have demonstrated a significant correlation between testis diameter and plasma LH concentration. Another possible approach that remains to be investigated in detail might be to assess the release of LH induced by the administration of LH-RH or oestrogens. The time interval from the injection of oestradiol benzoate to the onset of LH discharge has been found to be greater in lambs of a more prolific breed at 11 weeks of age (Thimonier et al., 1972). However the response to LH-RH, in relation to fecundity, has not been reported.

Clearly, further detailed knowledge of the physiological variables, responsible for genetic variation in ovulation rate, is desirable. The present studies have shown that ovarian sensitivity is unlikely to be responsible for controlling ovulation rate in sheep. This suggests that further studies should concentrate on other aspects of the ovarian-hypothalamo-hypophyseal axis. In this way a suitable selection criterion may be established. In addition to a sound scientific basis, for any selection procedure to be acceptable to the sheep farmer it must be simple to carry out and involve minimal trauma to his stock. A single blood sample or measurement of an external surface, such as testis diameter, would meet these requirements. In view of the many natural and experimental differences in reproductive activity that have been related to prolificacy, it appears hopeful that a selection procedure will be established in the future. By incorporation into sheep breeding programmes this could result in a more rapid improvement in reproductive performance within a flock.

TABLES

TABLE 2.1 Evaluation of Oestradiol-17β standard curve (results from six curves)

E_2 (pg)	% Bound			Estimated Mass (pg)		
	Mean	S.D.	C.V.	Mean	S.D.	C.V.
2.5	95.5	1.9	2.0			
5	91.6	1.9	2.1	5	1.0	20.2
10	82.7	1.6	1.9	10	1.1	11.2
20	72.1	2.4	3.3	20	2.7	13.6
50	52.8	1.7	3.2	50	3.5	6.9
100	35.7	1.8	5.1	100	8.9	8.9
200	24.2	1.5	6.1	200	21.5	10.8
400	16.2	1.1	6.8	400	37.5	9.4
500	13.7	1.3	9.8			

S.D. = Standard Deviation.

C.V. = Coefficient of Variation.

TABLE 2.2 Water and plasma blank values

	Volume (ml)	No. of Determinations	Amount of E_2-17β Measured		
			Mean (pg)	S.D.	C.V.
Water	0.5	40	7.0	2.2	31.4
"	4.0	20	6.5	2.1	32.0
Plasma	0.5	40	10.9	3.6	33.0
"	4.0	20	15.4	3.1	19.8

TABLE 2.3 Accuracy of radioimmunoassay

Amount of E_2-17β added to plasma (pg)	Volume of Plasma	Number of Determinations	Amount of E_2-17β Measured			% Recovery (Mean ± S.D.)
			Mean (pg)	S.D.	C.V.	
20	0.5	40	28.7	4.9	17.1	87.5 ± 16.8
50	"	40	54.5	7.3	13.4	84.9 ± 14.1
100	"	40	101.6	12.3	12.1	90.2 ± 12.6
20	4.0	10	31.9	4.0	12.7	82.5 ± 17.0
50	"	10	57.3	6.9	12.1	84.7 ± 10.6
100	"	10	92.2	11.6	12.6	77.4 ± 11.3

TABLE 2.4 Precision of radioimmunoassay - Inter-assay variation

Control Plasma	No. of Determinations	Amount of E_2-17β Measured		
		Mean (pg)	S.D.	C.V.
A	36	139.8	22.3	16.0
B	28	61.9	10.1	16.3

TABLE 2.5 Precision of radioimmunoassay - Intra-assay variation

Control Plasma	No. of Determinations	Amount of E_2-17β Measured		
		Mean (pg)	S.D.	C.V.
C	10	62.5	5.1	8.2
D	10	156.8	9.6	6.1

TABLE 2.6 Cross-reaction of some steroids with oestradiol antiserum

Steroid	% Cross-reaction
Oestradiol-17β	100.00
Oestrone	51.80
Oestriol	4.30
Cortisol	< 0.01
Progesterone	< 0.01
Testosterone	< 0.01

TABLE 2.7 Cross-reaction of various steroids (1 pg) with oestradiol antiserum as reported by Caldwell

Steroid	Oestradiol Equivalents (pg)	% Cross-Reaction
Oestradiol-17β		100
Oestrone		100
Oestriol		10
Dehydroepiandrosterone	15	< 0.01
Androstenedione	13	"
Adrenosterone	5	"
Cortisone	25	"
Cortisol	13	"
Deoxycorticosterone	30	"
Deoxycorticosterone acetate	6	"
Cholesterol	0	"
Pregnenolone	15	"
Progesterone	5	"
20α-hydroxy-4-pregnenone	2	"
20β-hydroxy-4-pregnenone	4	"
Dihydrotestosterone	8	"
Testosterone	7	"
17α-hydroxyprogesterone	3	"

TABLE 3.1 Mean litter sizes for Finnish Landrace and Scottish Blackface ewes*

Breed	Year	Litter Size (Mean ± S.D.)	Mean ± S.D.
Finnish Landrace	1973	2.64 ± 0.67	
	1974	1.96 ± 0.73	2.37 ± 0.88
	1975	2.55 ± 0.93	
Scottish Blackface	1973	1.42 ± 0.50	1.29 ± 0.46
	1974	1.13 ± 0.35	
Experimental Ewes	1975		
Finnish Landrace		2.61 ± 0.99	
Scottish Blackface		1.08 ± 0.29	

* These values include only the ewes that lambed.

TABLE 3.2 The concentration of total unconjugated oestrogens in the peripheral plasma of Finnish Landrace and Scottish Blackface ewes

Days of Oestrous Cycle	Finnish Landrace			Scottish Blackface		
	Number of Sheep	Oestrogens (pg/ml) (Mean ± S.D.)	Significance between Days	Number of Sheep	Oestrogens (pg/ml) (Mean ± S.D.)	Significance between Days
-4	15	4.7 ± 1.1		8	5.2 ± 1.4	
			N.S.*			N.S.
-3	22	5.4 ± 2.0		12	5.4 ± 2.0	
			N.S.			N.S.
-2	22	6.6 ± 2.3		15	7.7 ± 3.8	
			P <0.01			N.S.
-1	24	8.9 ± 3.2		15	9.1 ± 5.4	
			P <0.05			P <0.05
0	24	11.4 ± 4.1		17	5.9 ± 2.9	
			P <0.001			N.S.
1	24	5.3 ± 1.6		17	5.3 ± 2.4	
			N.S.			N.S.
2	24	5.8 ± 2.3		17	5.7 ± 2.8	
			N.S.			N.S.
3	24	6.6 ± 2.1		17	5.5 ± 1.6	
			N.S.			N.S.
4	24	6.6 ± 1.8		17	5.3 ± 1.4	
			N.S.			N.S.
5	24	6.4 ± 1.4		17	4.5 ± 1.6	
Base Level		5.1 ± 1.7			5.3 ± 1.7	

* N.S. = Not significant.

TABLE 4.1 The bodyweights and ovarian characteristics of control and PMSG-treated Scottish Blackface lambs during 1973

Treatment	Age (Days)	Lamb No.	Body-weight (kg)	Ovarian Volume (ml)	Large Follicles	
					Entire	Ovulated or Luteinised
Control	30	12	17.2	0.52	1	-
"	69	13R	17.7	1.57	-	-
"	71	79	21.3	0.62	-	-
"	94	10	21.8	2.08	-	-
"	123	67	31.3	0.74	-	-
"	127	62	21.8	1.42	-	-
250 iu PMSG	30	46	16.8	1.52	-	-
"	32	24	16.3	0.68	-	1
"	28	33S	13.2	0.53	-	-
500 iu PMSG	32	31	11.8	0.25	1	-
"	33	22	15.4	4.09	3	4
"	28	33R	13.2	1.07	-	2
"	67	31	15.4	0.87	-	2
"	68	22	21.8	Adhesions		
"	63	33R	21.3	1.57	-	2
"	70	16	24.5	3.54	2	3
"	71	7R	24.5	0.95	-	1
"	69	13S	20.0	1.29	-	1
"	94	7	16.3	1.81	7	-
"	93	8	19.0	1.20	2	-
"	95	9	23.6	1.07	1	-
"	133	55	35.6	1.41	-	1
"	130	57	38.5	0.86	-	1
"	128	60	32.7	1.35	-	1
1000 iu PMSG	28	35	14.3	18.57	50*	-
"	28	11	12.2	8.99	9	-
"	28	15	12.7	40.96	8	40*
"	137	51	34.9	1.52	2	1
"	130	56	29.9	1.33	6	1
"	131	58	38.1	2.26	2	5

* Estimated follicle counts.

- Indicates no large follicles.

TABLE 4.2 The bodyweights, ovarian characteristics and uterine horn diameters of control and PMSG-treated Finnish Landrace and Scottish Blackface lambs at 7 days of age during 1974

Breed	Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Large Follicles		Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	
Finnish	Control	28	1.5	0.03	-	-	0.4
	PMSG	172	3.0	0.12	-	-	0.5
	"	24	3.2	3.29	11	-	0.8
	"	26	4.1	0.11	-	-	0.5
	"	184	4.3	0.18	-	-	0.5
Mean $\pm$ S.D.			3.7 ± 0.6	0.92 ± 1.57			0.58 ± 0.15
Blackface	Control	26	4.0	0.11	-	-	0.7
	PMSG	19	5.7	0.09	-	-	0.6
	"	54	3.8	0.07	-	-	0.5
	"	16	5.2	0.54	2	-	0.7
	"	45	5.5	0.16	-	-	0.8
Mean $\pm$ S.D.			5.1 ± 0.9	0.22 ± 0.22			0.65 ± 0.13

- Indicates no large follicles.

TABLE 4.3 The bodyweights, ovarian characteristics and uterine horn diameters of control and PMSG-treated Finnish Landrace and Scottish Blackface lambs at 21 days of age during 1974

Breed	Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Large Follicles		Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	
Finnish	Control	174S	7.5	0.21	1	-	0.6
	PMSG	160S	5.9	0.22	4	-	0.8
	"	193G	4.1	0.06	-	-	0.5
	"	173	7.1	27.44	120*	-	0.6
	"	27R	8.0	1.88	2	2	0.7
Mean ± S.D.			6.3 ± 1.7	7.40 ± 13.39			0.65 ± 0.13
Blackface	Control	33	10.52	0.59	-	-	0.7
	PMSG	56	5.83	0.13	-	-	0.7
	"	40	9.99	3.90	10	-	0.6
	"	46	10.19	1.82	1	-	0.8
	"	15	9.34	3.93	4	-	0.8
Mean ± S.D.			8.8 ± 2.0	2.45 ± 1.83			0.73 ± 0.10

* Estimated follicle counts.

\- Indicates no large follicles.

TABLE 4.4 The bodyweights, ovarian characteristics and uterine horn diameters of control and PMSG-treated Finnish Landrace and Scottish Blackface lambs at 35 days of age during 1974

Breed	Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Large Follicles		Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	
Finnish	Control	197	9.2	0.69	-	-	0.6
	PMSG	22S	9.2	15.13	70*	-	0.7
	"	174R	9.0	42.30	200*	-	0.7
	"	193R	5.9	2.11	11	-	0.6
	"	27S	5.1	3.02	24	-	0.5
Mean ± S.D.			7.3 ± 2.1	15.64 ± 18.74			0.63 ± 0.10
Blackface	Control	39	11.68	4.03	-	-	1.1
	PMSG	53	11.75	0.53	3	-	1.1
	"	59	10.25	1.29	10	-	1.0
	"	47	9.39	10.33	45	-	1.0
	"	22	10.39	5.20	2	-	0.8
Mean ± S.D.			10.4 ± 1.0	4.34 ± 4.45			0.97 ± 0.13

* Estimated follicle counts.

\- Indicates no large follicles.

TABLE 4.5 The bodyweights, ovarian characteristics and uterine horn diameters of control and PMSG-treated Finnish Landrace and Scottish Blackface lambs at 49 days of age during 1974

Breed	Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Large Follicles		Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	
Finnish	Control	176	14.7	2.90	1	-	0.8
	PMSG	160R	12.8	3.61	15	-	0.9
	"	193S	13.2	2.69	12	-	0.8
	"	183	17.5	4.79	17	-	0.7
	"	22R	13.0	33.31	120*	-	0.7
Mean ± S.D.			14.1 ± 2.3	11.10 ± 14.83			0.78 ± 0.10
Blackface	Control	55	12.75	3.20	-	-	0.8
	PMSG	51	14.85	4.66	10	-	1.0
	"	58	15.75	11.52	45	-	0.9
	"	57	13.25	8.67	29	-	1.0
	"	52	15.20	4.40	9	-	0.8
Mean ± S.D.			14.8 ± 1.1	7.31 ± 3.42			0.93 ± 0.10

* Estimated follicle counts.

\- Indicates no large follicles.

TABLE 4.6 The epithelial height in the caruncular and intercaruncular regions of the uteri in Finnish Landrace and Scottish Blackface lambs during 1974*

Breed	Age (Days)	Height of Caruncular Epithelium (μ)		Height of Intercaruncular Epithelium (μ)	
		Control	PMSG	Control	PMSG
Finnish	7	10	9, 19	17	17, 27
	21	11	15, 13	16	18, 14
	35	16	23, 19	24	30, 33
	49	12	23	21	30
Blackface	7	10	10, 12	19	15, 21
	21	8	11, 33	8	17, 42
	35	11	22, 27	14	31, 28
	49	15	40, 35	15	45, 36
Mean ± S.D.		11.6 ± 2.7	20.7 ± 9.6	16.8 ± 4.8	26.9 ± 9.8

* The values represent the results for individual lambs.

TABLE 4.7 The bodyweights, ovarian characteristics and uterine horn diameters of control Finnish Landrace lambs during 1975

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
Control	184S	6.3	0.77	0.81	-	-	-	0.6
"	89R	10.5	0.76	0.86	-	-	-	0.6
"	169	8.3	0.60	0.72	-	-	-	0.6
"	156S	6.8	0.67	0.69	-	-	-	0.7
"	73R	7.0	1.80	1.76	-	-	-	0.5
Mean $\pm$ S.D.		7.8 ± 1.7	0.92 ± 0.50	0.97 ± 0.44	-	-	-	0.60 ± 0.07

- Indicates no large follicles.

TABLE 4.8 The bodyweights, ovarian characteristics and uterine horn diameters of control Scottish Blackface lambs during 1975

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
Control	63	7.0	0.13	0.15	-	-	-	0.6
"	53	4.3	0.13	0.13	-	-	-	0.5
"	65	9.0	0.12	0.17	-	-	-	0.9
"	66	6.0	0.26	0.24	-	-	-	0.6
"	67	5.5	0.06	0.07	-	-	-	0.6
Mean $\pm$ S.D.		6.4 $\pm$ 1.8	0.14 $\pm$ 0.07	0.16 $\pm$ 0.06	-	-	-	0.64 $\pm$ 0.15

- Indicates no large follicles.

TABLE 4.9 The number of oocytes in primordial, growing and vesicular follicles in the ovaries of Finnish Landrace and Scottish Blackface control lambs during 1975

Breed	No. of Lambs	No. of oocytes (Mean ± S.D.)		
		Primordial Follicles	Growing Follicles	Vesicular Follicles
Finnish Landrace	3	49,286 ± 6,283	1,306 ± 848	1,286 ± 429
Scottish Blackface	3	34,119 ± 7,559	1,700 ± 654	346 ± 307

TABLE 4.10 The bodyweights, ovarian characteristics and uterine horn diameters of PMSG-treated Finnish Landrace lambs during 1975

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
PMSG	86	11.5	4.91	5.58	12	2	14	1.0
"	153	8.0	12.98	9.89	21	18	39	0.9
"	151S	7.5	1.15	1.40	2	-	2	0.6
"	89S	9.7	3.38	2.83	6	3	9	0.9
"	165	6.0	0.17	0.20	3	-	3	0.9
"	161S	7.0	10.21	8.15	22	1	23	0.7
"	162	6.3	0.54	0.67	-	-	-	0.5
"	92	11.8	5.60	5.29	-	-	-	1.0
"	160S	8.3	7.64	7.12	8	18	26	0.7
"	156R	5.5	4.54	3.61	7	10	17	0.7
"	173S	9.2	18.00	14.82	22	16	38	0.9
"	152	9.5	9.33	8.31	11	-	11	0.9
"	176	9.3	20.55	16.11	30	11	41	0.9
Mean $\pm$ S.D.*		8.4 $\pm$ 2.0	7.62 $\pm$ 6.45	6.46 $\pm$ 5.03	13.1 $\pm$ 9.2	7.2 $\pm$ 7.6	20.3 $\pm$ 14.3	0.82 $\pm$ 0.16

* The means for bodyweights, ovarian volume, ovarian weight and uterine horn diameter include all treated lambs and the means for large follicles include only those lambs that responded to treatment.

\- Indicates no large follicles.

TABLE 4.11 The bodyweights, ovarian characteristics and uterine horn diameters of PMSG-treated Scottish Blackface lambs during 1975

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
PMSG	98	5.8	9.62	6.32	17	8	25	0.8
"	62	9.0	1.15	1.20	1	1	2	1.0
"	52	7.3	6.13	6.37	8	3	11	1.0
"	54	6.8	0.04	0.06	-	-	-	0.6
"	55	6.3	0.05	0.08	-	-	-	0.6
"	58	8.0	0.16	0.24	-	-	-	0.9
"	50	6.3	3.23	3.33	4	7	11	0.9
"	51	5.5	24.19	19.58	34	6	40	0.8
"	56	6.5	1.46	1.63	4	1	5	0.7
"	57	6.0	2.05	1.77	2	1	3	0.9
"	60	6.0	0.14	0.15	-	-	-	0.9
"	59	7.5	1.62	1.53	2	-	2	0.8
"	61	7.3	3.62	2.93	-	-	-	0.9
"	64	8.0	0.30	0.28	-	1	1	1.0
"	97	8.0	0.55	0.51	-	3	3	0.8
Mean $\pm$ S.D.*		7.0 $\pm$ 1.0	3.62 $\pm$ 6.28	3.07 $\pm$ 5.02	7.2 $\pm$ 10.7	3.1 $\pm$ 2.9	10.3 $\pm$ 12.7	0.84 $\pm$ 0.13

* The means for bodyweights, ovarian volume, ovarian weight and uterine horn diameter include all treated lambs and the means for large follicles include only those lambs that responded to treatment.

\- Indicates no large follicles.

TABLE 4.12 Plasma level of oestrogens on day 4 in PMSG-treated Finnish Landrace and Scottish Blackface lambs, during 1975, that responded by the development of large follicles

Finnish Landrace		Scottish Blackface	
Lamb Number	Oestrogens pg/ml	Lamb Number	Oestrogens pg/ml
86	145	98	337
153	555	62	60
151S	3	52	302
89S	98	50	376
165	4	51	983
161S	390	56	131
160S	355	57	47
156R	122	59	30
173S	436	64	35
152	43	97	31
176	820		
Mean $\pm$ S.D.	270 $\pm$ 263		233 $\pm$ 297

TABLE 4.13 The epithelial height in the caruncular and intercaruncular regions of the uteri in Finnish Landrace and Scottish Blackface lambs during 1975*

Breed	Height of Caruncular Epithelium (μ)		Height of Intercaruncular Epithelium (μ)	
	Control	PMSG	Control	PMSG
Finnish	10	23, 22, 36	16	33, 27, 36
	11	31, 10	12	37, 16
	11	19, 17	20	32, 28
	16	28, 27	19	31, 33
	10	9, 34	12	17, 38
Blackface	11	16, 24, 21	19	26, 36, 29
	13	25, 27, 22	17	36, 40, 30
	15	13, 25, 25	18	17, 36, 30
	14	11, 23, 28	20	18, 29, 29
	10	13, 15	18	16, 17
Mean $\pm$ S.D.	12.1 $\pm$ 2.2.	21.8 $\pm$ 7.3	17.1 $\pm$ 3.0	28.7 $\pm$ 7.7

* The values represent the results for individual lambs.

TABLE 5.1 The bodyweights, ovarian characteristics and uterine horn diameters of methallibure-treated Finnish Landrace lambs, with and without PMSG treatment

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
Methallibure	194S	6.8	0.40	0.50	-	-	-	0.5
"	191	5.8	0.79	0.87	-	-	-	0.5
"	18	8.8	2.42	2.18	-	-	-	0.7
"	171R	6.0	2.12	1.81	-	-	-	0.5
"	174S	7.0	0.79	0.72	-	-	-	0.5
"	198R	5.8	0.41	0.42	-	-	-	0.5
Mean $\pm$ S.D.		6.7 $\pm$ 1.2	1.16 $\pm$ 0.89	1.08 $\pm$ 0.74				0.53 $\pm$ 0.08
Methallibure + PMSG	194R	7.8	0.44	0.47	-	-	-	0.6
"	171G	5.0	0.10	0.13	-	-	-	0.7
"	174R	7.3	52.60	44.88	120*	10	130*	0.8
"	22	7.3	0.33	0.33	2	-	2	0.7
"	193	6.5	7.73	8.91	13	30	43	0.7
"	192	7.5	2.51	2.28	3	7	10	0.5
"	198S	5.3	0.35	0.41	1	-	1	0.7
Mean $\pm$ S.D.		6.7 $\pm$ 1.1	9.15 $\pm$ 19.35	8.20 $\pm$ 16.47				0.67 $\pm$ 0.09

* Estimated follicle counts.

\- Indicates no large follicles.

TABLE 5.2 The bodyweights, ovarian characteristics and uterine horn diameters of methallibure-treated Scottish Blackface lambs, with and without PMSG treatment

Treatment	Lamb No.	Bodyweight (kg)	Ovarian Volume (ml)	Ovarian Weight (g)	Large Follicles			Uterine Horn Diameter (cm)
					Entire	Ovulated or Luteinised	Total	
Methallibure	69	6.5	0.54	0.62	-	-	-	0.8
"	72	3.3	0.05	0.05	-	-	-	0.4
"	74	7.3	1.07	1.19	-	-	-	0.8
"	75	5.5	0.12	0.13	-	-	-	0.7
"	80	3.0	0.53	0.63	-	-	-	0.5
Mean $\pm$ S.D.		5.1 $\pm$ 1.9	0.46 $\pm$ 0.41	0.52 $\pm$ 0.46				0.64 $\pm$ 0.18
Methallibure + PMSG	73	5.5	0.63	0.75	-	-	-	0.8
"	76	7.3	2.93	2.59	-	-	-	1.1
"	77	5.8	0.11	0.13	-	-	-	0.5
"	78	5.3	27.86	20.01	30	30	60	1.0
"	79	6.0	3.59	3.35	7	2	9	0.9
Mean $\pm$ S.D.		6.0 $\pm$ 0.8	7.02 $\pm$ 11.74	5.36 $\pm$ 8.29				0.86 $\pm$ 0.23

- Indicates no large follicles.

TABLE 5.3 The mean bodyweights of normal and methallibure-treated lambs, including those that received PMSG

Breed	Treatment	Number of lambs	Bodyweight (kg) Mean ± S.D.
Finnish	Normal	18	8.2 ± 1.9
	Methallibure	13	6.7 ± 1.1
Blackface	Normal	20	6.8 ± 1.2
	Methallibure	10	5.5 ± 1.4

TABLE 5.4 The mean ovarian volumes and ovarian weights of control and methallibure-treated lambs

Breed	Treatment	Number of Lambs	Ovarian Volume (ml) Mean ± S.D.	Ovarian Weight (g) Mean ± S.D.
Finnish	Control	5	0.92 ± 0.50	0.97 ± 0.44
	Methallibure	6	1.16 ± 0.89	1.08 ± 0.74
Blackface	Control	5	0.14 ± 0.07	0.16 ± 0.06
	Methallibure	5	0.46 ± 0.41	0.52 ± 0.46

TABLE 5.5 The mean uterine horn diameters of control and methallibure-treated lambs

Breed	Treatment	Number of Lambs	Uterine Horn Diameter (cm) Mean ± S.D.
Finnish	Control	5	0.60 ± 0.07
	Methallibure	6	0.53 ± 0.08
Blackface	Control	5	0.64 ± 0.15
	Methallibure	5	0.64 ± 0.18

TABLE 5.6 The epithelial height in the caruncular and intercaruncular regions of the uteri in control lambs and methallibure-treated lambs, with and without PMSG treatment*

Breed	Height of Caruncular Epithelium (μ)			Height of Intercaruncular Epithelium (μ)		
	Control	Methallibure	Methallibure + PMSG	Control	Methallibure	Methallibure + PMSG
Finnish	10, 16	11, 11	11, 29	16, 19	15, 12	16, 41
	11, 10	11, 14	14, 17	12, 12	16, 18	24, 29
	11	11	12	20	21	19
Blackface	11, 14	16, 15	13, 31	19, 20	18, 21	17, 30
	13, 9	13, 15	19, 30	17, 18	18, 21	25, 40
	15	14	16	18	20	19
Mean ± S.D.	12.0 ± 2.4	13.1 ± 2.0	19.2 ± 7.8	17.1 ± 3.0	18.0 ± 3.0	26.0 ± 9.0

* These values represent the results for individual lambs.

TABLE 5.7 The absolute pituitary weights in control and methallibure-treated Finnish Landrace and Scottish Blackface lambs, with and without PMSG treatment

Breed	Absolute Pituitary Weight (g)			
	Control	PMSG	Methallibure	Methallibure + PMSG
Finnish	0.12, 0.25	0.23, 0.20	0.14, 0.13	0.15, 0.12
	0.21, 0.14	0.23, 0.15	0.13, 0.13	0.15, 0.10
	0.17	0.12, 0.19		0.14, 0.14
		0.11, 0.18		0.11
		0.24		
Mean $\pm$ S.D.	0.18 $\pm$ 0.05	0.18 $\pm$ 0.05	0.13 $\pm$ 0.00	0.13 $\pm$ 0.02
Blackface	0.14, 0.10	0.22, 0.18	0.27, 0.16	0.23, 0.25
	0.18, 0.16	0.21, 0.17	0.21, 0.18	0.17, 0.17
		0.25, 0.15	0.12	0.12
		0.13, 0.17		
		0.15, 0.17		
		0.21, 0.15		
		0.18, 0.15		
		0.14		
Mean $\pm$ S.D.	0.15 $\pm$ 0.03	0.18 $\pm$ 0.03	0.19 $\pm$ 0.06	0.19 $\pm$ 0.05

TABLE 5.8 The pituitary weights relative to bodyweights in control and methallibure-treated Finnish Landrace and Scottish Blackface lambs, with and without PMSG treatment

Breed	Relative Pituitary Weight - % ($\times 10^{-3}$)			
	Control	PMSG	Methallibure	Methallibure + PMSG
Finnish	1.9, 2.4	2.0, 2.2	2.1, 1.5	1.9, 2.1
	2.5, 2.1	2.6, 2.1	2.2, 1.9	2.4, 1.9
	2.4	2.3, 1.9		2.1, 2.2
		2.1, 2.6		1.4
		2.0		
Mean $\pm$ S.D.	2.26 $\pm$ 0.25	2.20 $\pm$ 0.25	1.93 $\pm$ 0.31	2.00 $\pm$ 0.32
Blackface	2.0, 3.0	2.5, 2.5	4.1, 3.3	4.2, 3.2
	2.4, 2.8	2.5, 2.8	4.9, 4.1	3.5, 1.9
		3.1, 2.8	2.9	2.9
		2.7, 2.1		
		3.1, 2.3		
		2.4, 1.9		
		2.3, 2.5		
		2.7		
Mean $\pm$ S.D.	2.55 $\pm$ 0.44	2.55 $\pm$ 0.33	3.86 $\pm$ 0.78	3.14 $\pm$ 0.84

TABLE 5.9 The mean number of oocytes in primordial, growing and vesicular follicles in the ovaries of methallibure-treated Finnish Landrace and Scottish Blackface lambs

Breed	Number of Lambs	No. of oocytes (Mean ± S.D.)		
		Primordial Follicles	Growing Follicles	Vesicular Follicles
Finnish Landrace	4	77,150 ± 36,148	1,415 ± 707	2,465 ± 1,212
Scottish Blackface	4	80,950 ± 43,879	1,255 ± 790	780 ± 832

FIGURES

FIG. 2.1 Extraction procedure (see text for details)

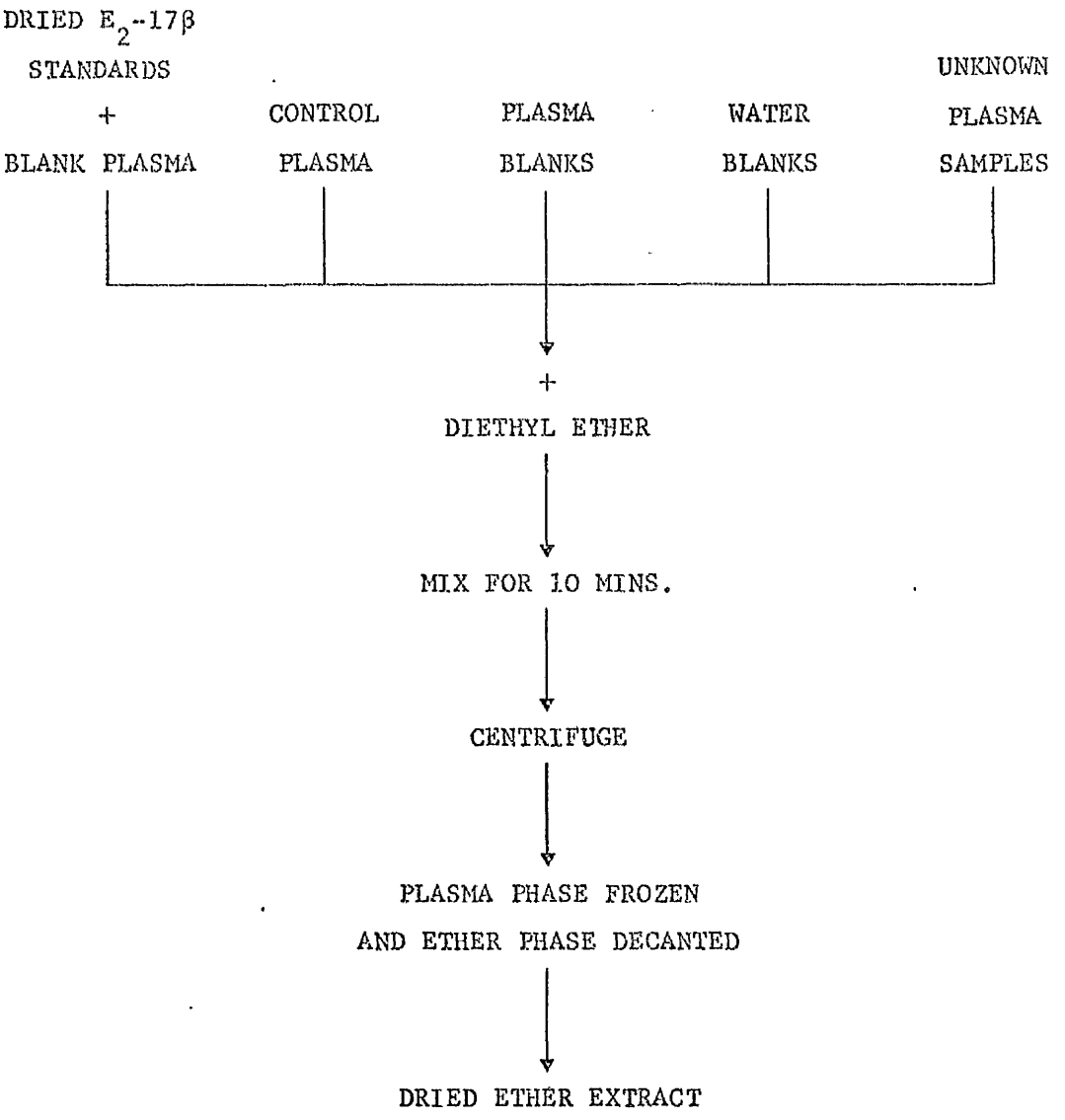


FIG. 2.2 Assay procedure (see text for details)

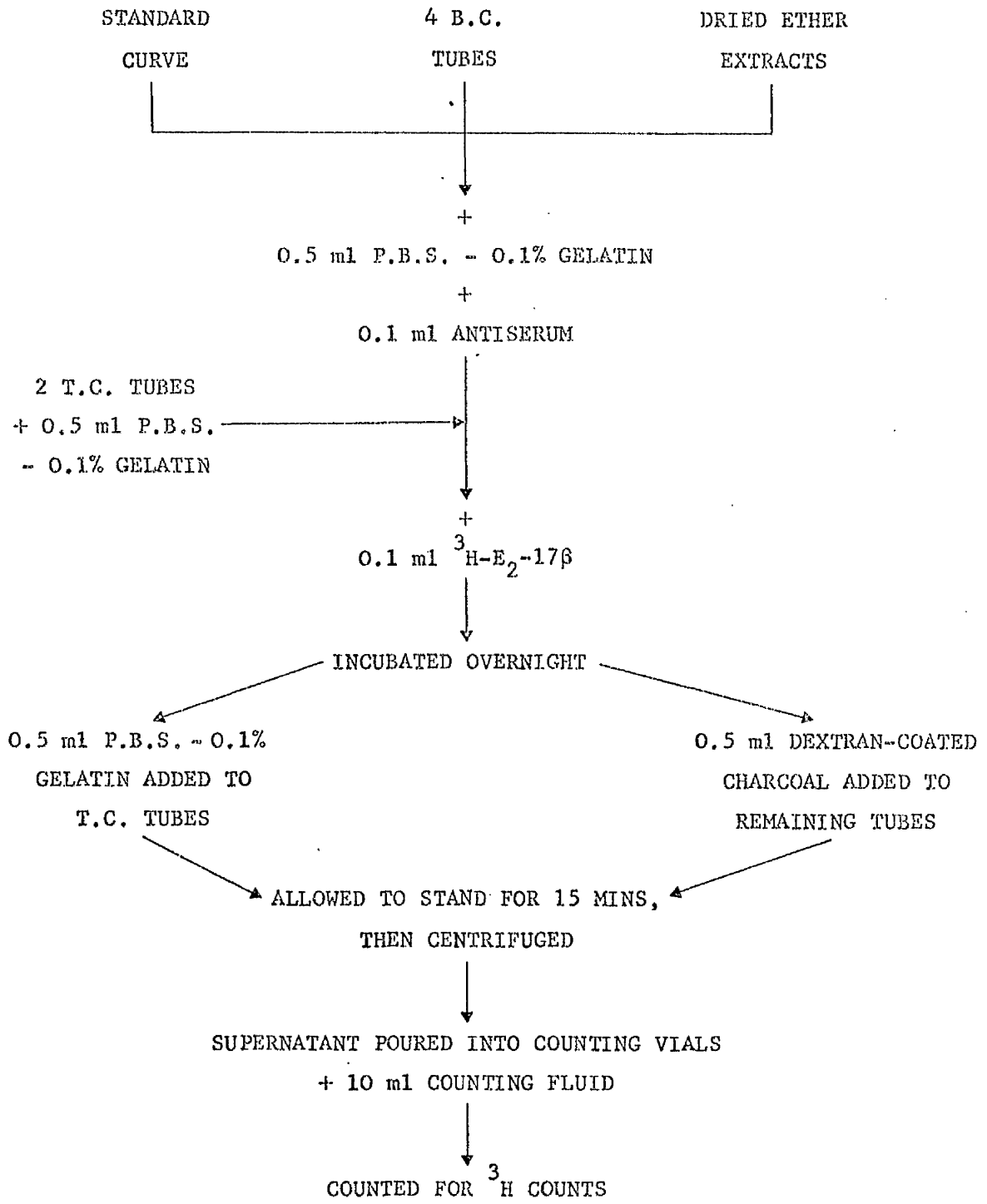


FIG. 2.3 Standard curve for oestradiol-17β radioimmunoassay (mean ± S.D. of results for six curves given in Table 2.1)

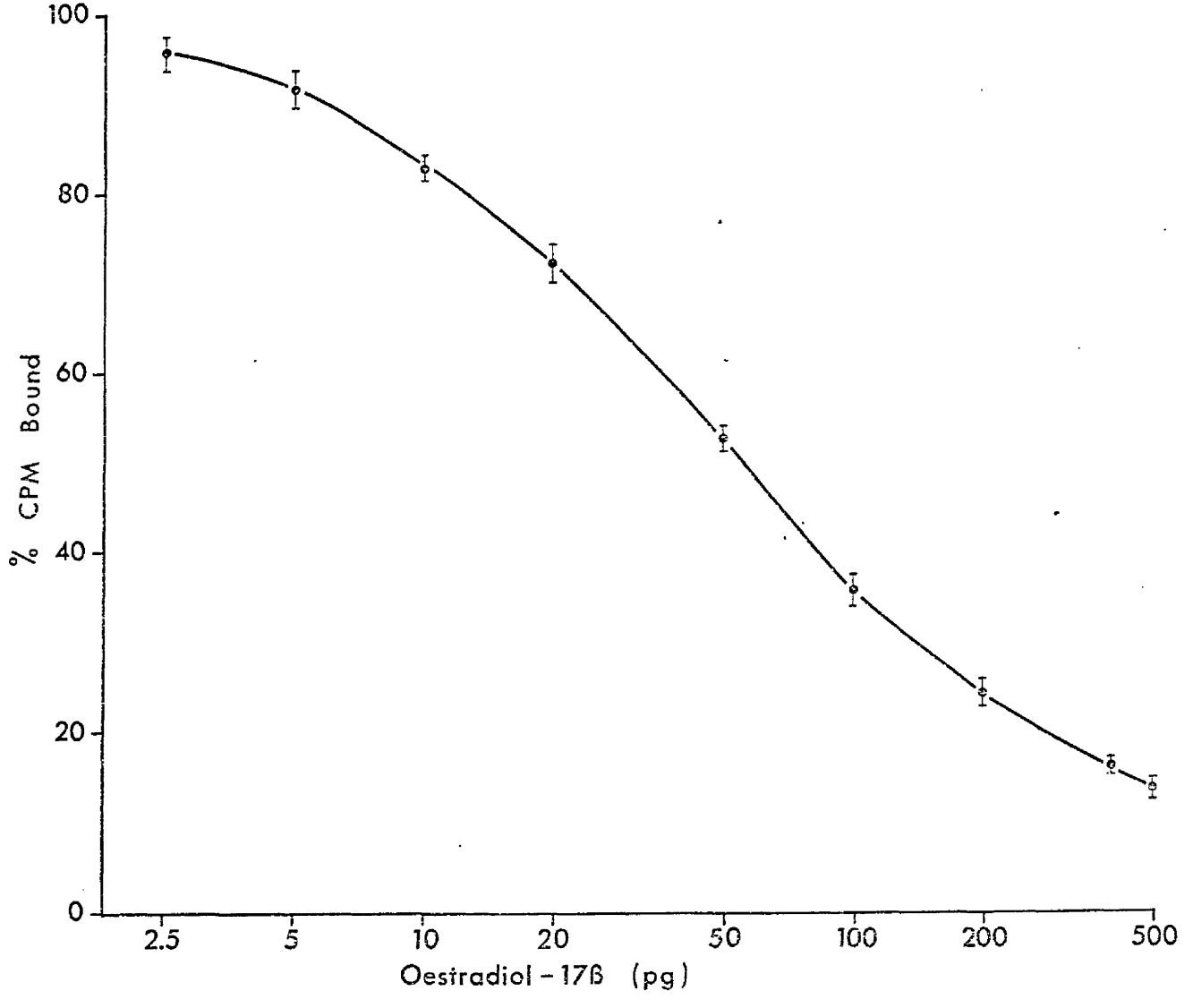
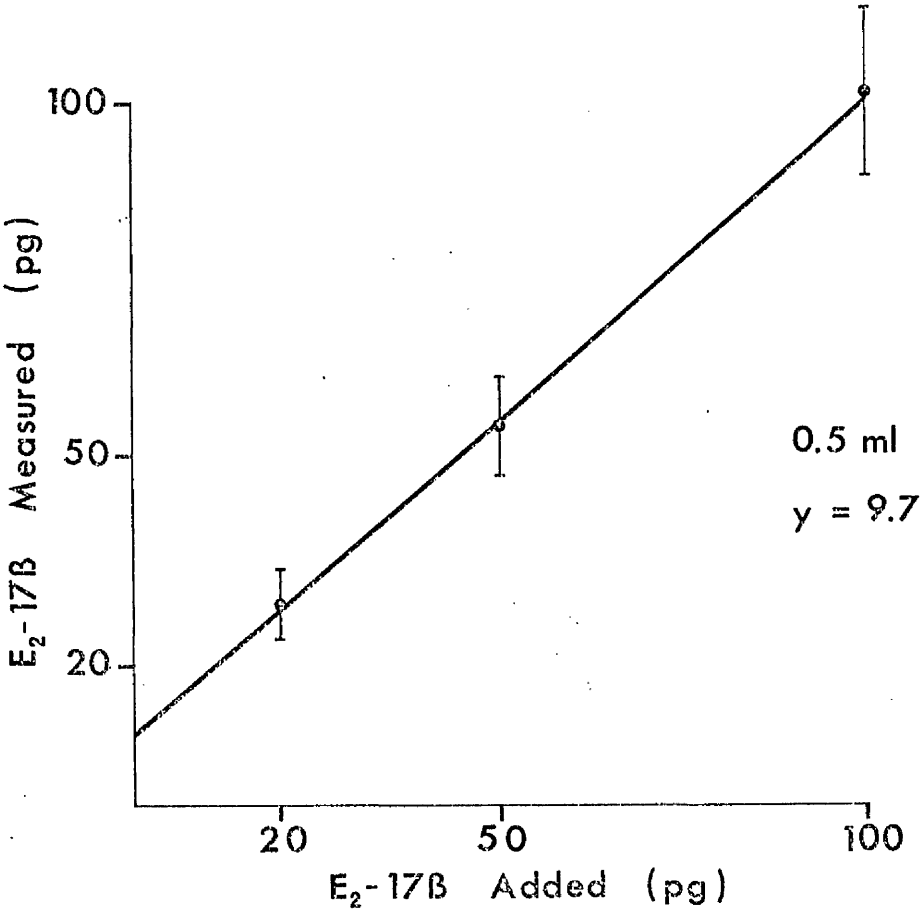

100
80
60
40
20
0
% CPM Bound
2.5
5
10
20
50
100
200
500
Oestradiol - 17ß (pg)

FIG. 2.4 The correlation between oestradiol-17β (E_2-17β) added to blank plasma and E_2-17β measured in the radioimmunoassay (0.5 ml plasma - mean $\pm$ S.D. of 40 determinations; 4.0 ml plasma - mean $\pm$ S.D. of 10 determinations)


100
50
20
E_2-17ß Measured (pg)
20
50
100
E_2-17ß Added (pg)
0.5 ml Plasma
y = 9.78 + 0.91x

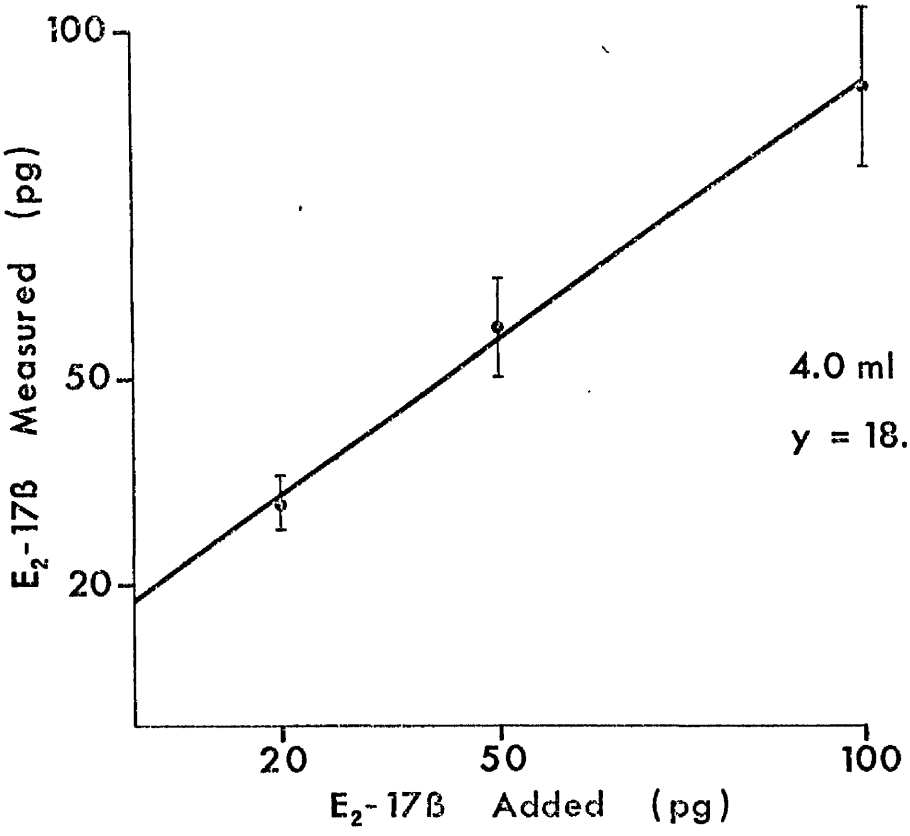

100
50
20
E_2-17ß Measured (pg)
20
50
100
E_2-17ß Added (pg)
4.0 ml Plasma
y = 18.04 + 0.75x

FIG. 2.5 Antibody dilution curve produced during March, 1973 (each point represents the mean of duplicate estimations)

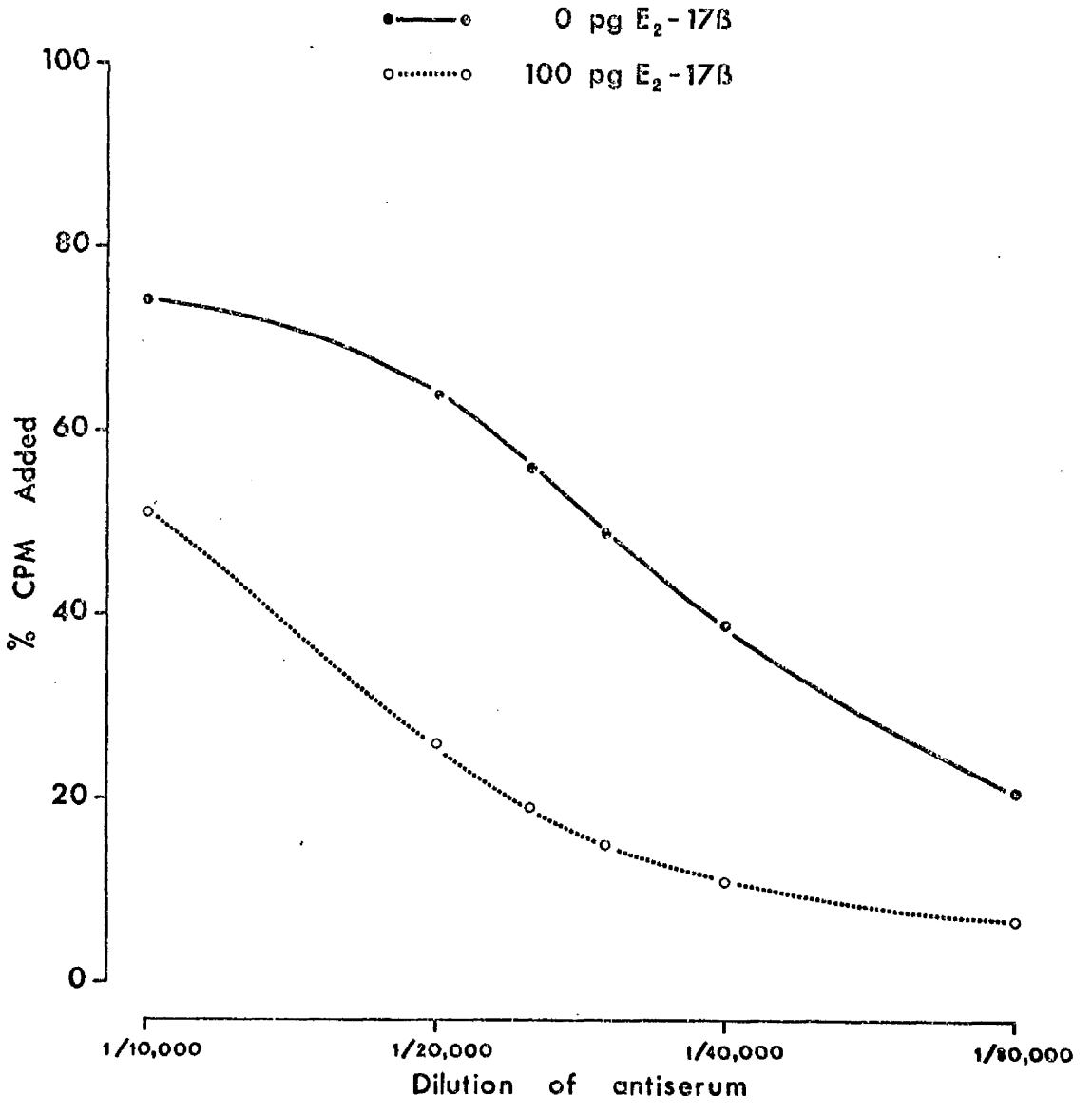

0 pg E_2-17β
100 pg E_2-17β
100
80
60
40
20
0
% CPM Added
1/10,000
1/20,000
1/40,000
1/80,000
Dilution of antiserum

FIG. 2.6 Antibody dilution curve produced during February, 1975 (each point represents the mean of duplicate estimations)

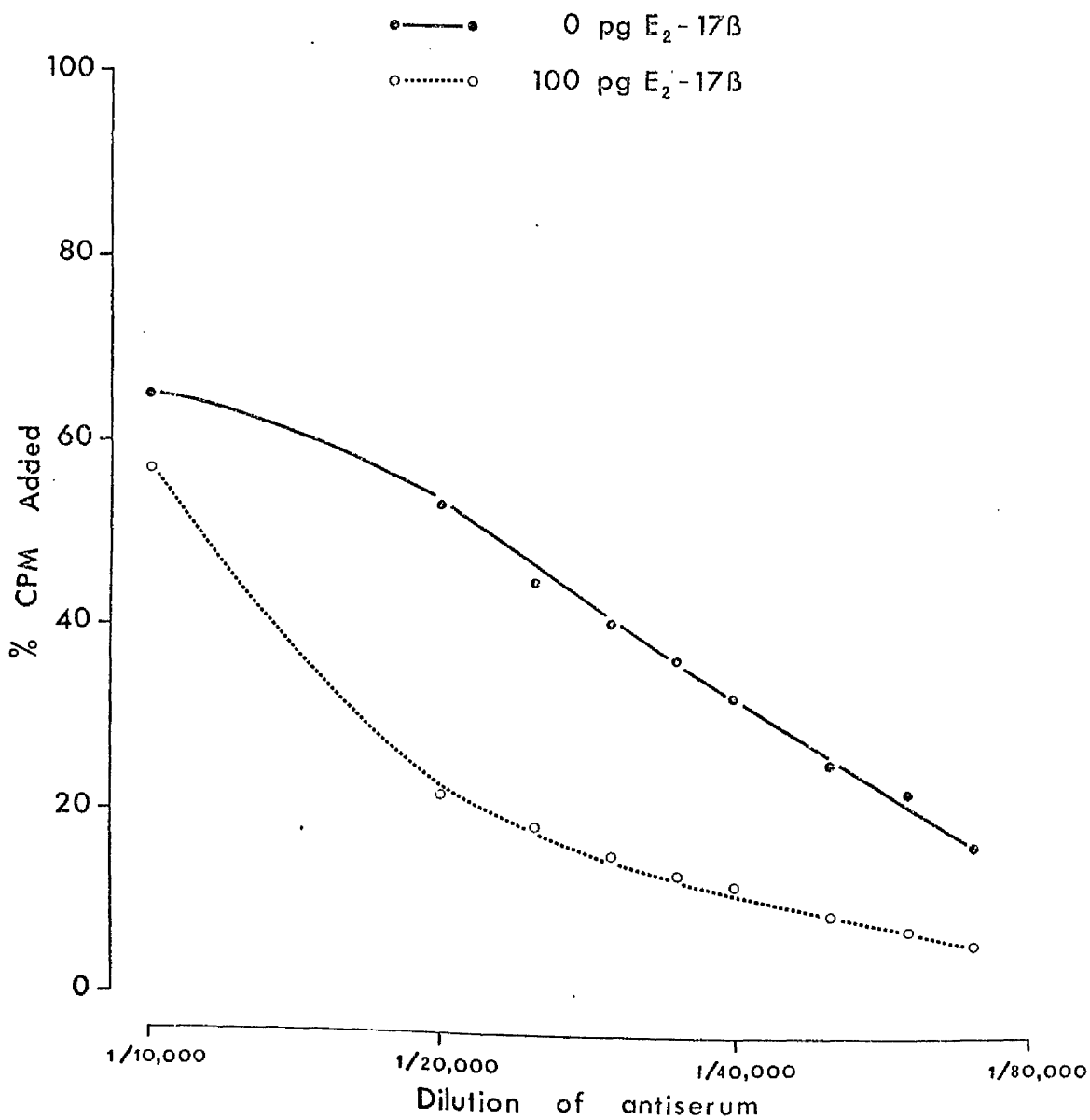

0 pg E_2-17ß
100 pg E_2-17ß
100
80
60
40
20
0
% CPM Added
1/10,000
1/20,000
1/40,000
1/80,000
Dilution of antiserum

FIG. 2.7 The cross-reaction of some steroids with the oestradiol antiserum (C = cortisol, T = testosterone, P = progesterone, E_1 = oestrone, E_2-17β = oestradiol-17β, E_3 = oestriol)

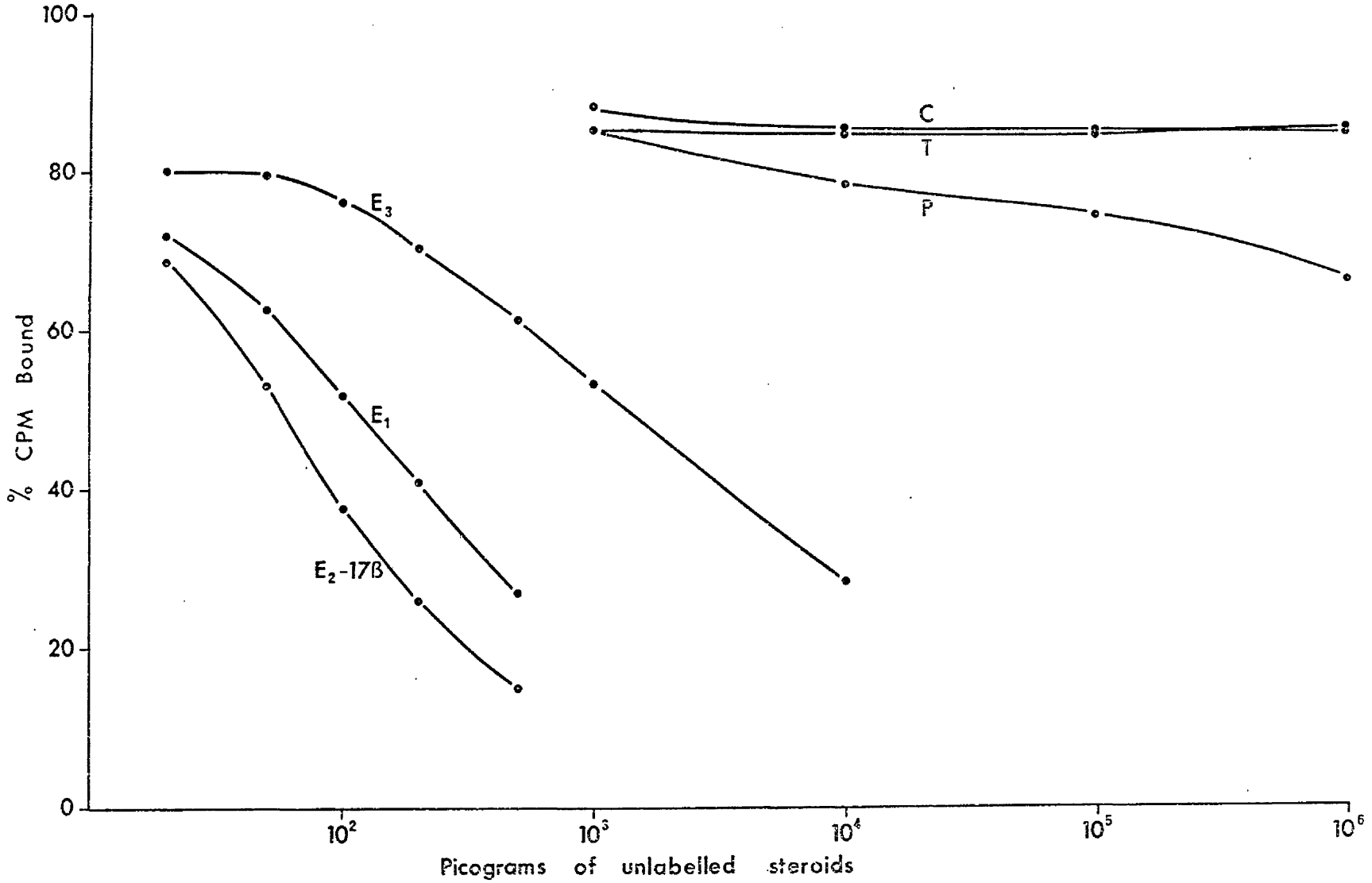

100
80
60
40
20
0
% CPM Bound
C
T
P
E3
E1
E2-17β
10²
10³
10⁴
10⁵
10⁶
Picograms of unlabelled steroids

FIG. 3.1 The pattern of the prooestrous peaks of oestrogens in Finnish Landrace and Scottish Blackface ewes around oestrus (each point represents the mean value for all animals sampled on that day)

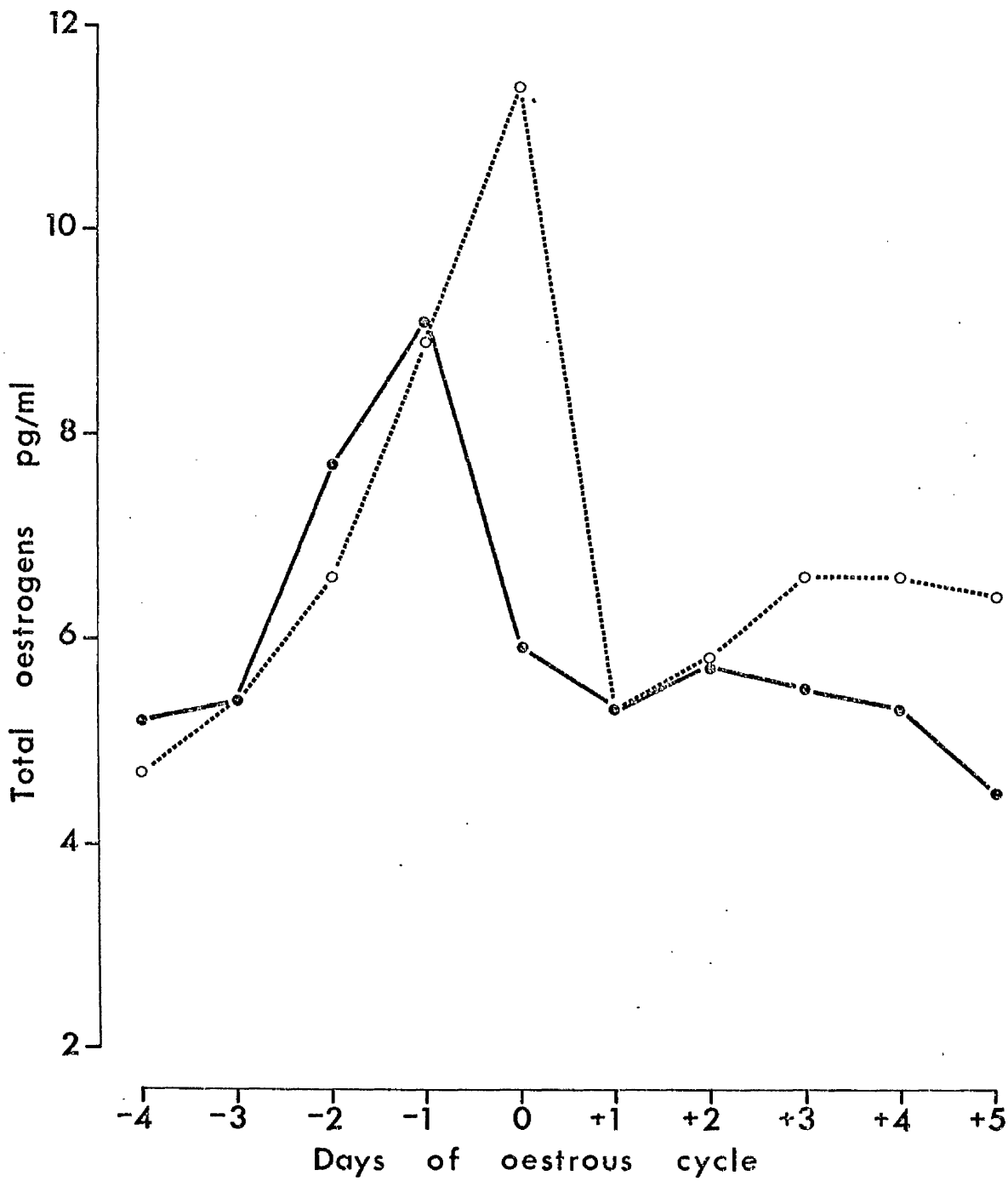

Finnish Landrace
Scottish Blackface
12
10
8
6
4
2
Total oestrogens pg/ml
-4
-3
-2
-1
0
+1
+2
+3
+4
+5
Days of oestrous cycle

FIG. 4.1 Plasma levels of oestrogens in Scottish Blackface control lambs and following treatment with 250 i.u. and 500 i.u. PMSG during 1975 (the values are the mean results for the groups shown in Table 4.1)

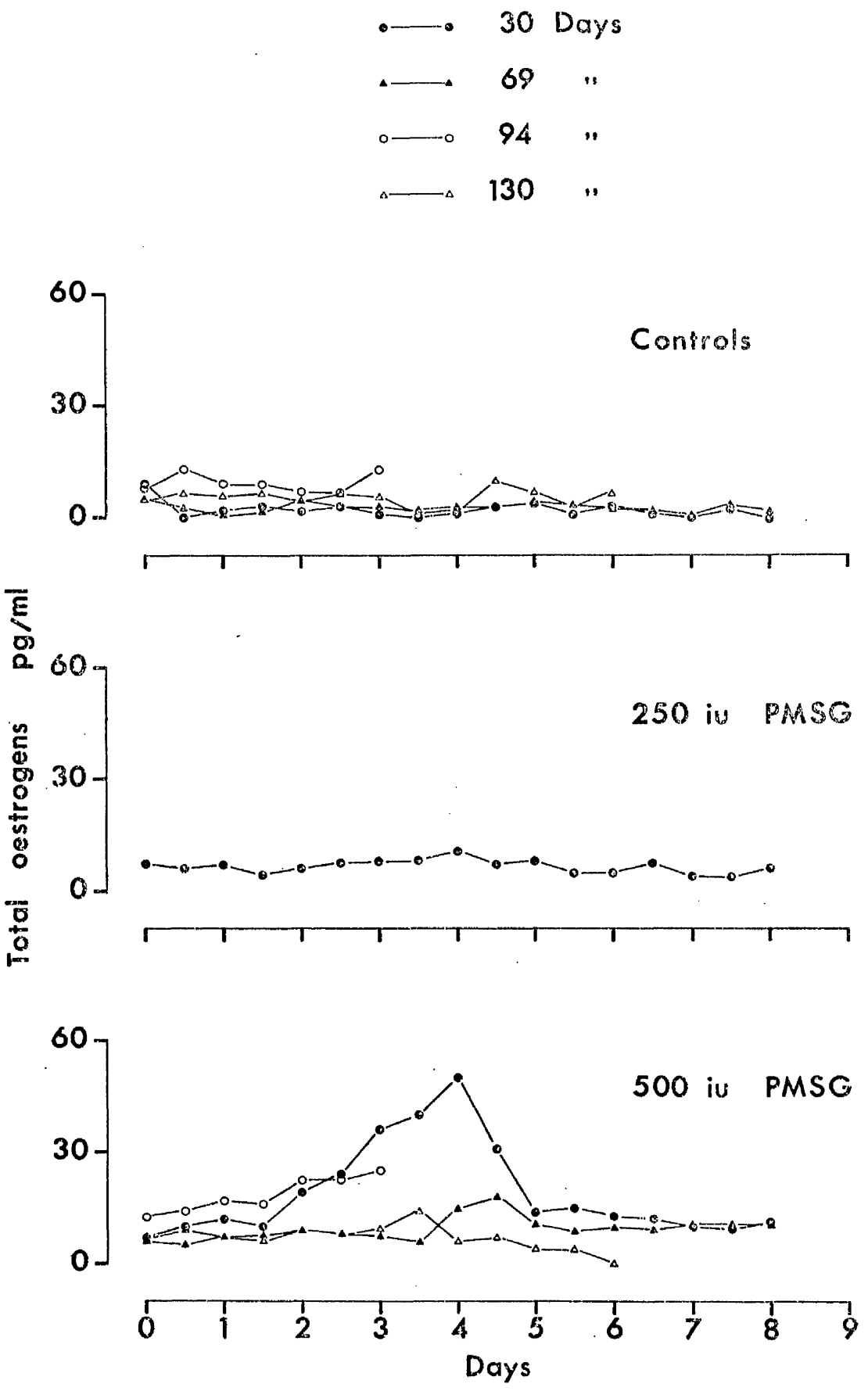

30 Days
69 "
94 "
130 "
Controls
250 iu PMSG
500 iu PMSG
Total oestrogens pg/ml
60
30
0
0 1 2 3 4 5 6 7 8 9
Days

FIG. 4.2 Plasma levels of oestrogens in Scottish Blackface lambs following treatment with 1000 i.u. PMSG during 1975 (the values are the mean results for the groups shown in Table 4.1)

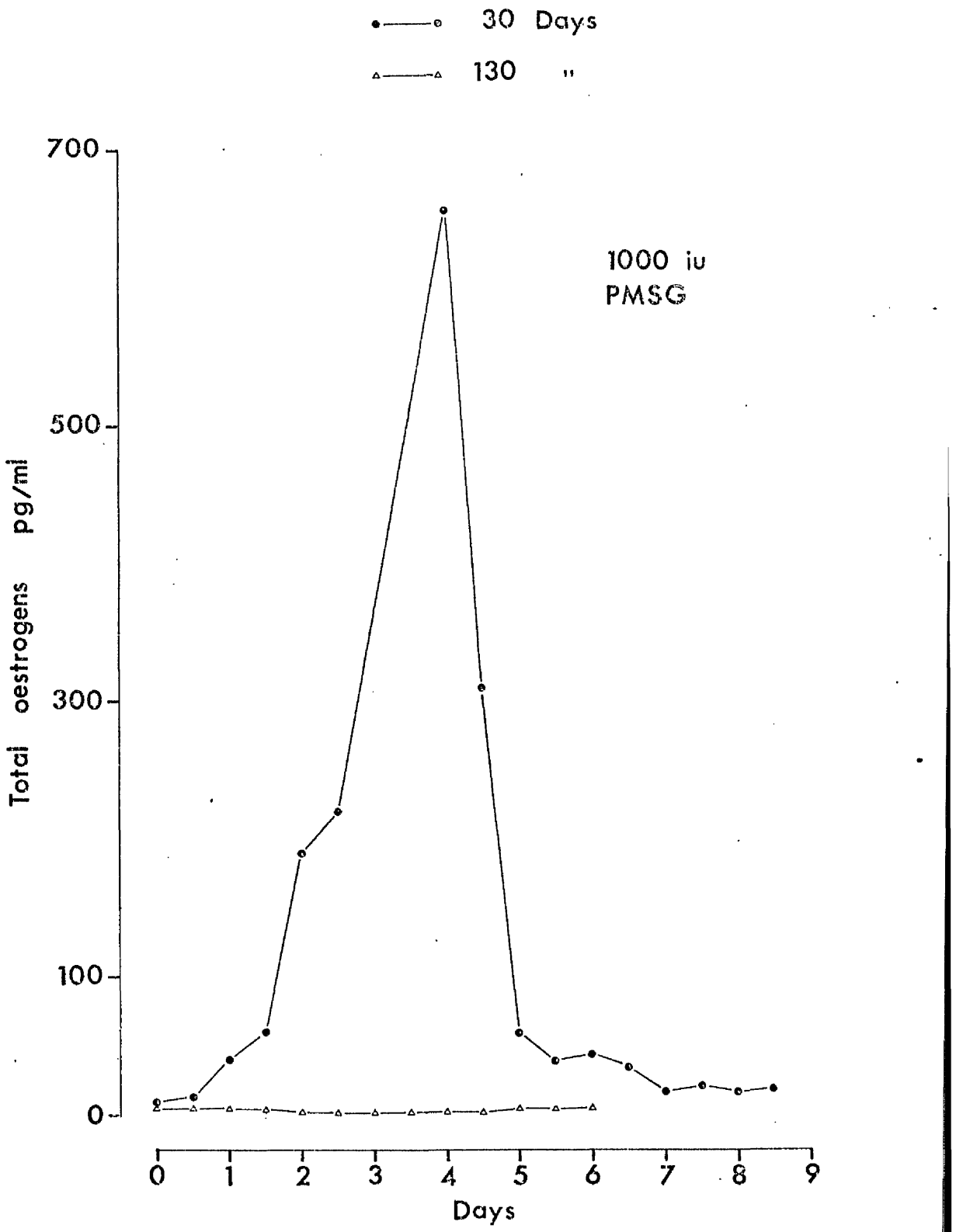

30 Days
130 "
700
500
300
100
0
Total oestrogens pg/ml
1000 iu
PMSG
0 1 2 3 4 5 6 7 8 9
Days

FIG. 4.3 Plasma levels of oestrogens in control and PMSG-treated Finnish Landrace lambs at 7 days of age during 1975

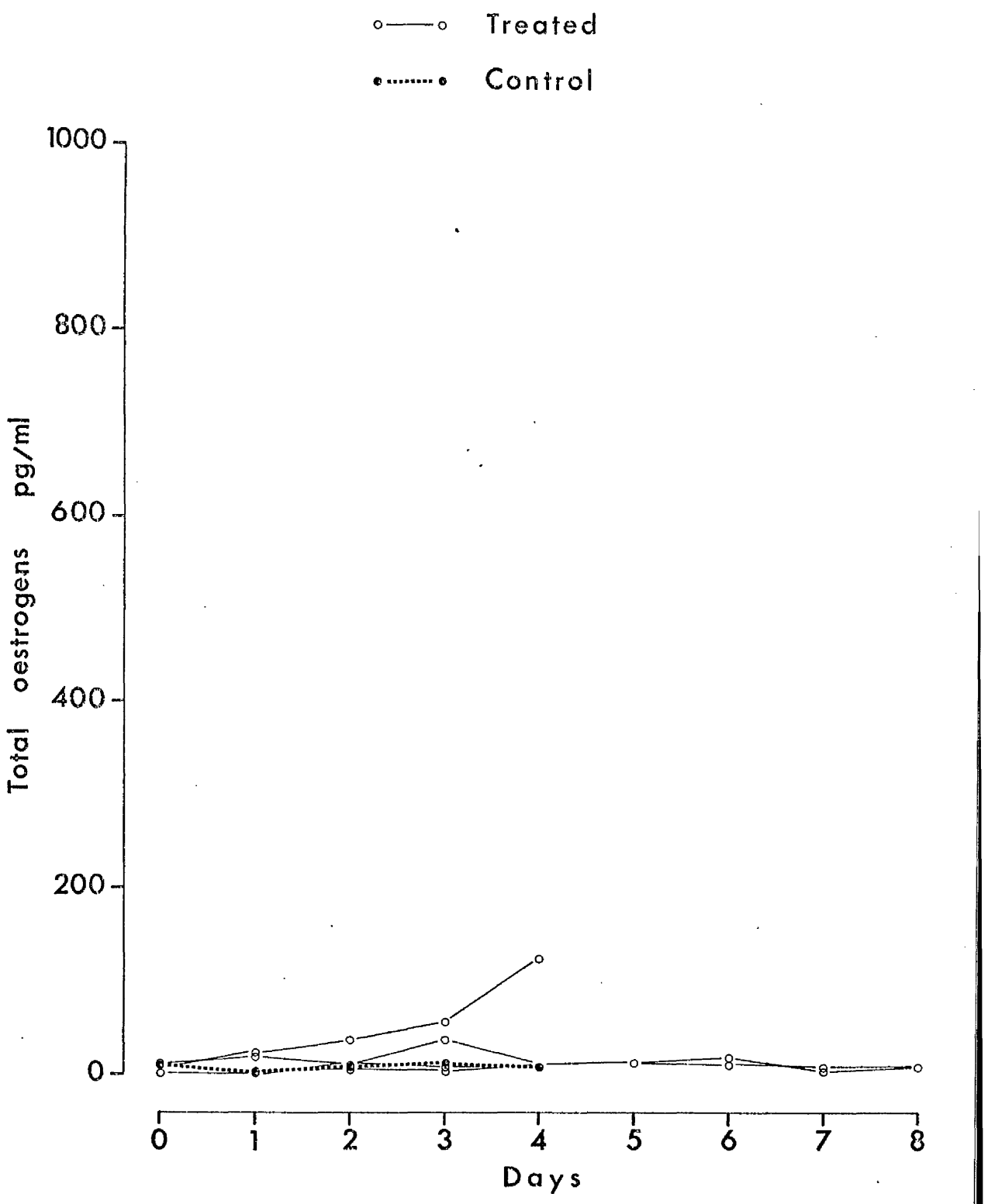

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.4 Plasma levels of oestrogens in control and PMSG-treated Scottish Blackface lambs at 7 days of age during 1975

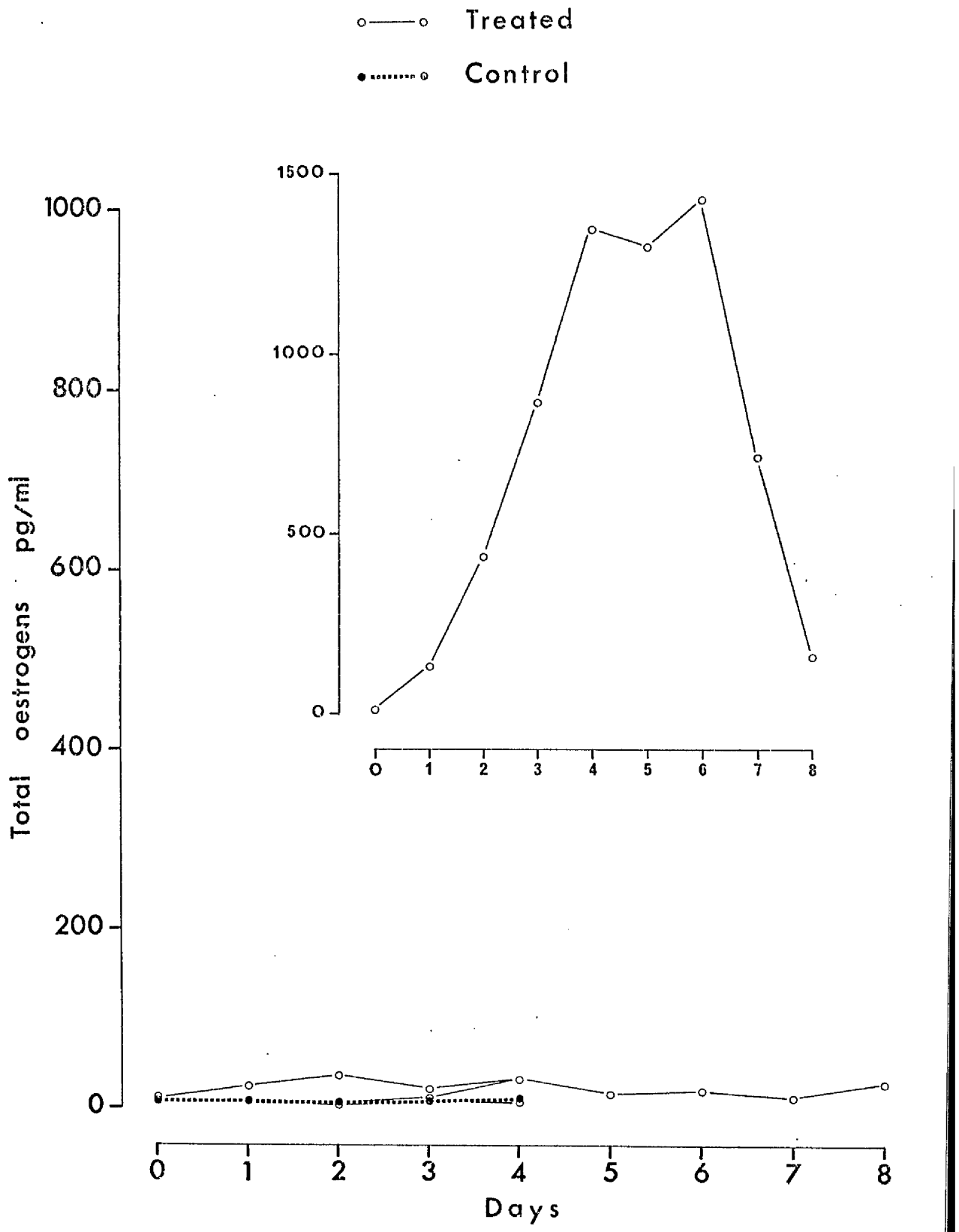

Treated
Control
Total oestrogens pg/ml
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8
Days
1500
1000
500
0
0 1 2 3 4 5 6 7 8

FIG. 4.5 Plasma levels of oestrogens in control and PMSG-treated Finnish Landrace lambs at 21 days of age during 1975

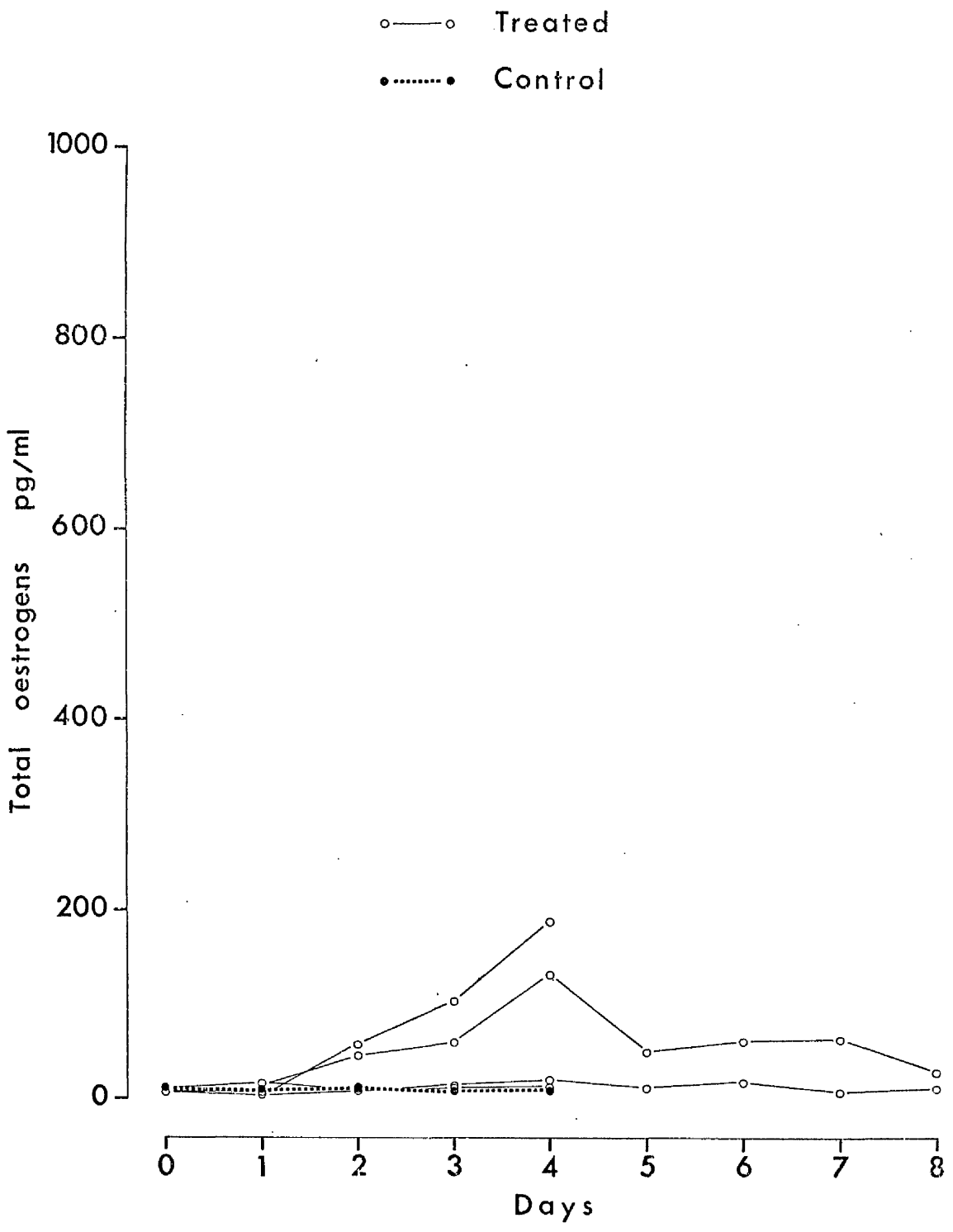

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.6 Plasma levels of oestrogens in control and PMSG-treated Scottish Blackface lambs at 21 days of age during 1975 .

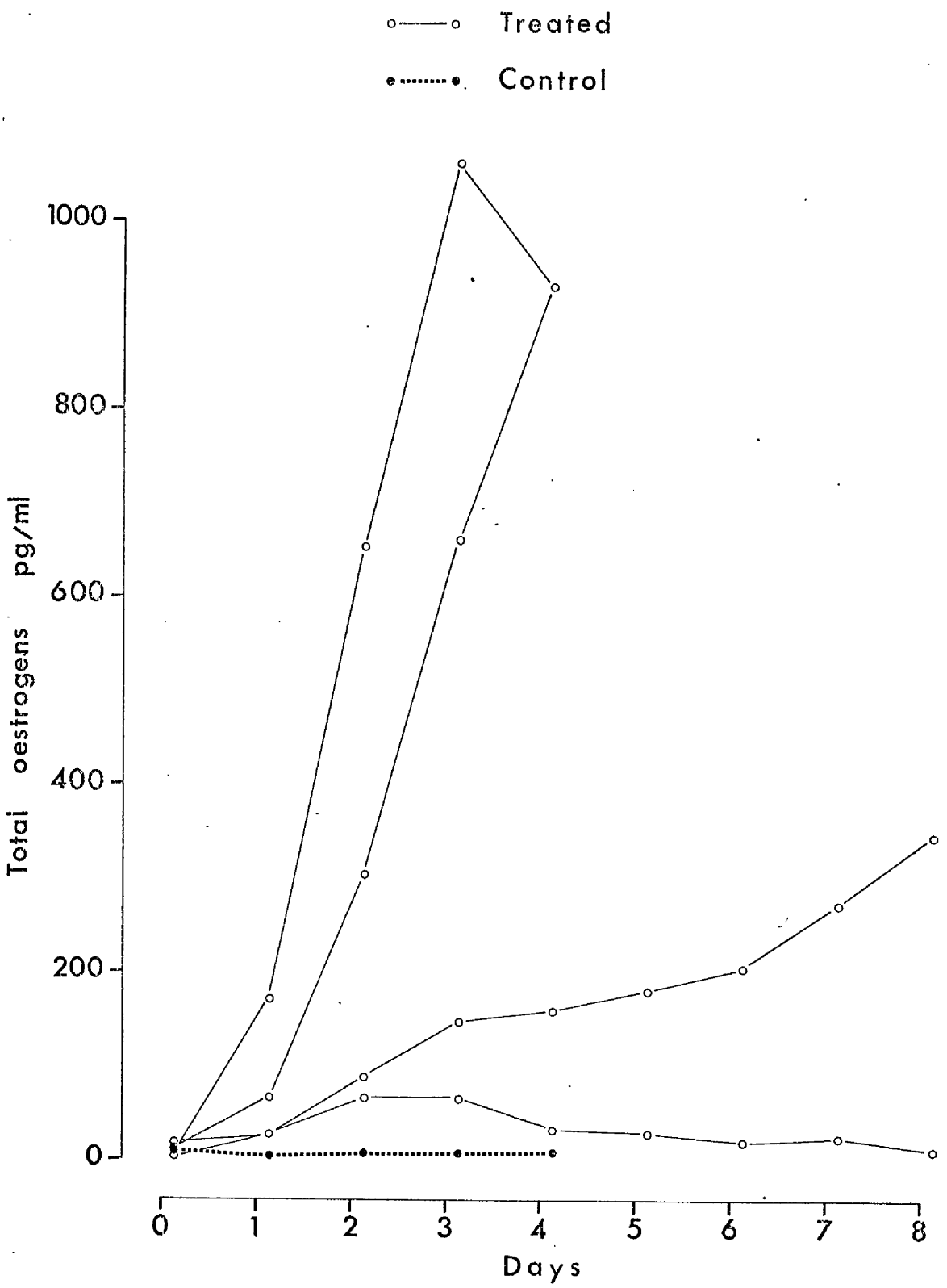

Treated
Control
1000
800
600
400
200
0
Totai oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.7 Plasma levels of oestrogens in control and PMSG-treated Finnish Landrace lambs at 35 days of age during 1975

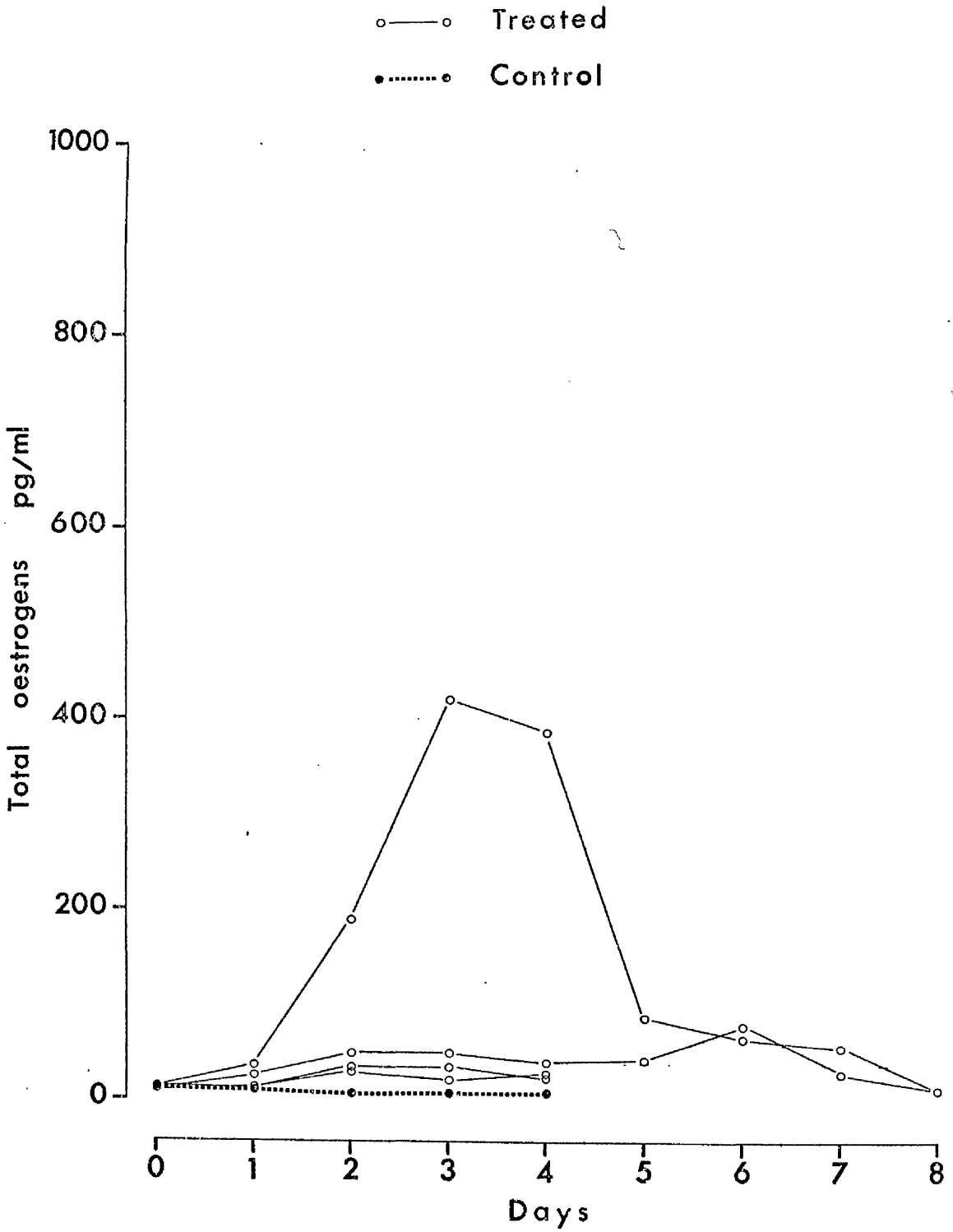

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.8 Plasma levels of oestrogens in control and PMSG-treated Scottish Blackface lambs at 35 days of age during 1975

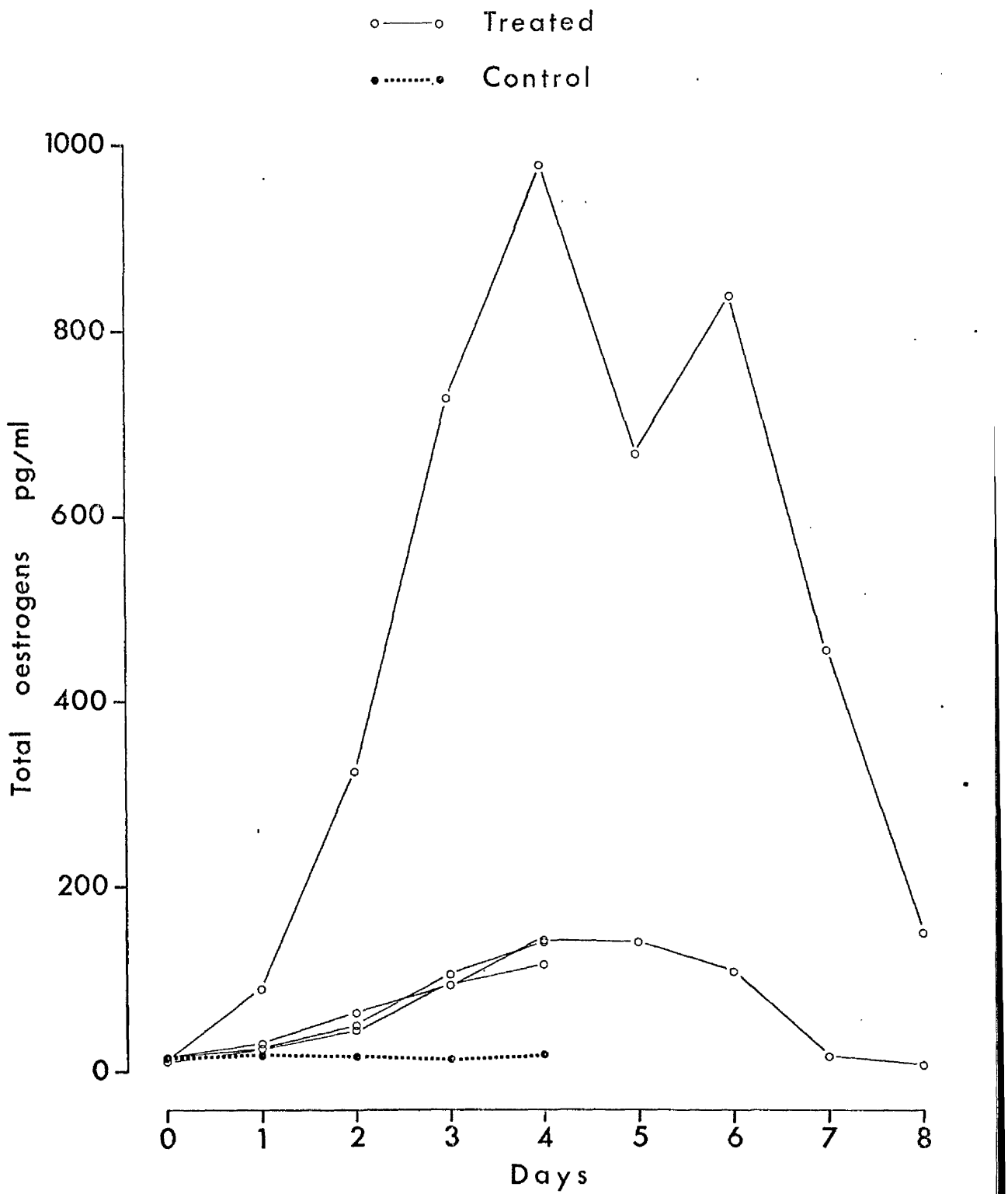

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.9 Plasma levels of oestrogens in control and PMSG-treated Finnish Landrace lambs at 49 days of age during 1975

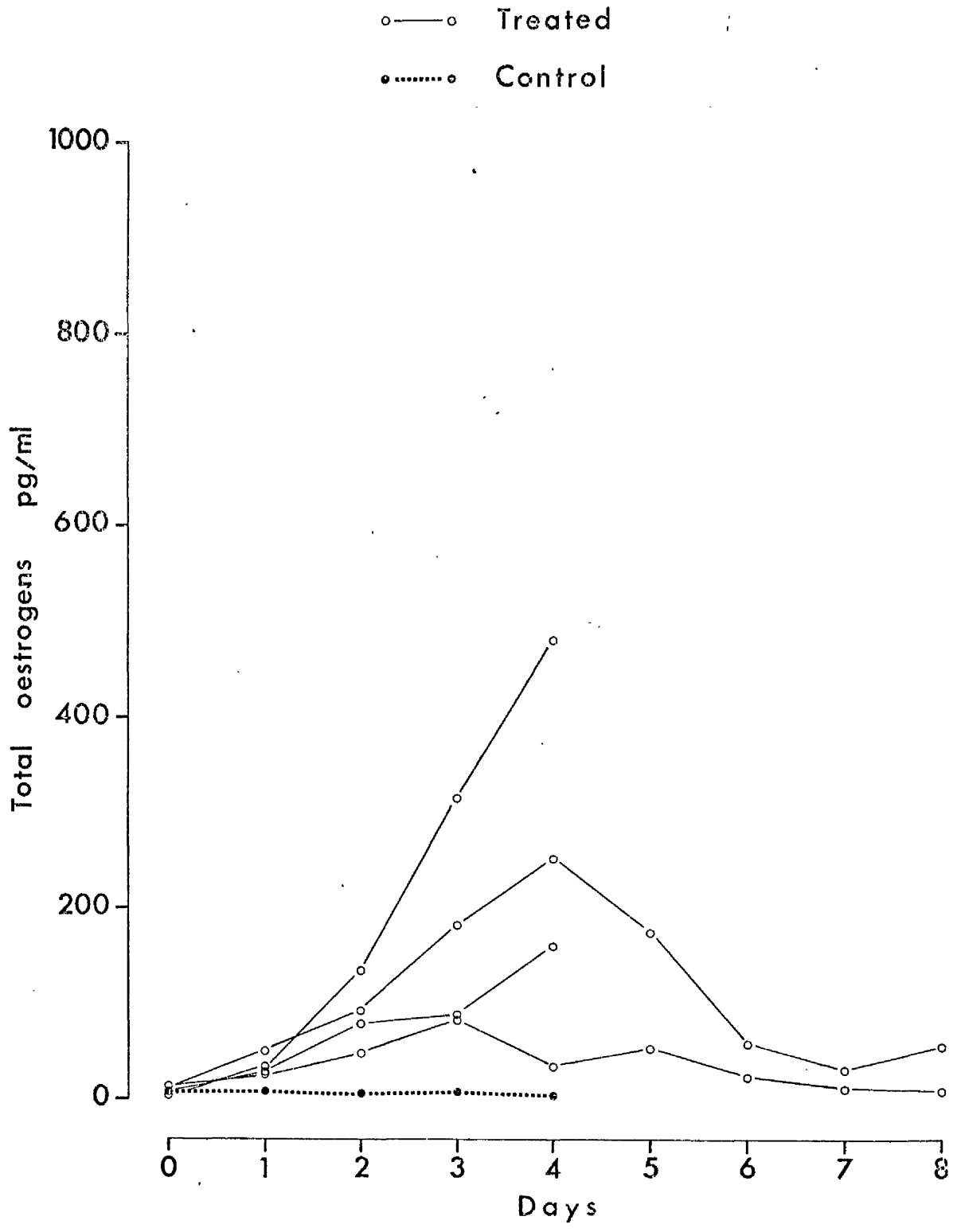

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.10 Plasma levels of oestrogens in control and PMSG-treated Scottish Blackface lambs at 49 days of age during 1975

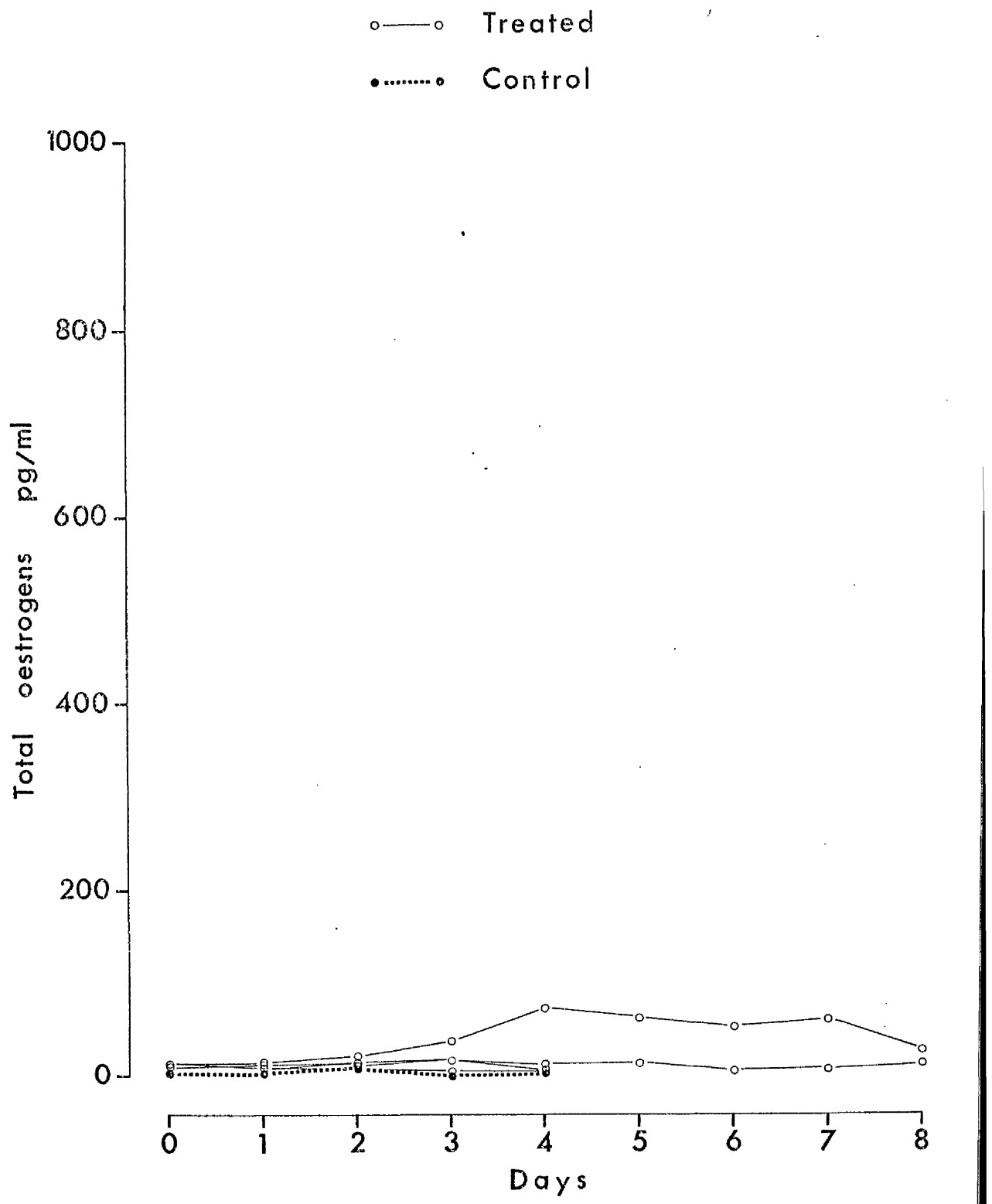

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
7
8
Days

FIG. 4.11 The correlation between the number of large follicles and the plasma level of oestrogens on day 4, in Finnish Landrace and Scottish Blackface lambs during 1974

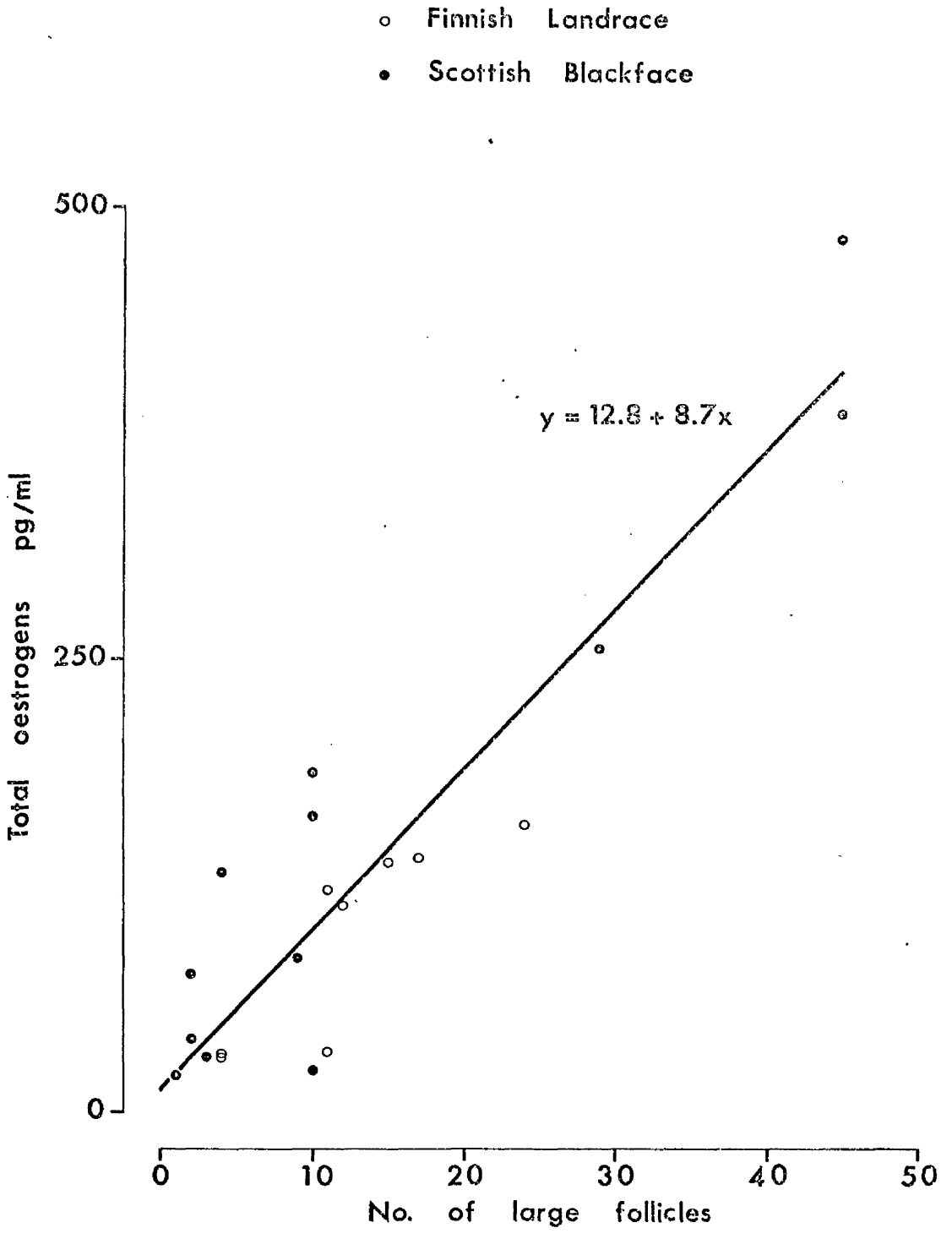

o Finnish Landrace
• Scottish Blackface
500
250
0
Total oestrogens pg/ml
y = 12.8 + 8.7x
0
10
20
30
40
50
No. of large follicles

FIG. 4.12 Plasma levels of oestrogens in Finnish Landrace lambs at 35 days of age during 1975 (individual values are given for treated lambs and mean values for the control lambs)

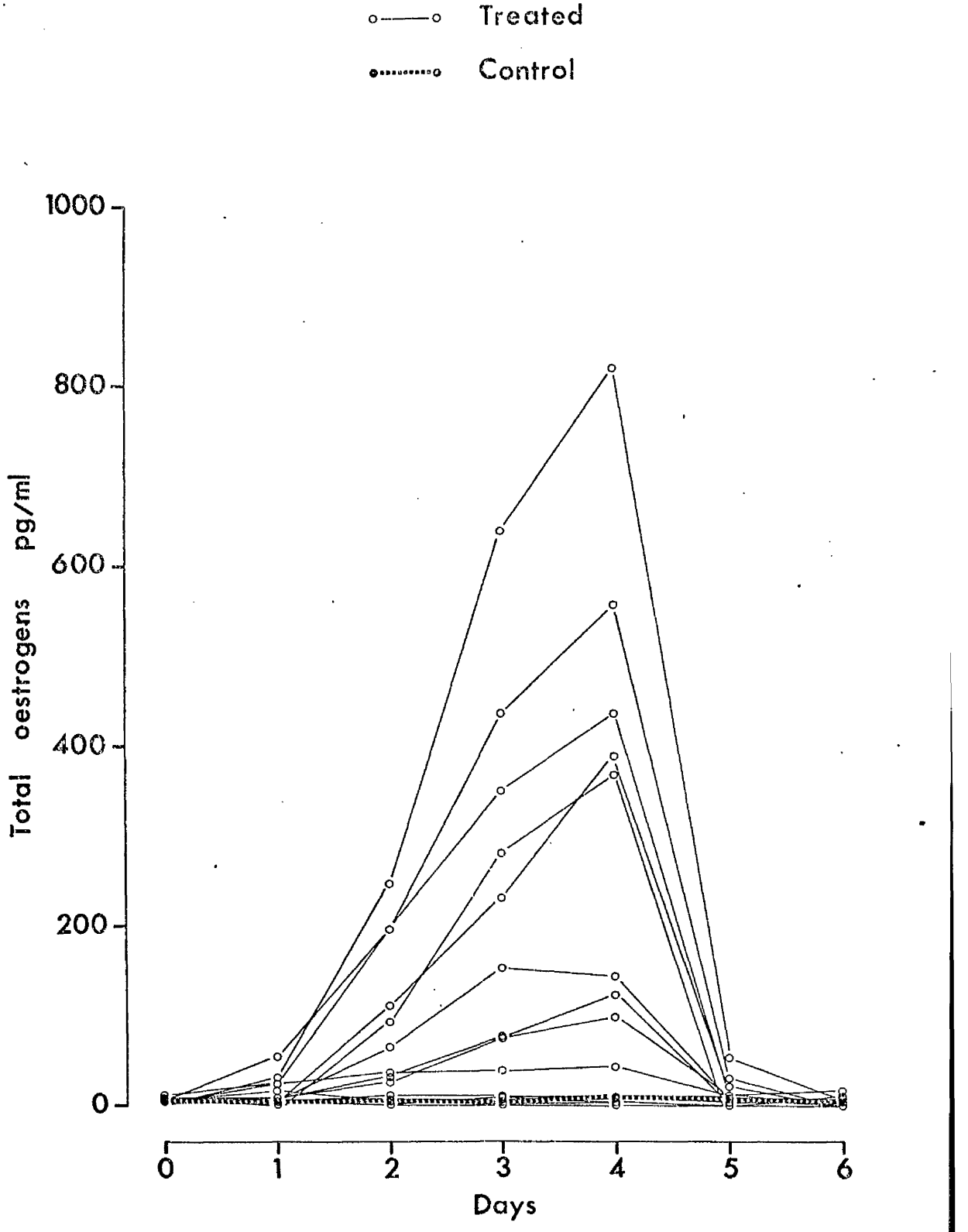

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
Days

FIG. 4.13 Plasma levels of oestrogens in Scottish Blackface lambs at 35 days of age during 1975 (individual values are given for treated lambs and mean values for the control lambs)

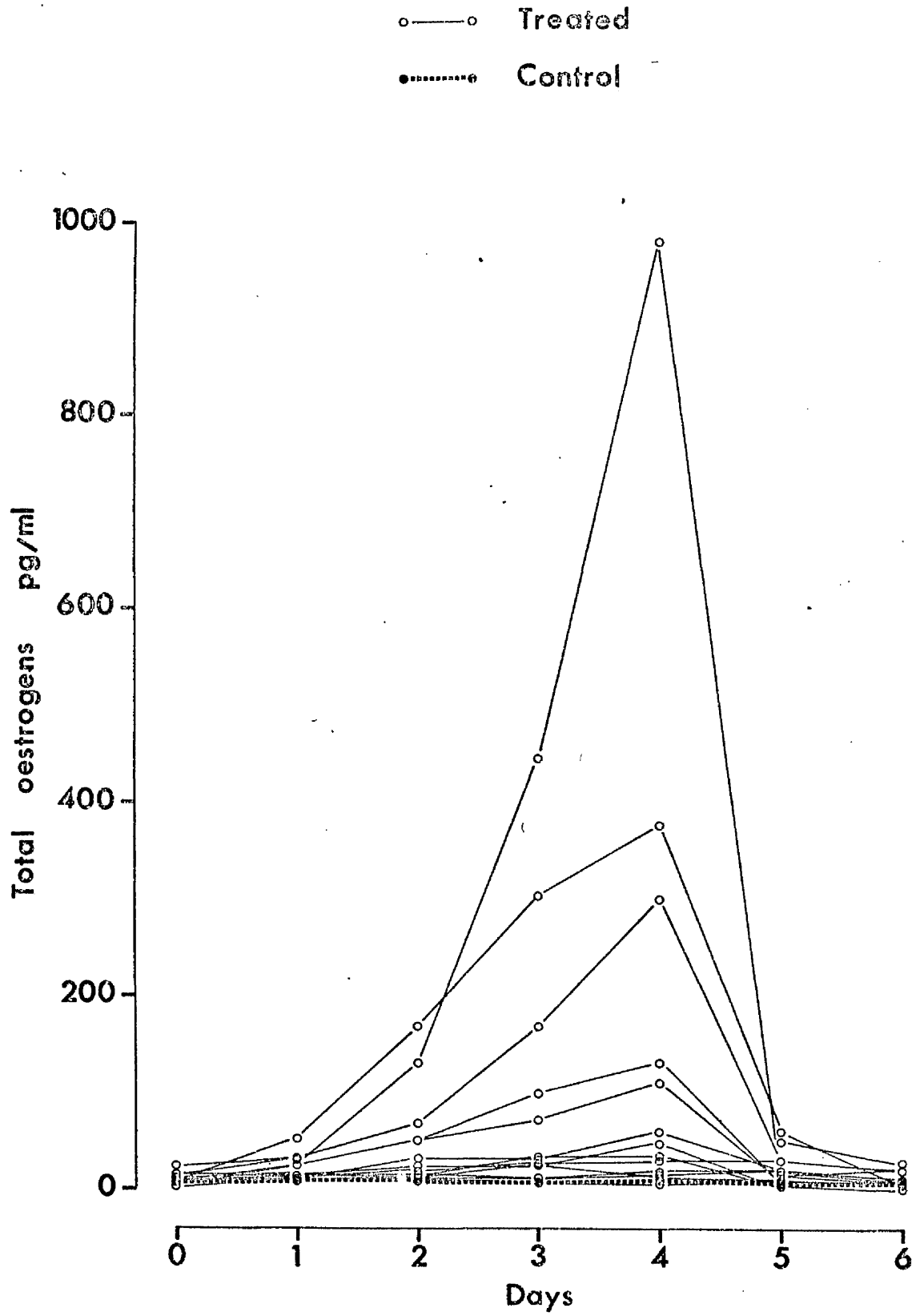

Treated
Control
1000
800
600
400
200
0
Total oestrogens pg/ml
0
1
2
3
4
5
6
Days

FIG. 4.14 The correlation between the number of large follicles and ovarian volume in Finnish Landrace and Scottish Blackface lambs treated with PMSG during 1975

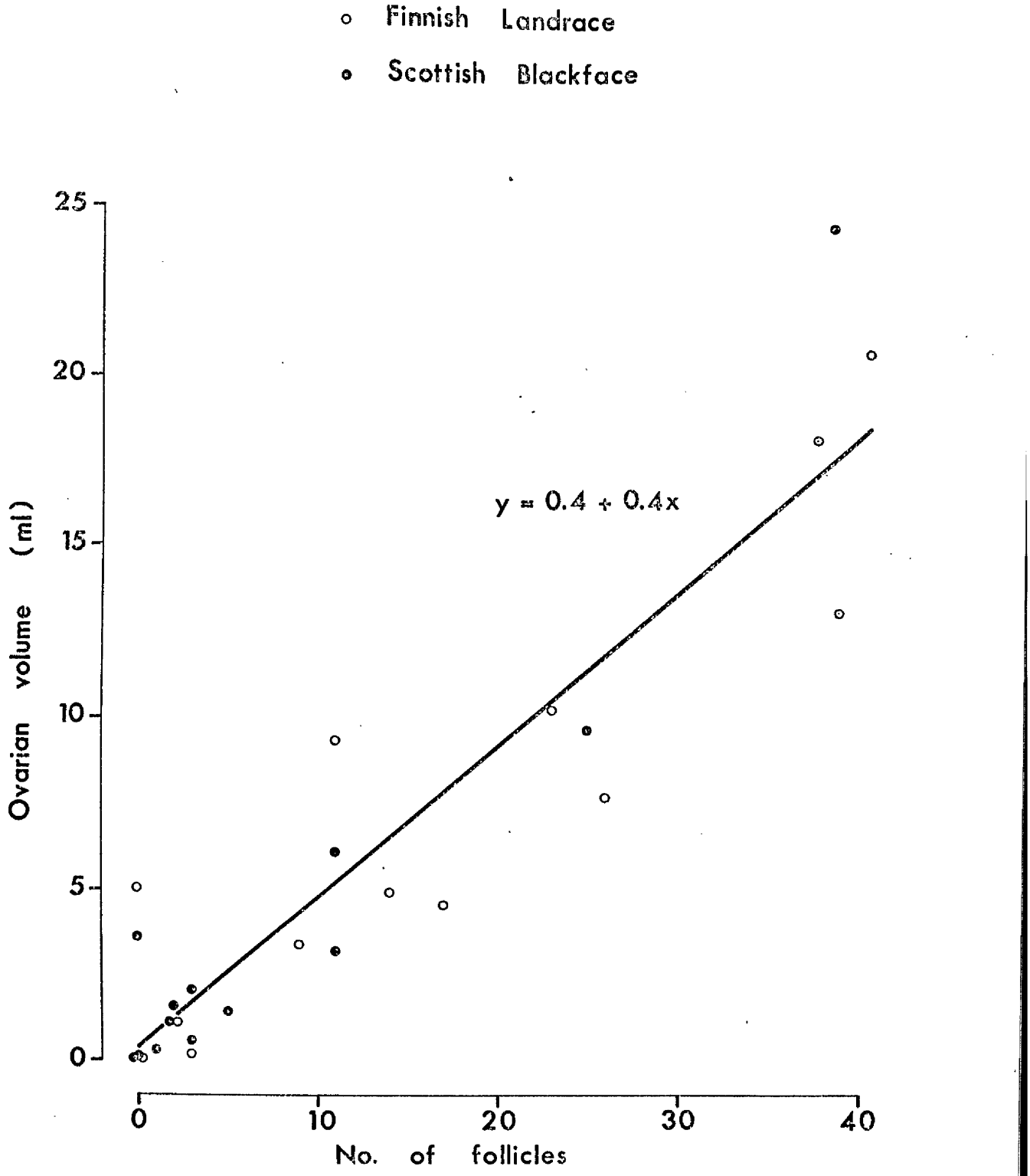

Finnish Landrace
Scottish Blackface
y = 0.4 + 0.4x
Ovarian volume (ml)
25
20
15
10
5
0
0
10
20
30
40
No. of follicles

FIG. 4.15 The correlation between the number of large follicles and ovarian weight in Finnish Landrace and Scottish Blackface lambs treated with PMSG during 1975

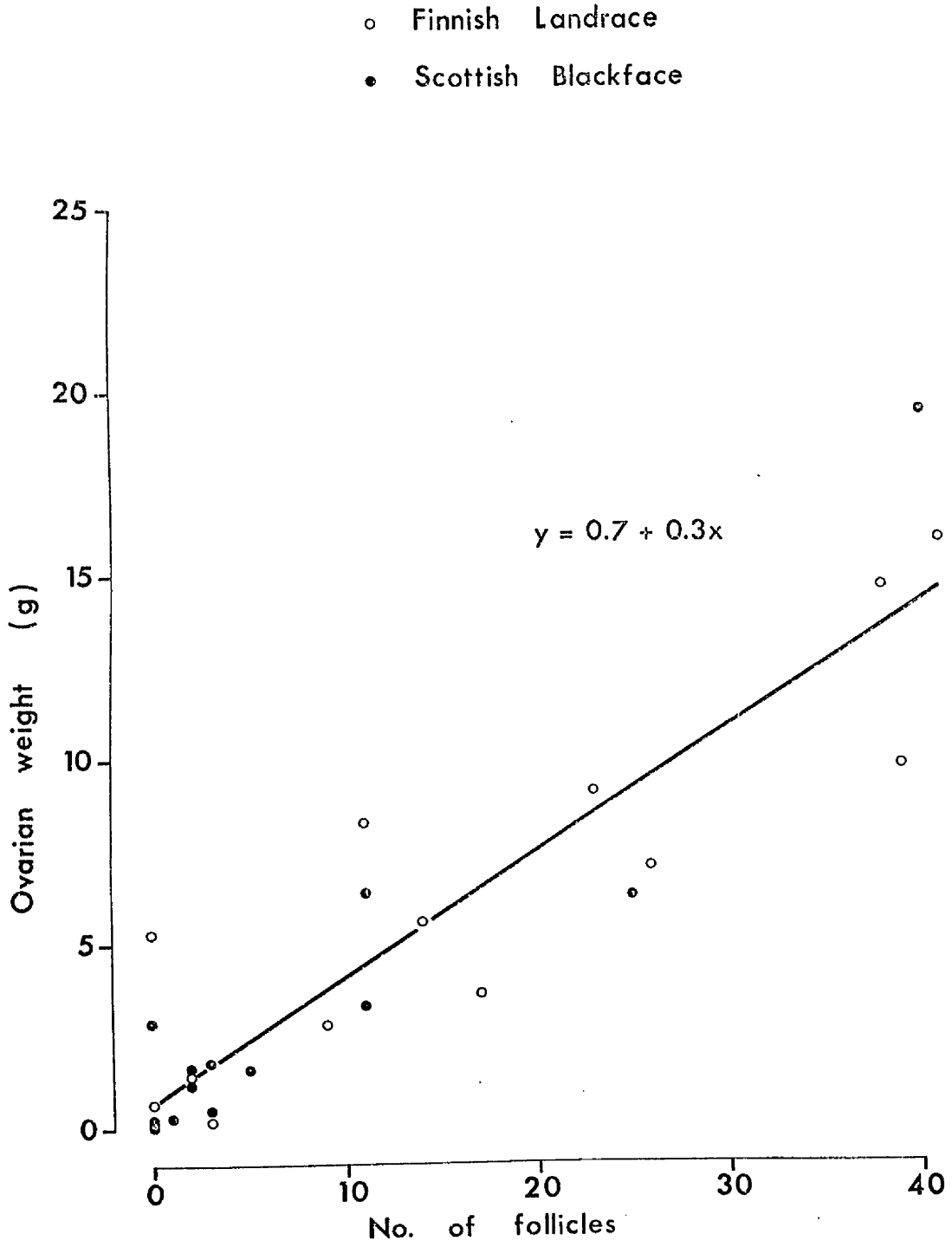

o Finnish Landrace
• Scottish Blackface
y = 0.7 + 0.3x
Ovarian weight (g)
25
20
15
10
5
0
0
10
20
30
40
No. of follicles

FIG. 4.16 The correlation between the number of large follicles and the plasma level of oestrogens, on day 4, in Finnish Landrace and Scottish Blackface lambs during 1975

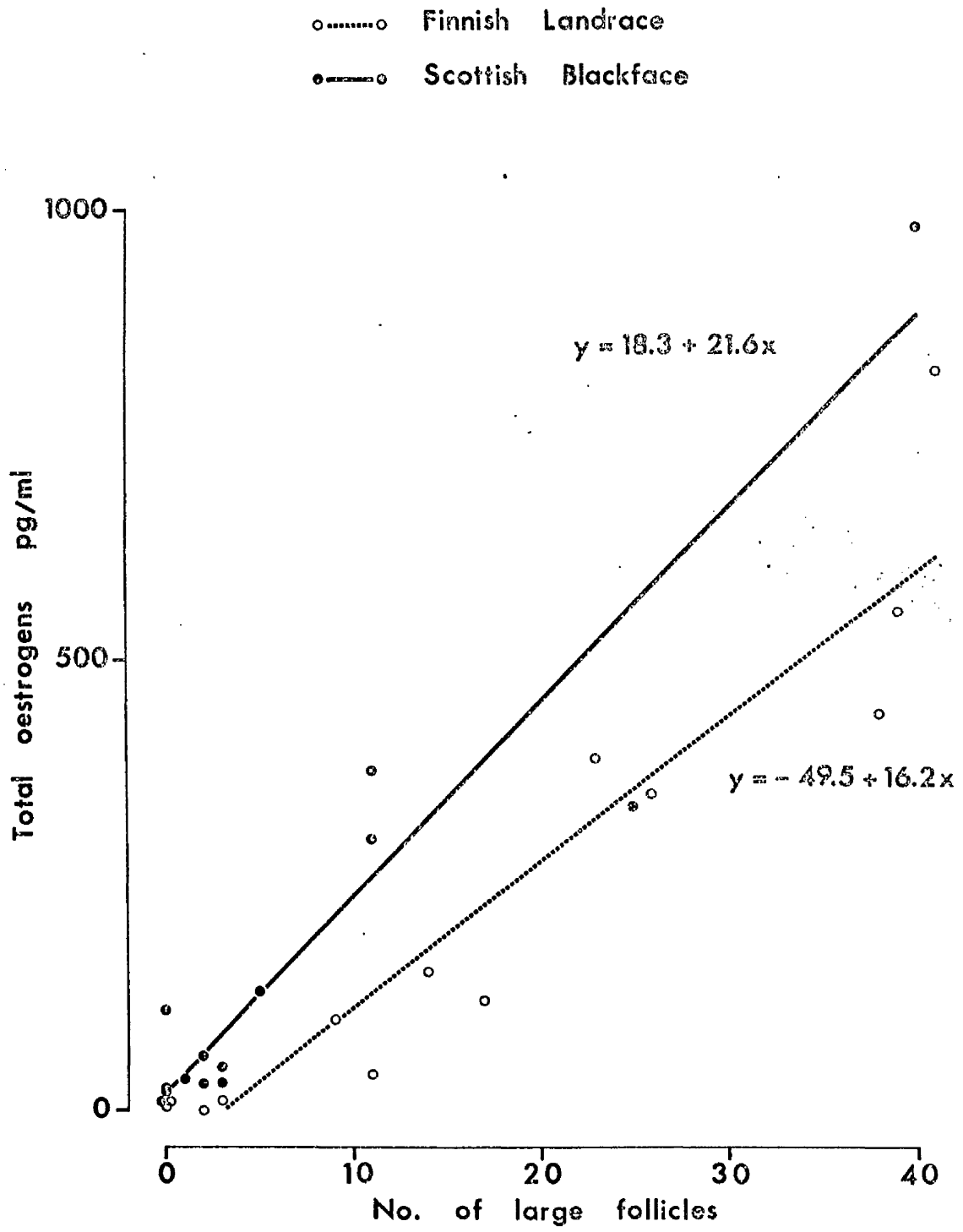

o.....o Finnish Landrace
o——o Scottish Blackface
1000
500
0
Total oestrogens pg/ml
y = 18.3 + 21.6x
y = - 49.5 + 16.2x
0
10
20
30
40
No. of large follicles

FIG. 4.17 The correlation between the number of large follicles and bodyweight in Finnish Landrace and Scottish Blackface lambs treated with PMSG during 1975

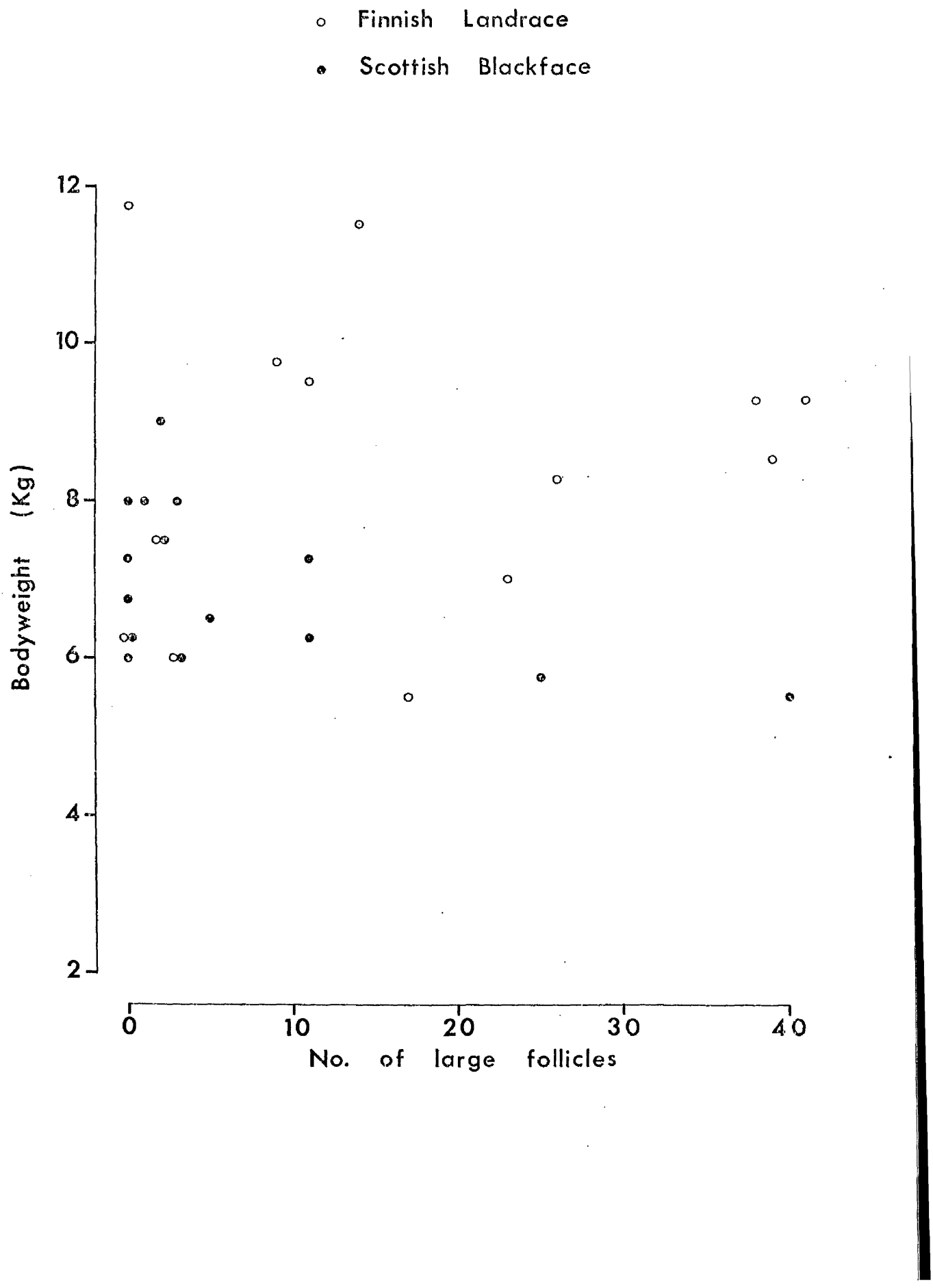

Finnish Landrace
Scottish Blackface
12
10
8
6
4
2
Bodyweight (Kg)
0
10
20
30
40
No. of large follicles

FIG. 5.1 Plasma levels of oestrogens in methallibure-treated Finnish Landrace lambs, with and without PMSG stimulation, at 35 days of age, (individual values are given for lambs that received methallibure and PMSG; mean values are given for lambs that received only methallibure)

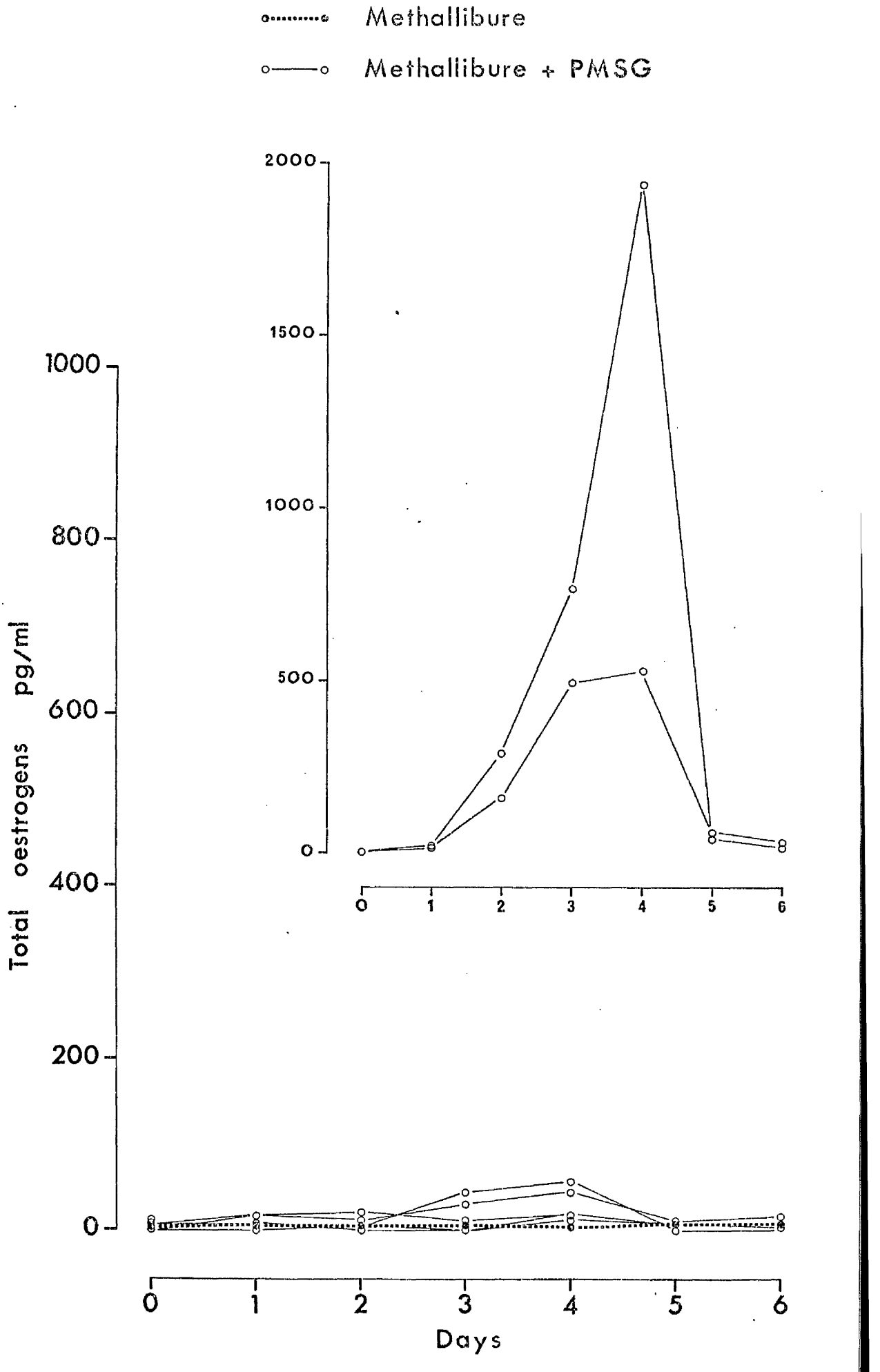

Methallibure
Methallibure + PMSG
Total oestrogens pg/ml
2000
1500
1000
800
600
500
400
200
0
0
1
2
3
4
5
6
Days

FIG. 5.2 Plasma levels of oestrogens in methallibure-treated Scottish Blackface lambs, with and without PMSG stimulation, at 35 days of age, (individual values are given for lambs that received methallibure and PMSG; mean values are given for lambs that received only methallibure)

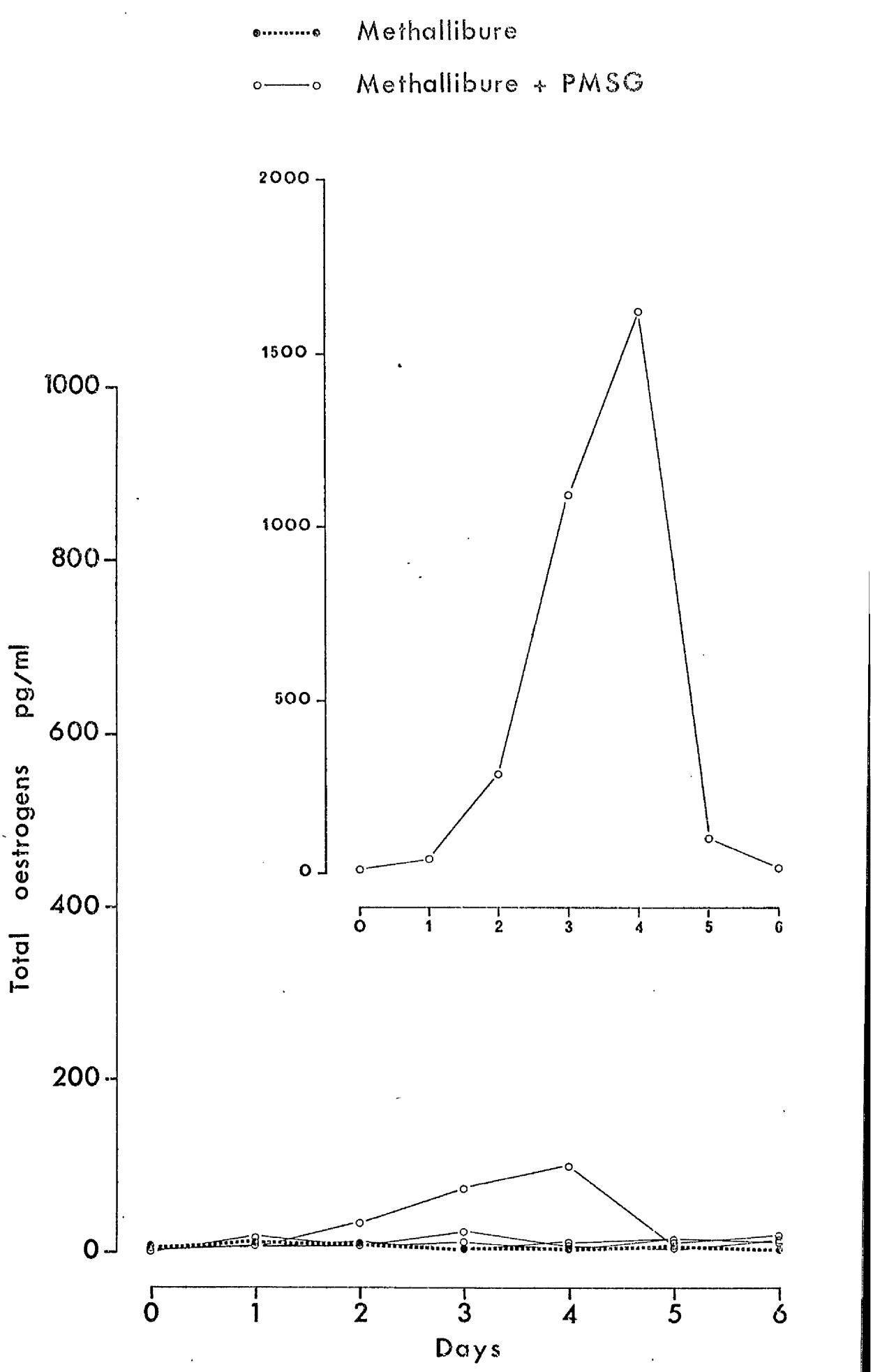

Methallibure
Methallibure + PMSG
Total oestrogens pg/ml
2000
1500
1000
500
0
1000
800
600
400
200
0
0 1 2 3 4 5 6
0 1 2 3 4 5 6
Days

FIG. 5.3 Plasma levels of LH in 45 day-old Finnish Landrace lambs treated with 5 µg LH-RH

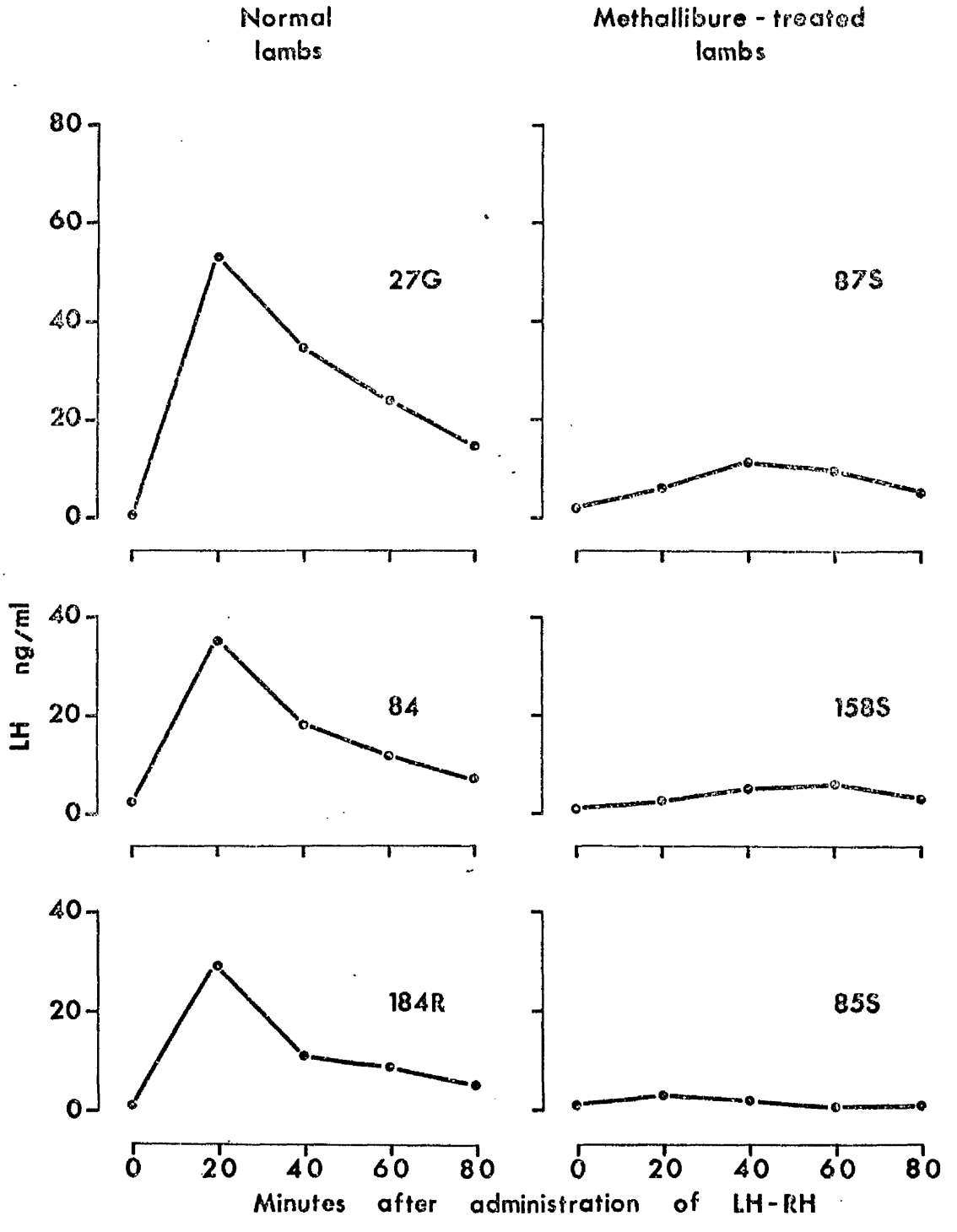

Normal lambs
Methallibure - treated lambs
80
60
40
20
0
27G
87S
40
20
0
84
158S
40
20
0
184R
85S
LH ng/ml
0
20
40
60
80
Minutes after administration of LH-RH

FIG. 5.4 Plasma levels of LH in 45 day-old Scottish Blackface lambs treated with 5 µg LH-RH

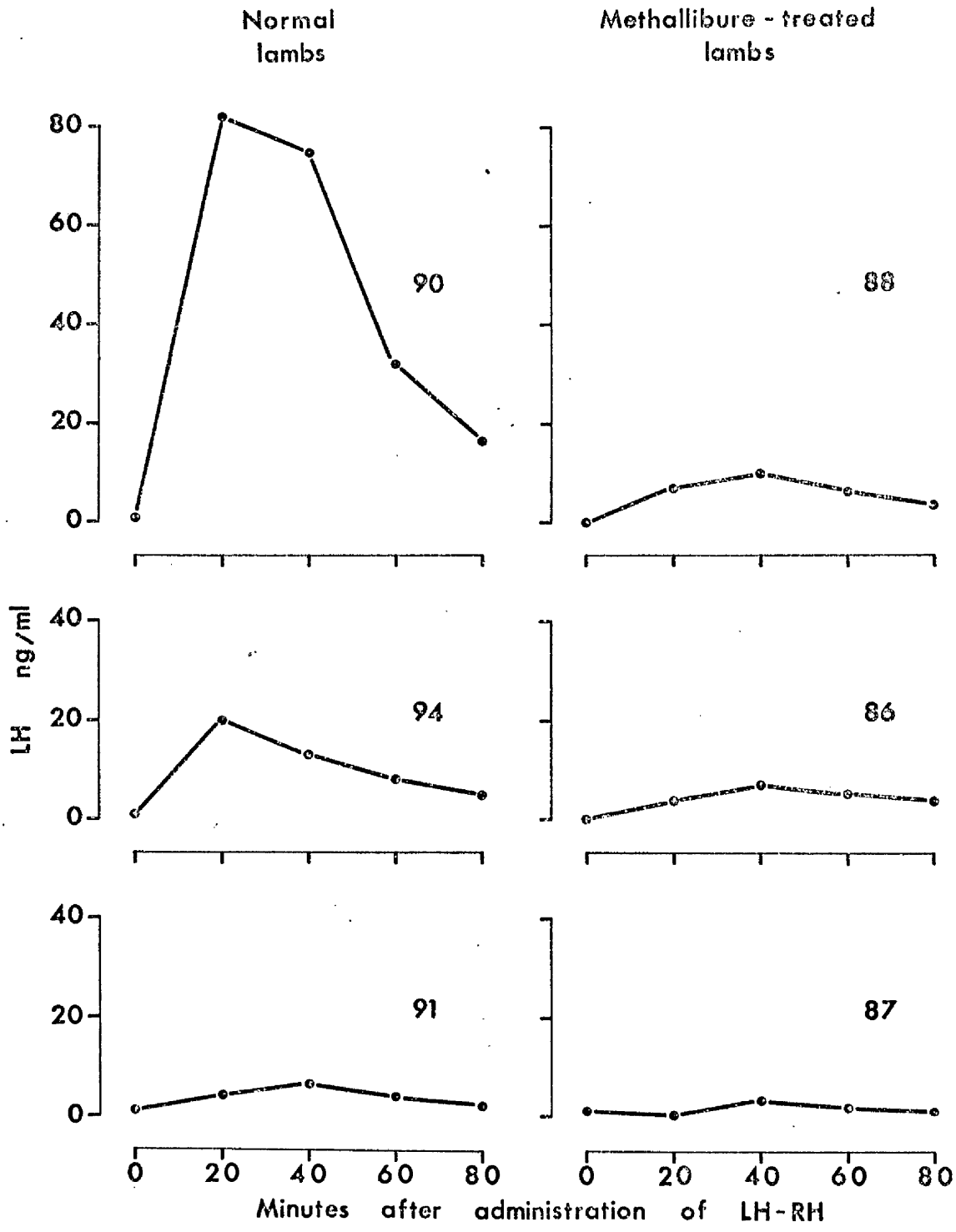

Normal lambs
Methallibure - treated lambs
LH ng/ml
80
60
40
20
0
90
88
40
20
0
94
86
40
20
0
91
87
0
20
40
60
80
Minutes after administration of LH-RH

FIG. 5.5 Plasma levels of LH in 43 day-old Finnish Landrace lambs treated with 25 μg oestradiol-17β

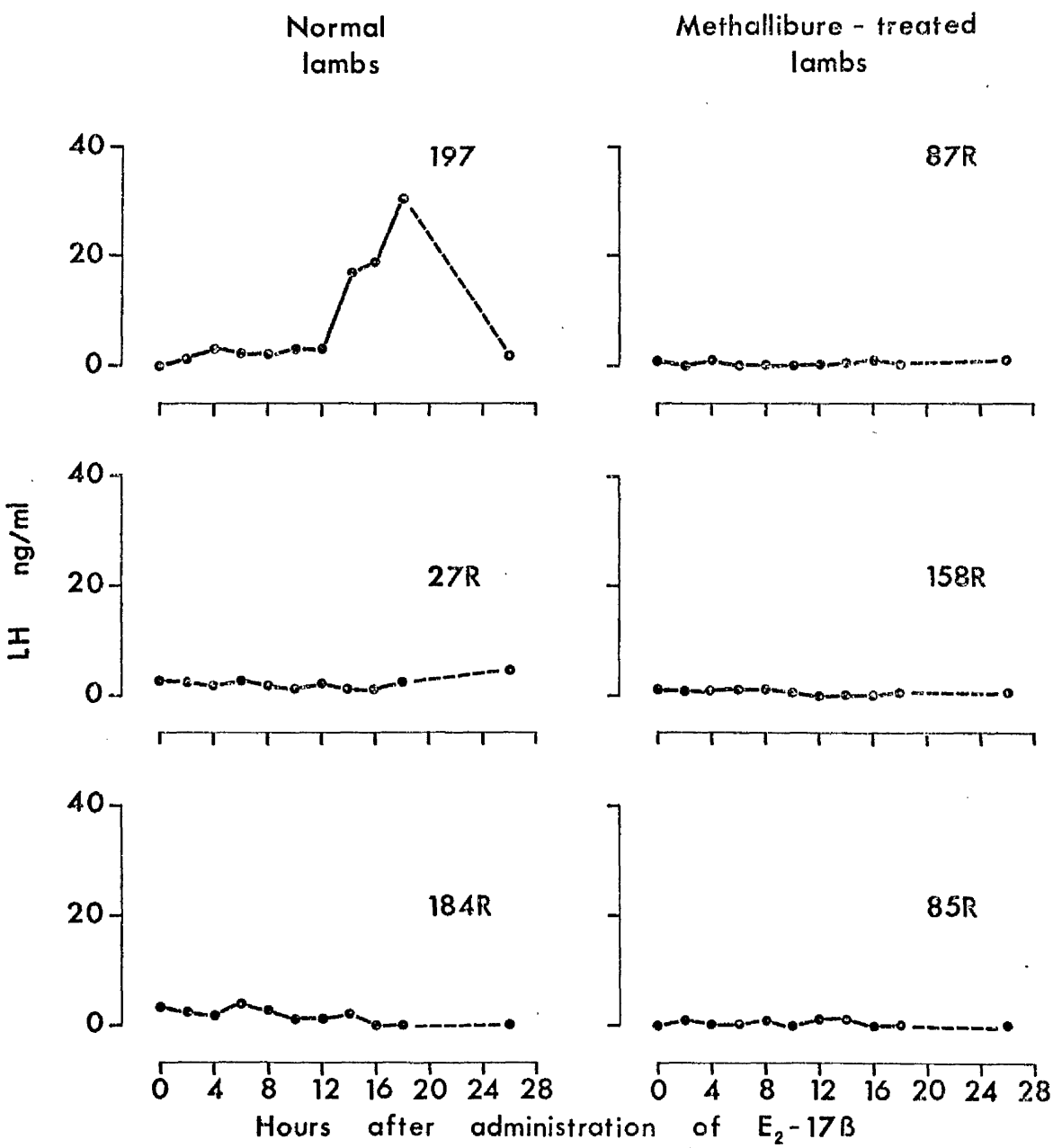
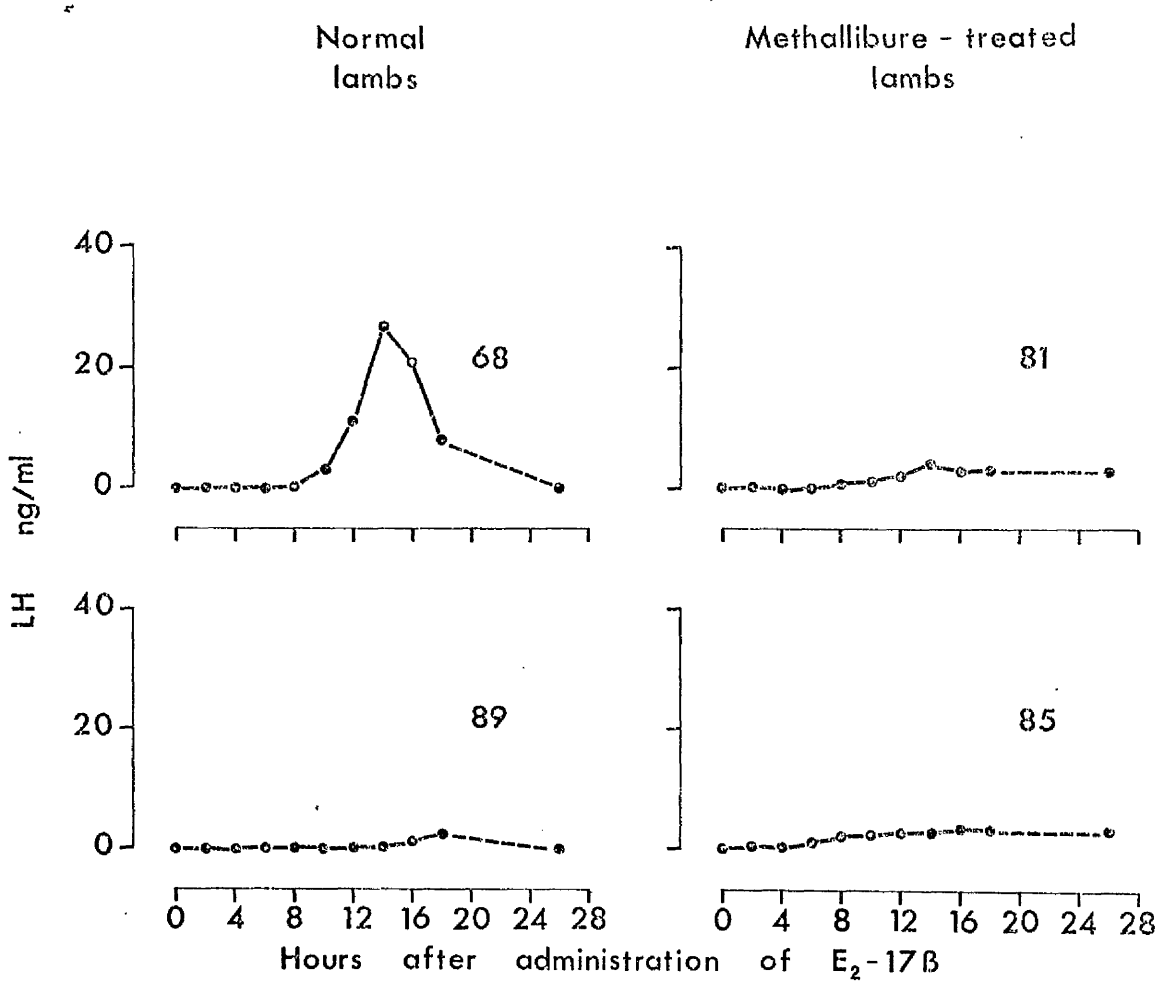

Normal lambs
Methallibure - treated lambs
197
87R
27R
158R
184R
85R
40
20
0
LH ng/ml
0 4 8 12 16 20 24 28
Hours after administration of E_2-17ß

FIG. 5.6 Plasma levels of LH in 43 day-old Scottish Blackface lambs treated with 25 μg oestradiol-17β


Normal lambs
Methallibure - treated lambs
LH ng/ml
40
20
0
68
81
89
85
0 4 8 12 16 20 24 28
Hours after administration of E_2-17ß

PLATES

PLATE 1.1 Section of ovary, from a 35 day-old Scottish Blackface lamb, to show the presence of primordial (P), growing (G) and vesicular (V) follicles in the ovarian cortex

x 52

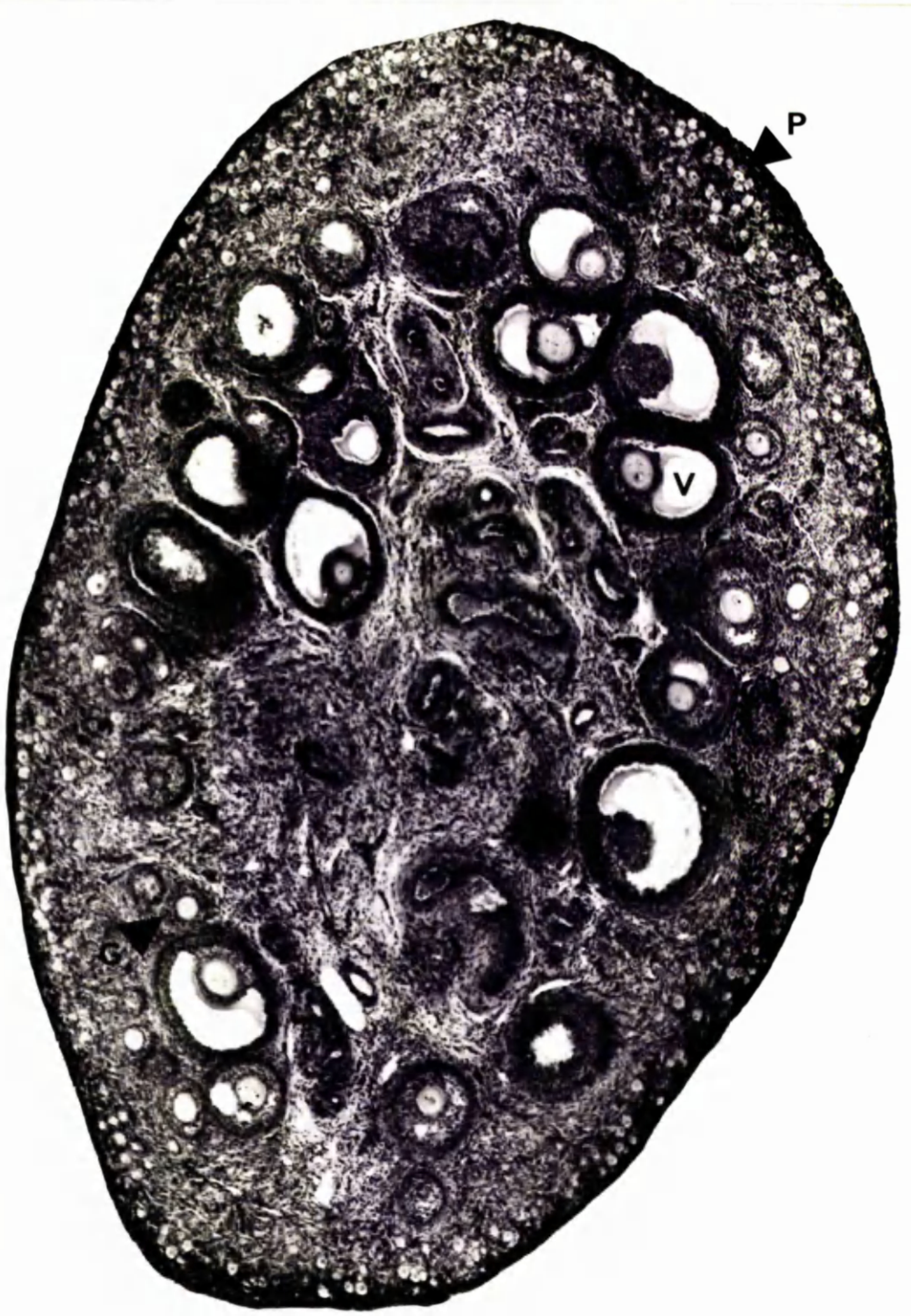


PLATE 1.2 Section of ovary, shown in Plate 1.1, to show several primordial follicles

x 450

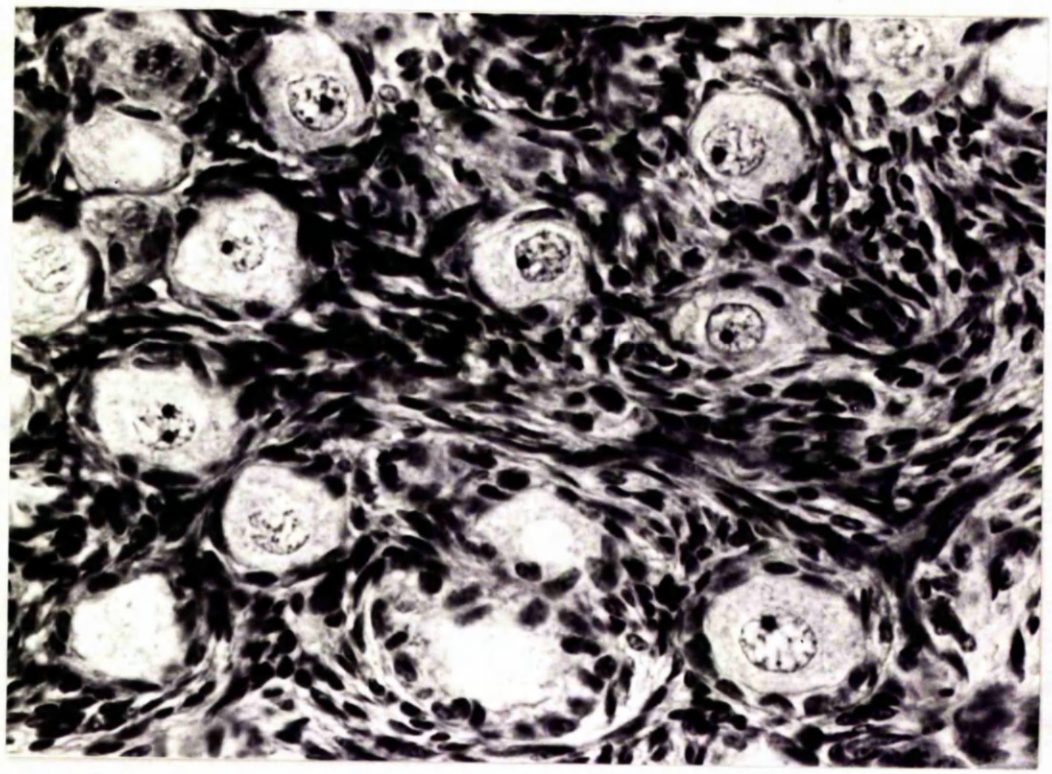


PLATE 1.3 Section of ovary, shown in Plate 1.1, to show two growing follicles

x 400

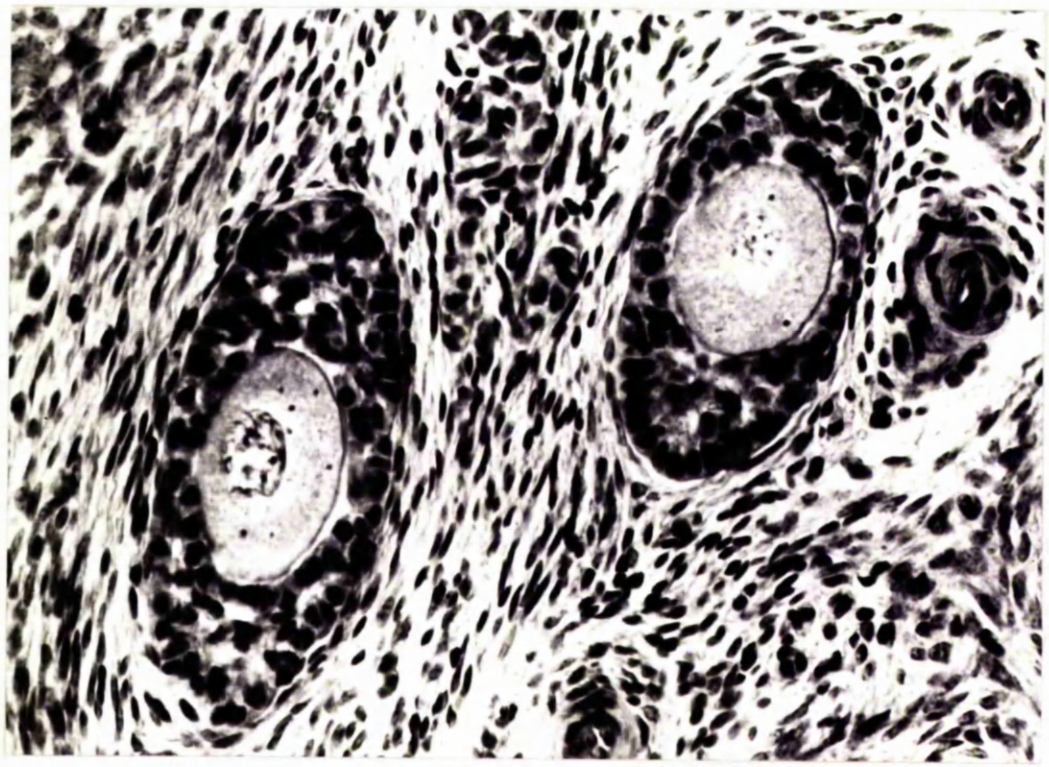


PLATE 1.4 Section of ovary, shown in Plate 1.1, to show a vesicular follicle

x 130

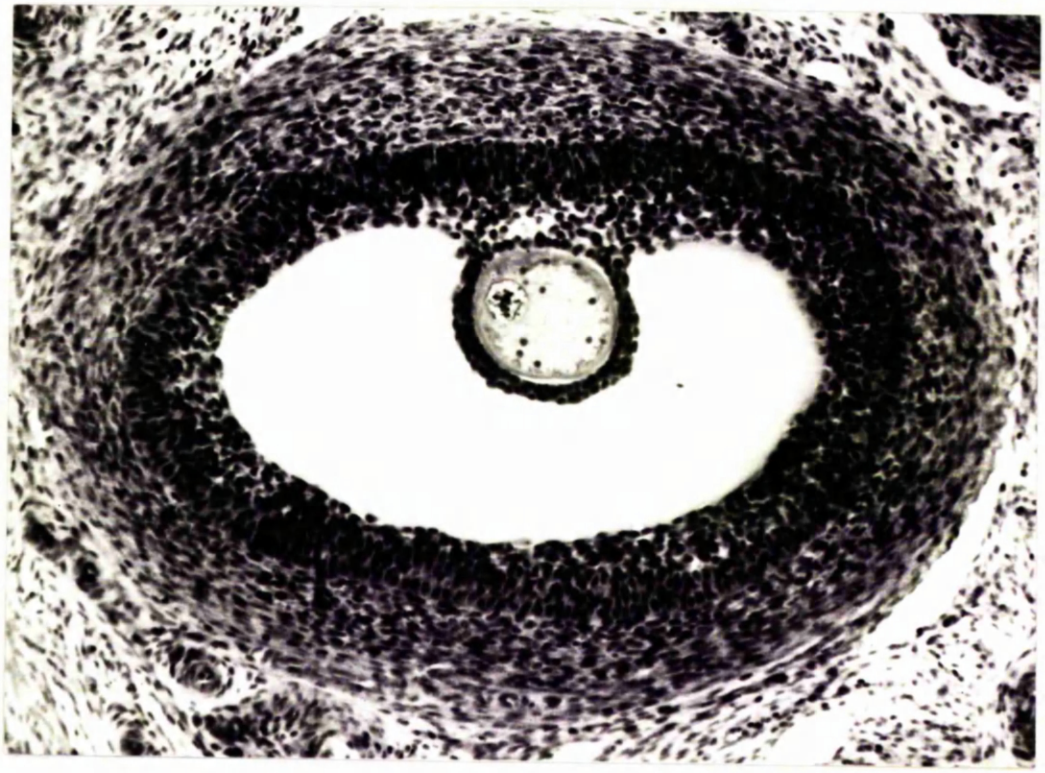


PLATE 1.5 A mature Finnish Landrace ewe with her pure-bred lambs

PLATE 1.6 A mature Scottish Blackface ewe with her pure-bred lamb

PLATE 4.1 The reproductive tract from a 7 day-old Scottish Blackface lamb (No. 26, 1974)

(a) External appearance of the ovaries and uterus

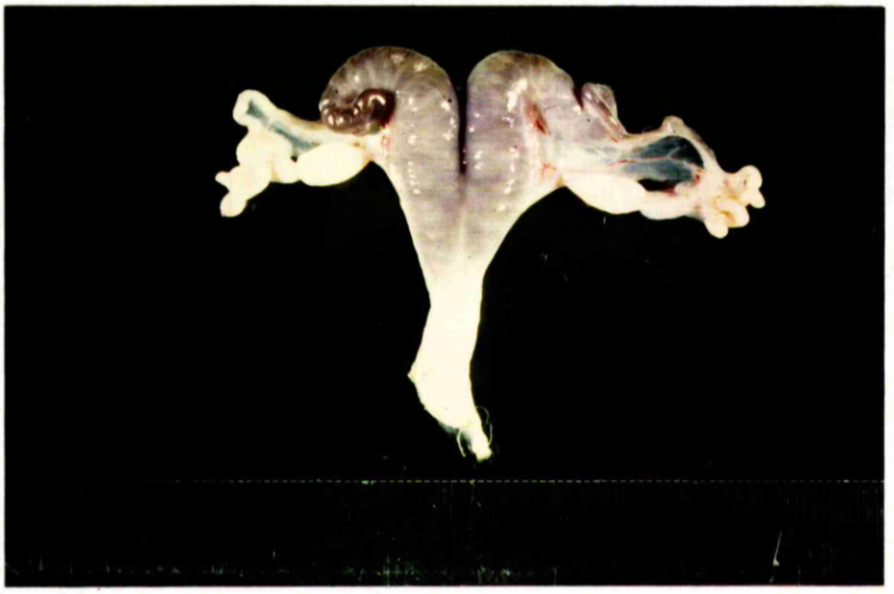


PLATE 4.1

(b) Cross-section of ovary shown in Plate 4.1 (a)

x 13

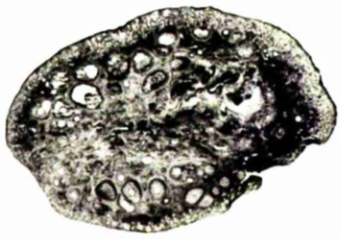


PLATE 4.1

(c) Cross-section of uterus shown in Plate 4.1 (a)

x 15

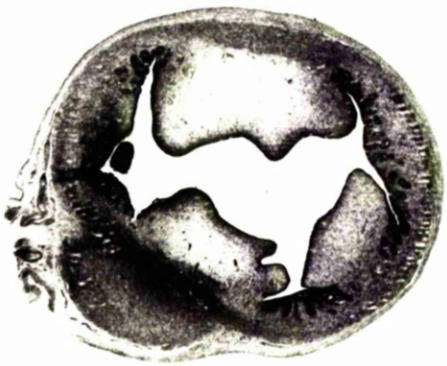


PLATE 4.2 The reproductive tract from a 21 day-old Scottish Blackface lamb (No. 33, 1974)

(a) External appearance of the ovaries and uterus

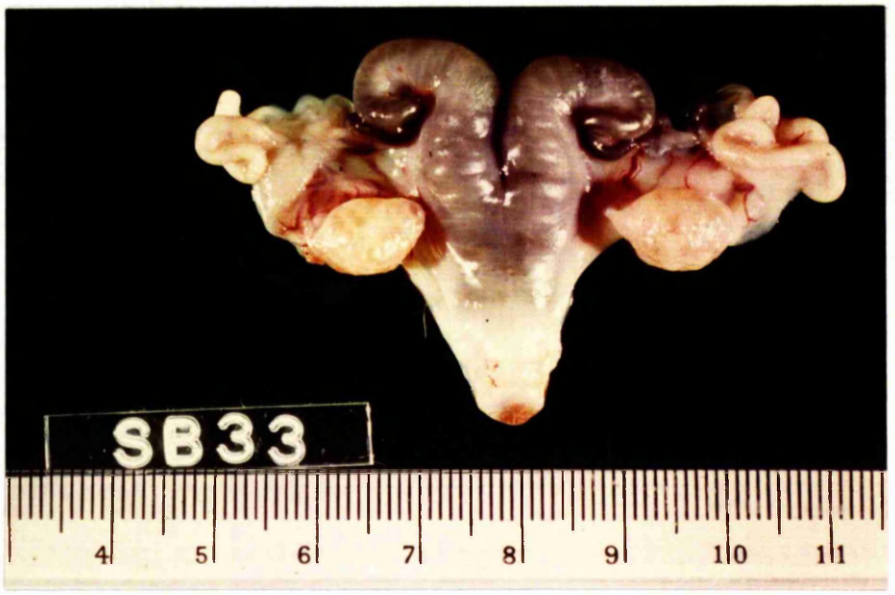


PLATE 4.2

(b) Cross-section of ovary shown in Plate 4.2 (a)

x 13

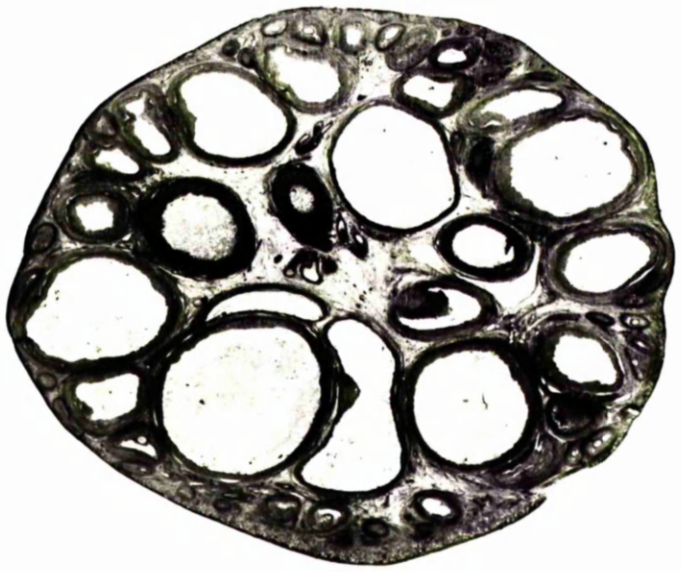


PLATE 4.2

(c) Cross-section of uterus shown in Plate 4.2 (a)

x 15

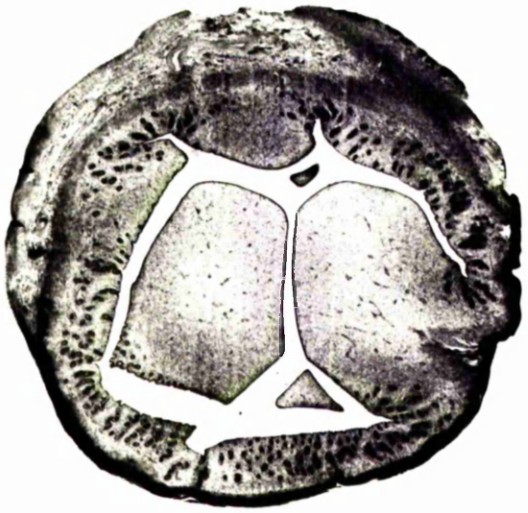


PLATE 4.3 The reproductive tract from a 35 day-old Scottish Blackface lamb (No. 39, 1974)

(a) External appearance of the ovaries and uterus

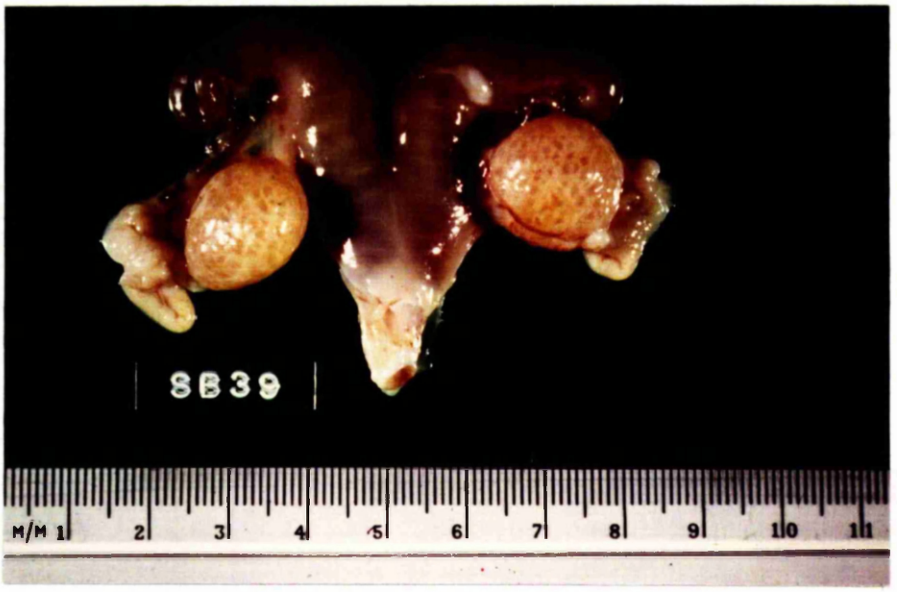


PLATE 4.3

(b) Cross-section of ovary shown in Plate 4.3 (a)

x 13

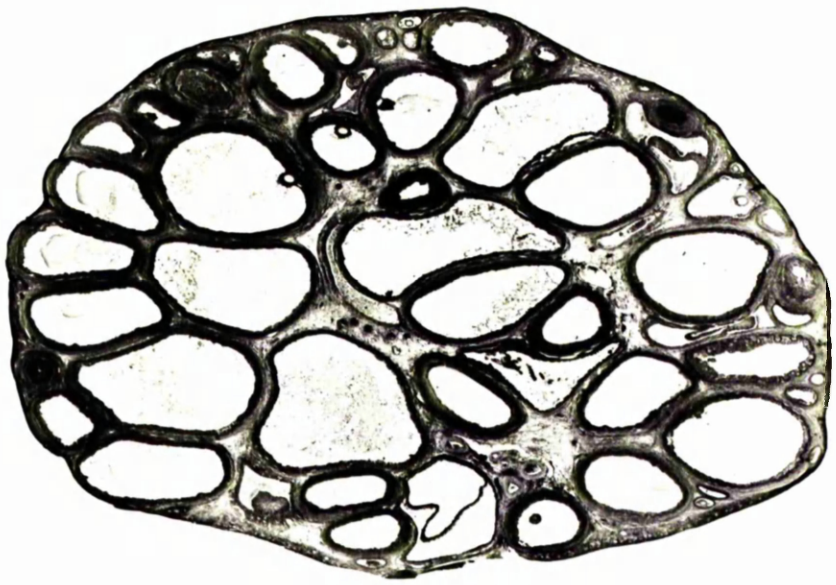


PLATE 4.3

(c) Cross-section of uterus shown in Plate 4.3 (a)

x 15

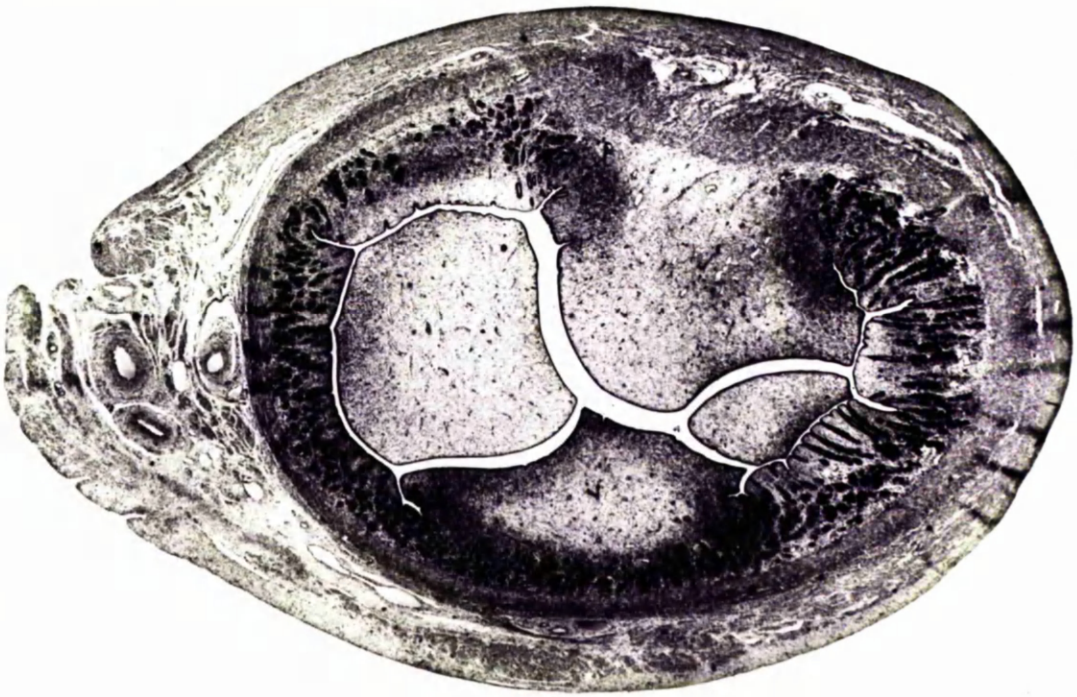


PLATE 4.4 The reproductive tract from a 49 day-old Scottish Blackface lamb (No. 55, 1974)

(a) External appearance of the ovaries and uterus

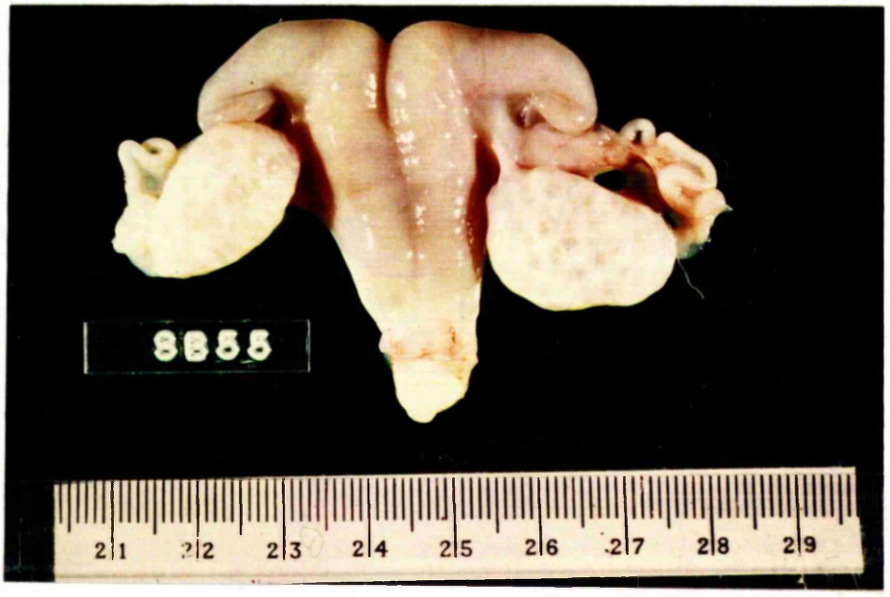


PLATE 4.4

(b) Cross-section of ovary shown in Plate 4.4 (a)

x 13

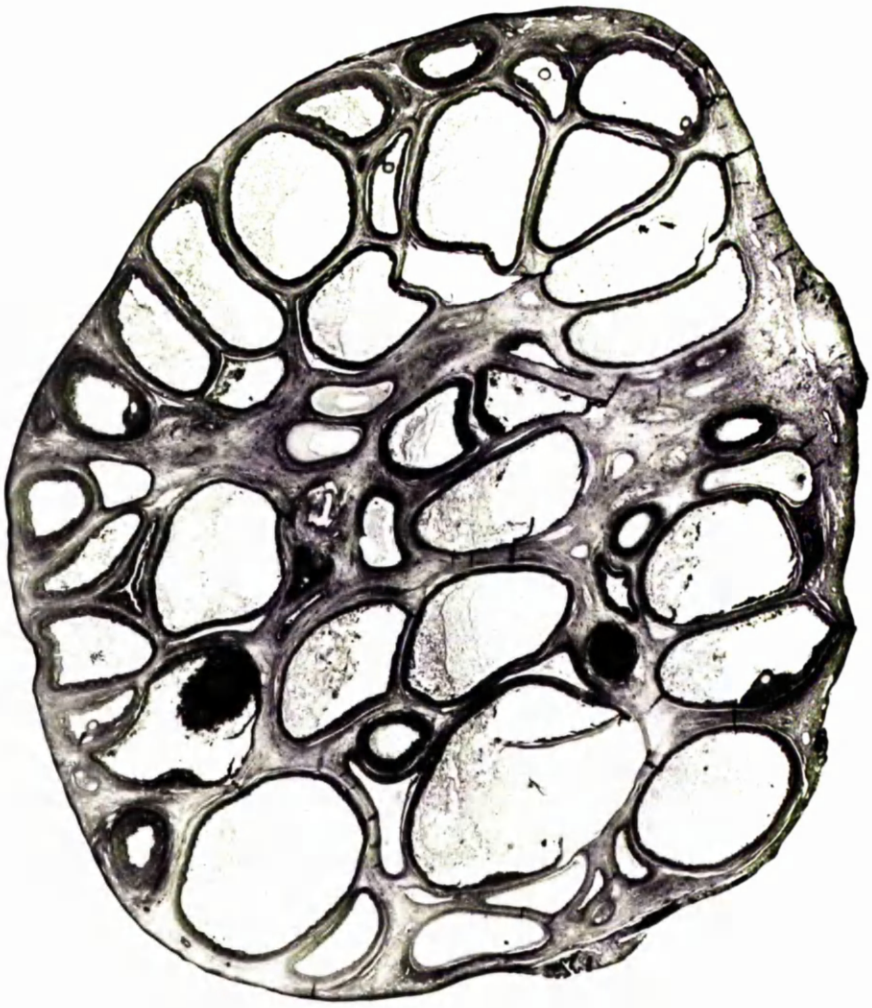


PLATE 4.4

(c) Cross-section of uterus shown in Plate 4.4 (a)

x 15

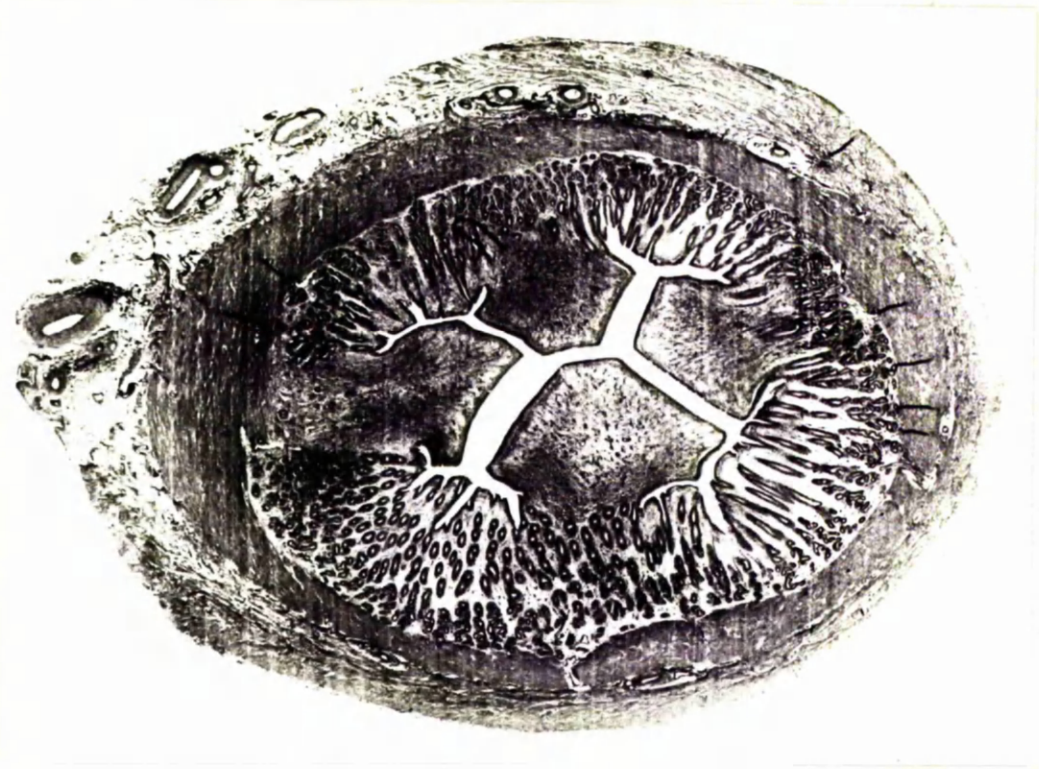


PLATE 4.5 The reproductive tract from a 35 day-old Finnish Landrace lamb (No. 92, 1975), after treatment with PMSG, showing an increase in ovarian size compared to the ovaries of control lambs of the same age

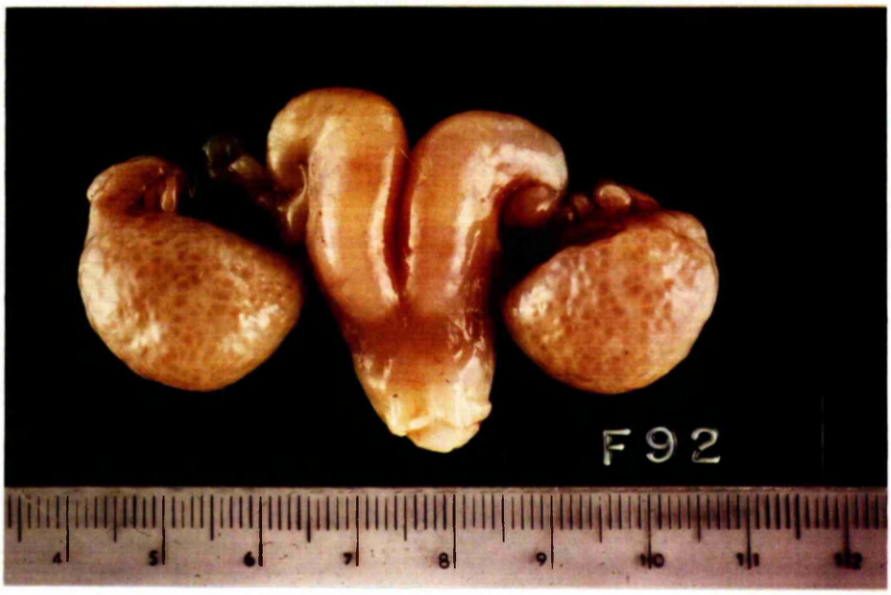


PLATE 4.6 The reproductive tract from a 35 day-old Scottish Blackface lamb (No. 57, 1975), after treatment with PMSG, showing a large vesicular follicle on one ovary

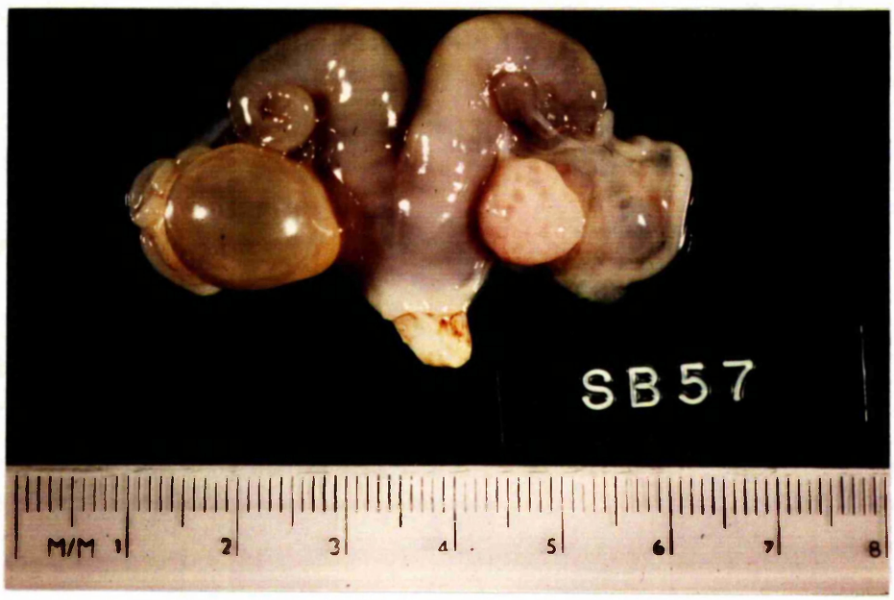


PLATE 4.7 The reproductive tract from a 35 day-old Scottish Blackface lamb (No. 51, 1975), after treatment with PMSG, showing the development of many large vesicular follicles in both ovaries

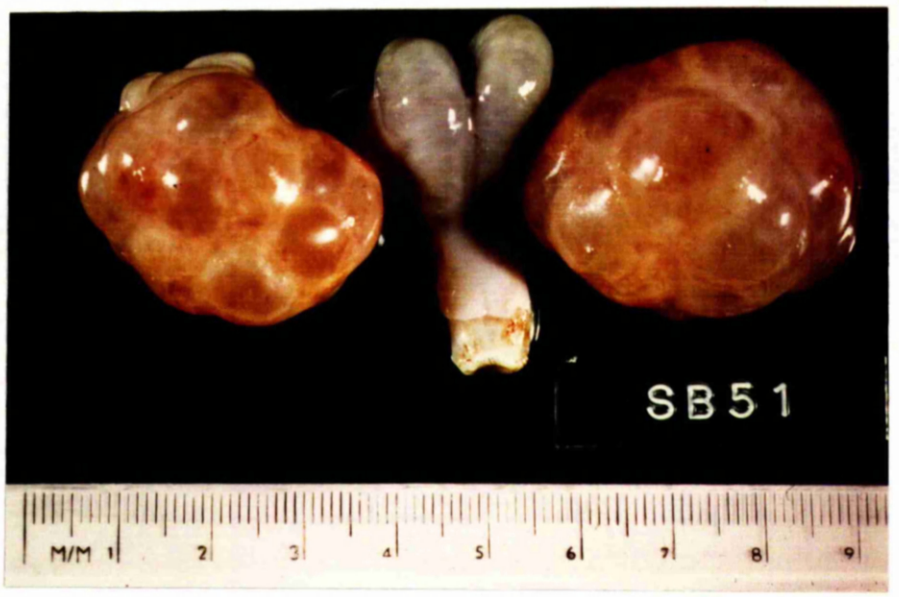


PLATE 4.8 The reproductive tract from a 35 day-old Finnish Landrace lamb (No. 160S, 1975), after treatment with PMSG, with some stimulated follicles showing signs of ovulation or luteinisation

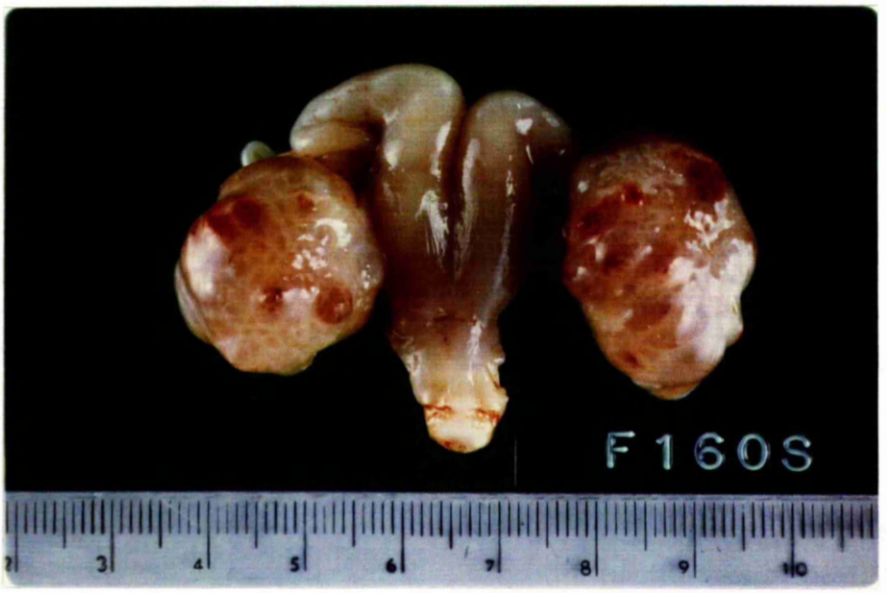


PLATE 4.9 Uterine sections from 35 day-old lambs to show the effect of PMSG on the uterine epithelium

(a) Intercaruncular epithelium from a control lamb

x 250

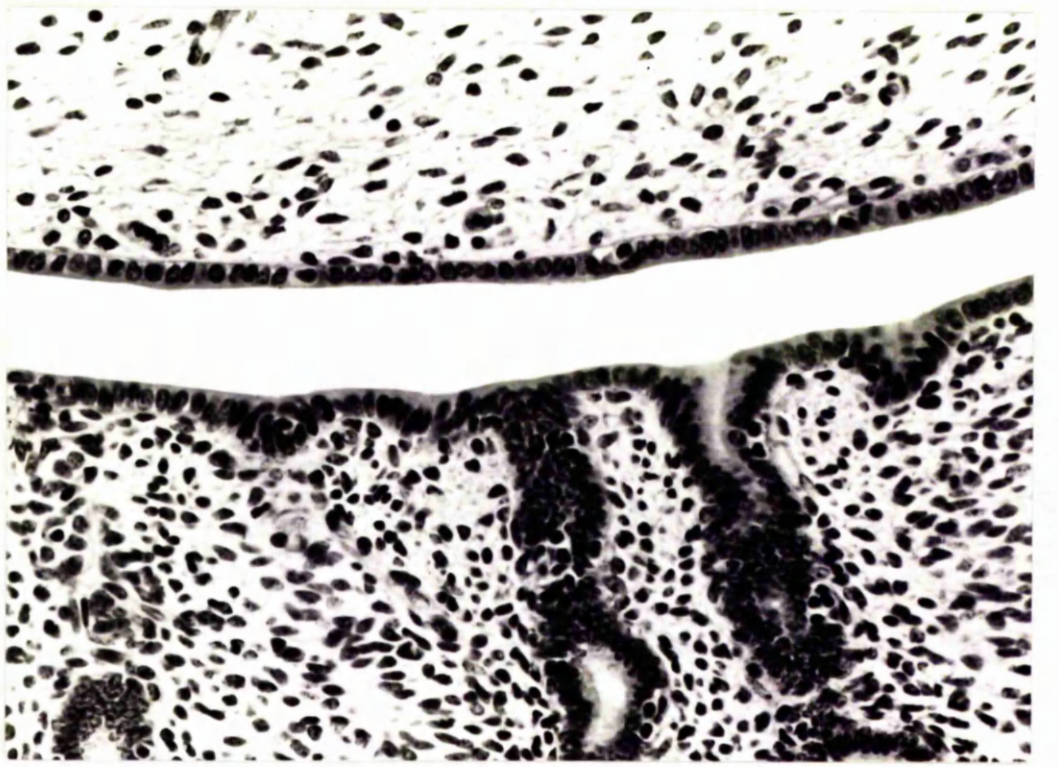
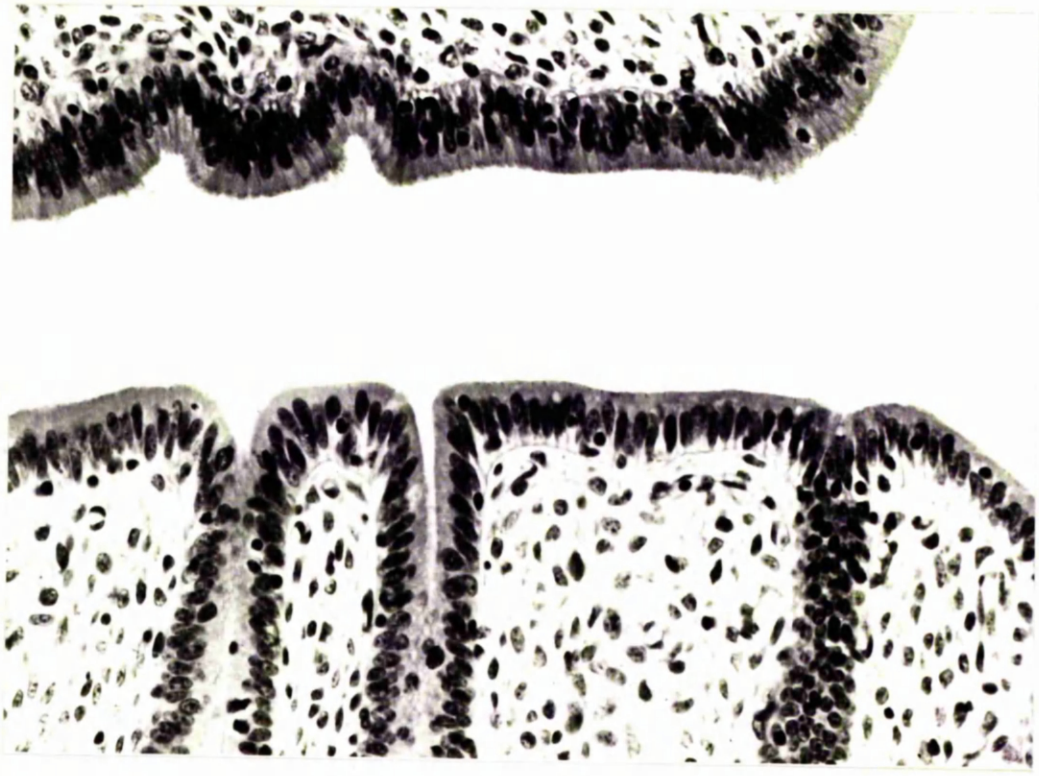


PLATE 4.9

(b) Intercaruncular epithelium from a PMSG-treated lamb x 250



APPENDICES

APPENDIX 1

TABLE 1 Peripheral plasma levels of oestrogens in Finnish Landrace ewes around oestrus (shown in Fig. 2.1)

Ewe Number	Days of Cycle									
	-4	-3	-2	-1	0	+1	+2	+3	+4	+5
18	3.7	4.3	5.1	6.7	5.2	3.6	3.6	4.7	4.1	5.0
22		5.1	7.6	10.4	13.8	6.3	5.7	7.6	7.6	6.6
27	5.1	6.1	7.4	11.4	7.5	5.9	5.1	7.8	8.7	7.4
28		5.4		8.9	13.7	5.3	5.3	6.5	7.3	6.4
84	5.3	11.6	13.1	20.4	5.0	7.6	5.4	7.1	8.1	7.8
85	4.3	5.6	9.1	9.6	19.3	7.6	6.2	13.6	6.9	6.3
86				9.1	12.4	4.3	3.9	4.5	4.6	7.3
87	2.4	2.4	5.3	9.5	8.3	3.9	4.7	5.1	3.5	4.9
92			9.2	8.1	5.1	4.6	4.0	5.9	5.7	4.6
150	4.9	3.6	5.2	6.8	7.3	5.2	4.9	4.6	6.2	7.1
156	6.5	8.3	6.9	9.4	20.2	6.3	5.9	6.7	8.4	9.5
160	4.2	7.9	5.7	10.3	12.0	6.9	3.9	4.9	5.6	7.7
164		5.6	4.7	5.4	8.7	4.5	5.6	6.1	4.2	3.7
171		4.7	4.5	8.3	10.4	2.6	5.1	5.5	4.6	3.9
172	4.6	5.2	4.5	7.9	12.5	3.5	5.4	6.1	7.5	6.9
173	4.8	3.8	8.0	4.9	10.6	6.6	9.9	8.4	8.9	5.9
174	5.3	5.2	6.7	8.9	14.7	6.8	6.8	6.5	7.3	6.6
176	6.1	4.1	6.4	7.5	13.5	3.8	4.6	7.2	7.1	5.7
184		5.3	4.5	7.6	16.5	7.9	13.8	8.8	7.5	6.7
193		5.4	5.1	5.4	11.8	5.4	6.9	8.5	10.7	6.8
194	4.9	5.4	9.8	9.8	9.8	5.2	7.3	5.6	6.4	7.6
196		7.0	8.3	14.2	14.1	6.3	7.6	7.9	7.8	6.9
197	5.5	4.0	4.2	6.8	8.6	4.2	5.7	4.8	5.8	7.5
198	3.1	2.6	4.0	6.8	12.5	2.5	2.7	3.0	4.9	4.3

APPENDIX 1

TABLE 2 Peripheral plasma levels of oestrogens in Scottish Blackface ewes around oestrus (shown in Fig. 2.1)

Ewe Number	Days of Cycle									
	-4	-3	-2	-1	0	+1	+2	+3	+4	+5
3	4.8	3.5	9.1	5.5	2.3	4.1	7.4	4.0	3.1	7.6
5					4.6	4.8	4.9	5.6	5.4	4.2
7		9.2	19.1	9.0	7.7	7.3	8.8	6.7	5.1	7.2
8	4.7	3.9	6.6	3.3	2.9	3.1	3.8	6.1	3.1	4.6
11	5.6	4.3	4.8	8.0	7.4	4.2	5.6	5.3	4.1	2.6
12			4.7	5.1	5.0	5.7	2.9	3.8	4.4	3.7
13		4.5	5.9	5.4	2.7	2.5	3.7	3.8	5.9	3.7
14	4.3	5.9	6.1	7.6	4.2	2.9	3.4	5.3	4.0	2.7
16	4.2	4.7	5.6	8.0	5.5	3.8	3.4	5.3	6.2	4.9
17	8.0	6.1	7.5	10.1	7.5	6.3	7.7	8.4	7.4	5.9
39			12.3	24.7	11.1	11.8	9.0	5.3	8.3	6.6
40		4.7	8.2	9.2	11.1	5.8	6.6	5.8	5.0	3.3
41	3.9	3.9	5.3	7.4	4.1	4.6	2.2	3.9	5.4	2.5
42					9.3	5.3	6.6	5.4	7.3	5.5
45	6.2	4.6	6.2	8.5	7.7	5.5	5.0	5.6	6.1	4.7
48			4.6	6.7	1.4	3.3	2.9	3.8	4.6	4.0
49		9.3	10.0	17.9	5.0	9.7	12.4	9.5	5.5	3.5

APPENDIX 2 Plasma levels of oestrogens in Scottish Blackface lambs during 1973 (shown in Figs. 4.1 and 4.2)

Treatment	Age (Days)	Lamb Number	Days																	
			0		1		2		3		4		5		6		7		8	
			am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
Control	30	12	9	0	2	3	2	3	1	0	1	2	4	1	3	1	0	2	0	
"	69	13R	6	0	0	0	6	2	2	2	0	0	1	2	3	2	1	3	4	
"	71	79	5	5	1	3	3	4	4	2	6	6	8	5	2	2	0	4	0	
"	94	10	8	12	9	9	7	7	12											
"	123	67	9	12	9	3	2	3	3	3	3	7	6	5	0					
"	127	62	0	0	2	10	6	10	8	0	2	12	8	2	12					
250 i.u. PMSG	30	46	12	11	5	1	3	8	5	7	9	3	8	3	4	7	2	1	10	
"	32	24	7	2	10	5	12	8	12	11	20	9	11	6	5	8	7	6	7	
"	28	33S	1	3	6	8	3	7	4	6	3	9	6	6	7	8	4	5	2	

APPENDIX 2 (continued)

Treatment	Age (Days)	Lamb Number	Days																	
			0		1		2		3		4		5		6		7		8	
			am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
500 i.u. PMSG	32	31	9	10	9	9	9	10	16	20	24	11	11	9	8	9	9	8	10	
"	33	22	11	11	15	10	34	44	63	70	93	48	21	20	21	18	15	16	19	
"	28	33R	1	9	13	11	14	18	29	32	33	34	10	18	10	11	7	5	6	
"	67	31	12	14	13	15	16	15	13	10	11	13	11	12	9	10	13	9	11	
"	68	22	8	7	9	6	3	4	5	5	5	6	10	8	9	10	3	10	4	
"	63	33R	3	5	5	5	5	6	4	1	5	12	14	9	11	12	15	10	10	
"	70	16	0	0	0	0	0	0	5	0	6	0	5	6	9	6	17	21	20	
"	71	7R	5	1	11	3	9	8	1	4	47	64	10	5	3	0	0	0	0	
"	69	13S	11	13	16	16	20	16	16	18	17	18	18	14	18	21	16	14	17	
"	94	7	16	27	22	24	32	32	44											
"	93	8	13	13	16	13	20	20	24											
"	95	9	9	2	13	11	16	16	7											

APPENDIX 2 (continued)

Treatment	Age (Days)	Lamb Number	Days																	
			0		1		2		3		4		5		6		7		8	
			am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm	am	pm
500 i.u. PMSG	133	55	7	5	4	6	5	9	9	6	8	13	0	0	0					
"	130	57	13	18	8	8	17	10	13	17	8	6	4	4	0					
"	128	60	1	4	10	5	5	6	7	11	3	3	9	9	2					
1000 i.u. PMSG	28	35	10	16	24	37	129	163			753	365	72	38	20	17	20	19	20	19
"	28	11	11	13	30	43	100	125			324	106	35	25	45	25	2	4	14	20
"	28	15	6	11	69	97	345	374			890	456	77	58	75	65	29	48	17	17
"	137	51	6	7	6	5	0	4	2	5	5	2	4	1	6					
"	130	56	7	2	0	2	4	5	4	1	4	1	7	11	8					
"	131	58	2	7	8	7	4	1	1	4	1	3	5	4	5					

APPENDIX 3

TABLE 1 Peripheral plasma levels of oestrogens in Finnish Landrace and Scottish Blackface lambs at 7 days of age during 1974 (shown in Figs. 4.3 and 4.4)

Breed	Treatment	Lamb Number	Days								
			0	1	2	3	4	5	6	7	8
Finnish	Control	28	9	3	8	15	7				
	PMSG	172	1	0	11	7	8				
	"	24	7	23	36	55	123				
	"	26	9	3	5	2	9	12	18	3	8
	"	184	13	18	10	37	9	13	10	8	8
Blackface	Control	26	6	6	11	2	6				
	PMSG	19	19	11	9	8	9				
	"	54	27	12	18	18	10				
	"	16	18	18	25	41	76	66	56	64	31
	"	45	14	16	16	19	16	16	8	10	15

APPENDIX 3

TABLE 2 Peripheral plasma levels of oestrogens in Finnish Landrace and Scottish Blackface lambs at 21 days of age during 1974 (shown in Figs. 4.5 and 4.6)

Breed	Treatment	Lamb Number	Days								
			0	1	2	3	4	5	6	7	8
Finnish	Control	174S	9	7	9	8	12				
	PMSG	160S	9	9	6	12	32				
	"	193G	8	7	4	7	6				
	"	173	11	133	436	865	1343	1290	1418	711	157
	"	27R	12	25	37	22	30	15	21	11	27
Blackface	Control	33	11	10	13	8	9				
	PMSG	56	11	15	10	12	13				
	"	40	7	7	58	104	187				
	"	46	6	5	8	14	20	12	18	9	11
	"	15	12	16	46	60	132	50	62	63	29

APPENDIX 3

TABLE 3 Peripheral plasma levels of oestrogens in Finnish Landrace and Scottish Blackface lambs at 35 days of age during 1974 (shown in Figs. 4.7 and 4.8)

Breed	Treatment	Lamb Number	Days								
			0	1	2	3	4	5	6	7	8
Finnish	Control	197	9	5	8	7	8				
	PMSG	22S	12	66	305	660	931				
	"	174R	13	171	654	1062	931				
	"	193R	3	28	66	65	33	28	19	24	10
	"	27S	18	27	88	147	158	179	205	272	346
Blackface	Control	39	15	10	8	9	9				
	PMSG	53	10	14	30	22	29				
	"	59	14	15	35	35	23				
	"	47	14	37	191	420	386	88	66	58	15
	"	22	12	26	30	30	40	43	81	25	14

APPENDIX 3

TABLE 4 Peripheral plasma levels of oestrogens in Finnish Landrace and Scottish Blackface lambs at 49 days of age during 1974 (shown in Figs. 4.9 and 4.10)

Breed	Treatment	Lamb Number	Days								
			0	1	2	3	4	5	6	7	8
Finnish	Control	176	14	15	15	11	14				
	PMSG	160R	14	24	48	103	138				
	"	193S	13	29	62	92	114				
	"	183	24	23	42	94	140	138	106	16	5
	"	22R	9	88	322	726	976	665	837	453	148
Blackface	Control	55	8	9	8	10	7				
	PMSG	51	10	30	81	91	163				
	"	58	6	37	136	317	483				
	"	57	17	52	94	186	256	176	60	34	58
	"	52	17	28	49	85	37	55	26	14	12

APPENDIX 4

TABLE 1 Peripheral plasma levels of oestrogens in Finnish Landrace lambs during 1975 (shown in Fig. 4.12)

Treatment	Lamb Number	Days						
		0	1	2	3	4	5	6
Control	184S	3	2	5	3	5	5	5
"	89R	8	8	12	15	12	12	7
"	169	5	5	6	5	5	7	5
"	156S	5	5	5	3	10	12	6
"	73R	5	0	0	0	5	5	5
PMSG	86	7	7	66	153	145	8	0
"	153	6	24	195	437	555	29	10
"	151S	5	5	0	0	3	0	0
"	89S	2	8	25	74	98	16	0
"	165	6	2	0	8	4	1	11
"	161S	9	9	90	231	390	22	1
"	162	6	6	10	11	9	5	9
"	92	6	16	6	3	6	0	11
"	160S	8	0	93	279	355	0	0
"	156R	0	7	31	70	122	9	1
"	173S	9	54	195	352	436	22	7
"	152	13	24	37	38	43	11	18
"	176	0	30	247	640	820	53	13

APPENDIX 4

TABLE 2 Peripheral plasma levels of oestrogens in Scottish Blackface lambs during 1975 (shown in Fig. 4.13)

Treatment	Lamb Number	Days						
		0	1	2	3	4	5	6
Control	63	5	5	5	6	3	2	1
"	53	14	13	18	21	11	8	8
"	65	8	4	6				
"	66	5	14	3	2	19	7	6
"	67	9	0	11	9	9	7	11
PMSG	98	5	8	28	93	337	32	14
"	62	6	10	33	30	60	19	7
"	52	24	33	68	168	302	32	22
"	54	8	13	25	25	13	11	10
"	55	16	12	12	17	18	30	8
"	58	11	17	15	14	19	19	12
"	50	12	53	169	304	376	61	10
"	51	17	31	130	447	983	43	29
"	56	10	25	52	99	131	17	22
"	57	13	10	14	27	47	6	1
"	60	14	11	11	7	7	8	10
"	59	9	15	20	29	30	28	20
"	61	6	25	51	73	110	17	13
"	64	3	12	15	36	35	3	1
"	97	4	17	19	18	31	5	6

APPENDIX 5

TABLE 1 Peripheral plasma levels of oestrogens in methallibure-treated Finnish Landrace lambs during 1975 (shown in Fig. 5.1)

Treatment	Lamb Number	Days						
		0	1	2	3	4	5	6
Methallibure	194S	5	0	5	1	7	0	10
"	191	8	5	8	8	4	8	8
"	18	6	12	10	10	7	6	10
"	171R	7	14	8	18		18	18
"	174S	0	8	6	0	6	8	8
"	198R	2	2	0	0	0	3	0
Methallibure + PMSG	194R	2	8	0	0	12	8	6
"	171G	10	18	23	12	18	10	8
"	174R	3	17	290	765	1941	40	17
"	22	0	2	5	0	16	6	9
"	193	0	14	161	495	530	54	32
"	192	9	0	8	44	58	0	0
"	198S	14	17	14	30	45	12	17

APPENDIX 5

TABLE 2 Peripheral plasma levels of oestrogens in methallibure-treated Scottish Blackface lambs during 1975 (shown in Fig. 5.2)

Treatment	Lamb Number	Days						
		0	1	2	3	4	5	6
Methallibure	69	6	9	10	6	3	6	3
"	72	3	9	8	3	11	10	13
"	74	11	16	8	6	0	1	3
"	75	5	1	7	3	4	18	2
"	80	15	25	32	11	5	12	11
Methallibure + PMSG	73	9	10	13	5	14	15	14
"	76	9	11	9	13	5	15	15
"	77	3	17	10	26	10	6	15
"	78	9	42	286	1092	1621	98	15
"	79	3	11	35	74	101	11	20

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