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CERVICAL MUCUS ARBORIZATION : A PRELIMINARY STUDY
OF ITS USE IN ASSESSING OVARIAN HORMONE LEVELS IN
THE EWE

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I INTRODUCTION

Many of the gross physiological processes affecting reproduction in animals have been elucidated. The availability of the ovarian hormones in relatively pure form has further allowed the confirmation of their role. It is apparent that there is general agreement and confidence in the findings of much of this work, as is shown by the increasing application of steroids to animal production. However, in the main the investigations so far reported have been of a qualitative nature only. It is thus apparent that for a thorough understanding of the basic mechanisms controlling reproduction it will be necessary also to study in detail the quantitative changes in hormone levels and their associated effects.

Progress in the investigation of the quantitative changes has been slow, owing to the lack of suitable techniques of study. A number of criteria have however been utilized to study ovarian activity and ovarian hormone levels. Unlike other large species, palpation of the ovaries per rectum is not possible with the ewe. Oestrous behaviour and visual observation of the ovaries after laparotomy thus remain the methods for assessment of ovarian activity. Biological assay of body fluids and excretory products using small animals has been somewhat successful. To avoid the attendant problems of the bioassay chemical methods are being developed but as yet

have met with limited success. In addition, biological endpoints obtained from within have been used. The characteristic vaginal smear changes found in the ewe are favoured by some workers, although there is not entire agreement on the application of this method. So far no work has been reported for the ewe utilizing the rheological properties or the arborization phenomenon of cervical mucus. However, the results obtained with other species suggest their possibilities. It thus seemed apparent that the investigation of one of these phenomena would be worthwhile.

Cervical mucus arborization, first reported by Papanicolaou (1945) and later used by Zondek (1954) as a biological test in the human was suggested as an endpoint for determining ovarian activity. The object of this study was to investigate cervical mucus arborization and to demonstrate its application as a method for study of ovarian function and hormone levels. Thus, as will be described presently, a preliminary study of the normal occurrence of this phenomenon in the entire ewe was made. In addition, cervical mucus along with the criteria of oestrous behaviour and vaginal smear changes was used to study some effects of progesterone and oestrogen injected into castrate ewes. The results of these trials were subsequently related to studies made with entire ewes.

II REVIEW OF LITERATURE

A. Cervix

1. Morphology

"The cervix or neck of the uterus consists of a powerful sphincter like segment of the genital tract, serving to separate anatomically and physiologically, the uterus from the vagina. It is continuous anteriorly with the uterine body via the os uteri and posteriorly with the vagina by the os externum which leads into the ventral floor of the vagina" (Sisson, 1953).

The comparative anatomy of this organ has been described for the ewe, sow and mare (Trautman, 1947). Williams (1917) has described that for the cow.

The cervix uteri of the ewe is remarkable for the great length and complexity of its lumen, as shown by the diagrams of Marshall and Hammond, (1937). The dimensions of the cervix have been stated by Grant (1933), Cloete (1939), and Sisson (1953). During periods other than at or close to parturition, the length of the cervix is 5 cm. approximately. The lumen of the cervix was greatly constricted by annular or tongue-like folds and ridges projecting inwards. These interleaved with one another when the cervix was contracted and completely blocked the passage. Because of this it was difficult to insert any instrument very far into the cervical canal of the ewe (Walton, 1933; Grant, 1934; Gunn, 1936; Inkster, (1956). However Grant (1934) did note that relaxation of the cervix was evident at oestrus. Four or five folds were effective in closing the cervix in the

ewe (Gunn, 1936) and the cow (Hammond, 1927). With the human and mare these folds were less apparent, while the tongue-like projections were absent.

The complexity of the external orifice of the cervix (os externum) was observed by Grant (1934), who noted one or more muscular folds to constitute the os externum. Dunn (1955) studied the vaginal - cervical junction, before and after slaughter. It was found that the structure of the os externum became more complex, as the number of parturitions increased. The annular folds appeared to be split by pregnancy and "tags" of musculature projected out through the canal opening. The passage or canal was more difficult to locate in aged ewes which had had more pregnancies than in maiden ewes. Dunn found that the os externum could be located in 100 per cent of maiden ewes, but in only 70 per cent of older sheep.

2. Histology

a. Sexual season

Casida and McKenzie (1932) Grant (1934), Cole and Miller (1935), and McKenzie and Terrill (1937) found the mid-cervical epithelium region to secrete mucus throughout the cycle. However, most mucus appeared to be produced and stored during dioestrus and later liberated at prooestrus and oestrus. It was evident that the changes in height of the epithelial layer was closely associated with the amount of mucus in the cells. Thus a "spent appearance" during the late luteal and follicular

phases was observed (McKenzie and Terrill, 1937).

b. Anoestrus and pregnancy

Grant (1934) detected mucus in the epithelial cells throughout anoestrus but noted that active secretion began approximately six weeks before the commencement of the breeding season. Cole and Miller (1935) found that mid anoestrus was characterized by a lower cell height of the superficial layer, less active underlying epithelial cells and reduced complexity of the cervical glands.

The changes occurring during pregnancy have been noted by Grant (1934). A tenacious, stringy mucus was secreted during most of pregnancy. Since little escaped into the vagina a cervical mucous plug was formed, which flattened and disorganized the epithelial cells.

B. Characteristics of Cervical Secretion

1. Origin and amount of cervical mucus

Pommerenke and Viergiver (1946 a) investigated the origin of cervical mucus in women. During pregnancy, the chorion, decidua and amnion effectively sealed the cervical canal from the uterine cavity, hence the mucus was likely to be entirely of cervical origin. These workers could not demonstrate that supracervical hysterectomy resulted in a lower output of mucus, than from intact non-pregnant women. However, they considered that for non-pregnant women the evidence was insufficient to determine the origin of the cervical secretion.

In the human the cervical canal was relatively open and mucus was aspirated easily. Abarbanel (1946 b) determined the volume and noted a relationship with the menstrual cycle. Mucus was found in greatest quantity near the time of ovulation (approximately the middle of the menstrual cycle), while a greatly reduced amount was apparent at other times. Similar findings have been reported by Pommerenke (1946), Pommerenke and Viergiver (1946 a, b.), and Zondek and Rozin (1954).

The difficulty in obtaining all of the mucus contained in the cervix of the cow and ewe appears to have prevented accurate measurement of the volume in the living animal. However, Woodman and Hammond (1925) with slaughter material from cows, noted a

gradual increase in amount of mucus until oestrus, but a decrease occurred just after heat.

Pregnancy was marked by a rise in total volume, over that of the oestrous cycle. Roark and Herman (1950) similarly found the greatest volume of mucus within the first three hours of oestrus in the live animal.

Grant (1934) reported the absence of mucus secreting glands in the vagina of the ewe. The mucus volume he measured originally came from the cervix. Further, it was observed that the volume of mucus secreted followed a cyclic pattern. A copious flow commenced at prooestrus and continued during oestrus but this was greatly diminished during the rest of the cycle.

In conclusion it is apparent that considerable variation in the amount of cervical mucus secreted does exist between species. The findings of Viergiver and Pommerenke (1944), and Pommerenke and Viergiver (1946 a) with intact and hysterectomised women shows that a large variation also exists within a species.

2. Chemical properties

Detailed examination of human cervical mucus has revealed that the chemical composition varied according to the stage of reproduction (Pommerenke and Viergiver, 1946 b; Viergiver and Pommerenke, 1947; Atkinson et. al., 1948; Pederson and Pommerenke, 1950; Bergmann and

Werner, 1951; Breckenridge and Pommerenke, 1951; Shettels, 1951; Shettels et. al., 1951).

Thus during the intermenstrum, the water content rose while the concentration of carbohydrate, amino acids, cholesterol and lipid phosphorus decreased.

Changes in composition of cervical mucus from the cow during the oestrous cycle and pregnancy have also been reported (Woodman and Hammond, 1925; Scott Blair et. al., 1941 b; Boyland, 1946). Thus the latter worker noted that cervical mucin present at oestrus was mainly a carbohydrate and possibly a mucopolysaccharide. However, during dioestrus and pregnancy both polysaccharide and protein were present.

The literature does not indicate any analysis made for mucus from the ewe.

3. Physical properties

a. Rheological

Woodman and Hammond (1925) have described the character of the cervical secretion of the cow. They noted the secretion to be extremely fluid at oestrus, but more viscous during mid-cycle. Pregnancy, was characterized by large amounts of a thick, tenacious, almost rubber-like mucus which effectively sealed the entrance to the cervix. This phenomenon was later proposed as the basis for a pregnancy test (Marshall and Hammond, 1937).

The rheological properties of human cervical secretion have been studied extensively. Scott Blair

et. al. (1941 a, b) and Clift (1945) also measured the flow elasticity* of cervical secretion from cows. Maximum values occurred near the time of ovulation, while much lower values were obtained during the rest of the cycle and in pregnancy. Urine contamination of the mucus caused erroneous values for viscosity measurements, but did not appear to affect flow elasticity. The additional characteristics of spinnbarkeit,**plasticity and tact have also been investigated for the human (Clift, 1945; Cohen et. al., 1952).

Further work (Clift et. al., 1950; Glover and Scott Blair, 1951; Clift and Hart, 1953; Glover and Scott Blair, 1953; Scott Blair, 1953) using mucus from cows and women, indicated that the variations in flow properties were closely correlated with the physiological changes of the cycle and pregnancy.

*When a column of fluid is being extruded from a capillary tube and the pressure is suddenly released the column will recoil. The amount of recoil is a measure of the flow elasticity of the fluid, (Scott Blair et. al., 1941 b).

**Spinnbarkeit - the capacity of liquids to be drawn into threads, (Clift, 1945).

b. Arborization phenomenon

Papanicolaou (1945), 1946) observed that cervical mucus collected from women near the time of ovulation "crystallised" and formed a typical arborization pattern* when allowed to dry on a microscope slide. Under the microscope, the smear had a striking pattern of flowers and leaves resembling fern or palm-leaves. Zondek (1954) described this as "fern or palm leaf (PL) formation. However the type of pattern was dependent partly upon the thickness of mucus; arborization often failed with thin smears, whereas flower patterns and palm leaf patterns were found with thicker mucus.

Arborization has also been shown to be characteristic of cervical mucus from the cow (Garm and Skjerven, 1952; Colluzzi and Battistacci, 1953; Higaki and Awai, 1953; Bone, 1954; Fedrigo, 1955; & Lora 1955) and the ewe (Raeside, unpublished data, 1955).

1. Mechanism of arborization - Papanicolaou (1946)

observed that the abundance of arborization coincided with increased cervical secretory activity and a change in viscosity of the fluid. However Zondek (1954) stated that arborization and elasticity of mucus were independent.

Rydberg (1948) suggested that the phenomenon was caused by crystals of sodium chloride. The characteristic

*Zondek (1954) has suggested that true crystals may not be involved in the phenomenon and preferred to term the process arborization.

forms which these assumed were thought to be dependent upon the presence of mucin-like substances secreted by the cervical glands. An intensive investigation reported by Zondek (1954) showed that arborization resulted when a protein complex was mixed with electrolytes and allowed to dry. Examination of many body fluids including nasal mucus, cerebrospinal fluid, follicular fluid, ovarian cystic fluid and that from the hydrosalpinx all showed arborization when completely dry. Detailed examination of the constituents of mucus showed that mucin was not the essential factor as suggested by Rydberg (1948). Thus tears which did not contain mucin or similar substances showed a typical PL reaction. Further, it was found that albumin, fibrinogen, and globulins, and the degradation products of protein (including peptones, dipeptides, tripeptides and polypeptides and certain amino acids), as well as monosaccharides and polysaccharides all showed arborization when mixed with electrolytes.

11. Factors affecting arborization - In the literature several factors which were likely to influence the PL reaction have been discussed.

Electrolyte status: Landerstrom-Lang produced typical arborization by drying a mixture of egg albumin and 0.9 per cent sodium chloride. Zondek (1954) showed that neither the sodium or chloride ions were specific to the PL reaction, since potassium permanganate also

facilitated arborization when mixed with a protein complex. Some salts failed to cause a PL reaction, notably calcium chloride, barium chloride, sodium bromide, potassium nitrate, sodium sulphate and sodium iodide.

The presence of electrolyte was essential to the arborization process since dialysis of cervical mucus inhibited arborization, whereas subsequent addition of electrolyte allowed PL formation (Zondek 1954). Further, it was noted that cellular type cervical mucus (negative smear) from premenstrual, menstrual, postmenstrual, pregnant, menopausal and castrate women developed arborization when electrolyte solution was added.

High concentrations of electrolyte were found to facilitate the PL reaction better than low concentrations (Zondek, 1954). However, arborization was still apparent even when low concentrations of both protein complex and electrolyte were present. Zondek concluded that for cervical mucus any protein or carbohydrate will produce arborization, provided a minimum concentration of electrolyte was present. The possibility that still unknown factors present in cervical mucus might be involved in the phenomenon, was also stressed.

Temperature: Papanicolaou (1945), Rydberg (1948), Roland (1952), Zondek (1954), Bone (1954), Zondek et. al., (1955) observed arborization after cervical mucus

was dried at air temperature. The atmospheric temperature was not stated. Campos da Paz (1953), and Zondek (1954), dried the smears in a flame and found arborization persistent even when the smear turned brown at high temperatures. No recordings appear to have been made of low temperature effects on arborization.

Foreign materials: Bone (1954) in a preliminary study of cervical mucus of the cow observed that the blood chlorides normally present in serum or tissue fluids failed to promote arborization. Zondek (1954) noted a similar condition with human cervical mucus. Further, he found that sperm and prostatic secretion inhibited the PL reaction. Similarly, boiled sperm prevented arborization and thus excluded the possibility that enzyme action was responsible.

No investigation of the mechanism by which these contaminants inhibit arborization has so far been reported. Zondek (1954) suggested an inhibition of arborization by a "mechanical effect" of the contaminants. It should be mentioned that this was in contrast to hormonal inhibition of arborization, to be discussed later.

C. Endocrine Control of Cervical Secretion

1. Amount

Seguy and Simmonet (1953) found a relationship between the secretion of cervical mucus and elimination of oestrogen in urine. Moricand (1936), and Abarbanel (1946 a, b, c) demonstrated that the cyclic changes in amount of cervical mucus secreted during the menstrual cycle, were under hormonal control. Thus it was noted that oestrogens administered to ovariectomised and hysterectomised women caused an increase in volume within 48-72 hours. Progesterone caused a rapid decrease in volume of mucus secreted. These findings were similar to the work of Sjoval1 (1938) who reproduced the cycle in the cervix and cervical mucus in the castrated guinea pig, by the use of oestrogen and progesterone.

It appears likely that during the follicular phase of the cycle and up to ovulation, oestrogen caused an increase in the volume of mucus, while in the luteal phase progesterone from the corpus luteum inhibited the production or release of mucus.

2. Chemical properties

Several workers have observed the "water phase" (high water content) of the cycle and noted its occurrence near the time of ovulation (Pommerenke and Viergiver, 1946 b; Bergmann, 1953).

Seguy and Simmonet (1933) noted that this period was associated with maximum penetrability of mucus by spermatozoa. Abarbanel (1946 a) indicated that the character of non-penetrable mucus could be changed by oestrogen therapy. His results showed that a clear, glairy, watery, easily penetrable mucus in ovariectomised and hysterectomised women, could be produced soon after oestrogen treatment. This resembled that found in normal women, at the ovulatory or water phase of the cycle. Progesterone had an antagonistic effect and appeared to reduce the water content of the mucus.

3. Physical properties

a. Rheological

It was previously noted that the values for viscosity and flow elasticity characteristics were correlated with the physiological changes of the oestrous cycle and pregnancy (Glover and Scott-Blair, 1953).

Clift and Hart (1953) suggested that the mechanism for secretion and the nature and quantity of cervical mucus was probably controlled by receptors in the cervix. These appeared sensitive to circulating hormones, as shown by administration of ovarian hormones. Abarbanel (1946) increased the quantity of clear, thin, cervical secretion from castrate women, by oestrogen

injections, while oestrogen plus progesterone therapy caused a reduced flow of "tougher secretion". Clift and Hart (1953) gave progesterone to non-pregnant women, and produced a cervical plug almost indistinguishable from the high consistency plug of pregnancy.

Glover and Scott Blair (1953) explain their results in the cow on the basis that low values for viscosity at the time of ovulation correspond to a high level of oestrogen, while high values indicate a high progesterone level. Further support for this contention was given by Clift and Hart (1953). They found that at menstruation a decreased quantity and high consistency of mucus was related to decreased oestrogen and raised progesterone concentration.

The clinical application of viscosity measurements appears to be of value in the determination of hormonal upsets, for example, secondary amenorrhoea and threatened miscarriage (Glover and Scott Blair, 1953; Clift and Hart, 1953).

b. Arborization phenomenon

Abundant arborization at the follicular phase of the cycle and the absence of the phenomenon during the luteal phase and menopause, indicated a relationship between arborization and ovarian activity (Papanicolaou, 1945). Holand (1952) noted arborization to occur from

day 5 or 7 until day 20 or 22 of the normal menstrual cycle. During the remainder of the cycle a cellular smear, comprised mainly of epithelial cells and leucocytes, was observed. Similar findings were reported by Zondek (1954), Zondek and Rozin (1954), Urdan and Kurzon (1955), and Zondek et al. (1955). Bone (1954) found arborization to occur during the normal oestrous cycle of the cow from three days prior to oestrus until nine days post oestrus. Garm and Skjerven (1952) showed the PL reaction was persistent up till the eleventh day post oestrus.

The change from the postmenstrual cellular type smear to a PL pattern of the proliferative phase appeared to be gradual (Zondek et al. 1955). Further, Campos da Paz (1953) found PL formation was most abundant at the peak of oestrogen activity. Associated with the development of the corpus luteum there was a rapid disintegration and disappearance of crystallisation and a replacement by a cellular smear (Urdan and Kurzon, 1955). During these transition periods, both cellular and PL mucus was observed. Garm and Skjerven (1952) with bovine cervical mucus illustrate on the same slide both cellular and fern-like material taken 11 days post oestrus. However mucus collected on day 13 was completely cellular.

Papanicolaou (1945) and Campos da Paz (1953) showed that oestrogen therapy induced or increased the

PL reaction during the cycle. Further, Roland (1952) found that oestrogens would induce arborization during menopause and even after castration of postmenopausal women. However, the PL patterns ceased two or three weeks after the oestrogen injections were stopped, but a second period of treatment allowed further arborization. Similar results with ovariectomised women have been noted (Zondek, 1954). Campos da Paz (1953), Di Paola and Lelio (1953) and Zondek (1954) observed that arborization occurred to some extent in cases of primary amenorrhea.

However in mucus from secondary amenorrhoeic women no arborization was detected until after oestrogen therapy (Di Paola and Lelio, 1953).

The cellular type pattern characteristic of the premenstrual, menstrual and postmenstrual periods of the human, also persisted during pregnancy, the menopause and after ovariectomy (Papanicolaou, 1945; Rydberg, 1948; Roland, 1952; ~~Roller~~, 1954; Zondek, 1954; Zondek and Rozin, 1954; Lewis, (1955)).

Neumann and Lehfelat, 1955; Pierce and Cope, 1955; Urdan and Kurzon, 1955; Zondek and Cooper, 1955; Zondek et. al. 1955). Also the inhibition of the PL reaction after progesterone therapy has been reported (Campos da Paz, 1951; Roland, 1952; Greenblatt, 1954; Zondek 1954).

Roland (1952) has commented on the relationship

between the endocrinology of the organism and arborization of cervical mucus. He was in agreement with the postulate of Landerstrom-Lang that the fern leaf patterns were the result of oestrogen activity on sodium chloride and mucin metabolism. This however is doubtful, since Zondek (1954) has shown that both mucin and sodium chloride were not essential to the process. Instead, Zondek postulated that oestrogen increased the electrolyte status of the cervical mucus secreting cells, perhaps by allowing increased permeability to electrolytes.

For those phases when arborization was absent, a deficiency of electrolyte was suggested. This may result from:

- i. An absence of oestrogen as in the postmenstrual phase, during the menopause, in ovariectomised individuals, or during secondary amenorrhea.
- ii. Inhibition of any oestrogen effect by a high level of circulating progesterone, as is probable during the premenstrual phase, the luteal phase of the oestrous cycle and during pregnancy.

Reference has been made that oestrogen injections to individuals showing a cellular smear, produced arborization, except during pregnancy. Zondek (1954) found that salt solutions added to the pregnant cellular smear allowed arborization. Thus he concluded that during pregnancy the

cervical glands lost the ability to produce mucus containing electrolytes. Further, large doses of oestrogen at this time did not restore this ability. Subsequent work, Zondek et al. (1955) showed that arborization may occur during pregnancy and suggested placental insufficiency (low progesterone production) as the factor responsible. When arborization was intense, abortion or intra uterine death of the foetus often occurred. However, after the PL reaction ceased a normal pregnancy proceeded. It was suggested that a change in the progesterone - oestrogen balance could explain arborization during pregnancy. Nevertheless it was stated that the exact mechanism of PL production or inhibition during this period was not clearly understood.

The cervical mucus arborization phenomenon has been used as a measure of the oestrogen level in the human (Zondek, 1954). The author injected a known quantity of oestrogen into postmenopausal women and castrated women and noted the minimum quantity of hormone necessary to produce arborization. A cervical mucus unit (C.M.U.)* for each oestrogen used (oestrone, oestradiol and others) was calculated and the following relationships established:

Oestrone 1 C.M.U.	= 1 mg.
Oestradiol 1 C.M.U.	= 0.3 - 0.4 mg.
Oestradiol benzoate 1 C.M.U.	= 0.2 mg.

*Cervical mucus unit - A C.M.U. was defined as the quantity of oestrogenic hormone inducing the production of cervical mucus which crystallised when dried 72-120 hours after a single injection (Zondek, 1954).

Increased sensitivity to ODB was noted when lower quantities of hormone caused arborization, after injections for three consecutive days.

D. Ovarian Changes and their Relation to the Oestrous Cycle in the Ewe

The relationship between the gross ovarian changes and the physiological functioning of the ovary was suggested as early as 1905 by Marshall and Jelly. They stated that:

"the Mammalian ovary in addition to its function of producing ripe ova, is an organ elaborating an internal secretion which reacts on the general metabolism of the animal, as shown by the effects brought about by its removal. The secretion is probably formed in greater or less quantity at all times, but it is produced in greatest abundance at certain more or less regularly recurrent periods, when it brings about those conditions which characterize the pro-oestrus and oestrus..... It is at these periods also that the ova mature and the follicle discharges.

After ovulation, which takes place during oestrus, the corpus luteum is formed and this organ provides a further secretion whose function is essential for the changes taking place during the attachment and development of the embryo in the first stages of pregnancy."

The gross changes of the graafian follicle during growth, rupture and subsequent development of the corpus luteum have been described (Marshall, 1904; Hammond, 1921; Kupfer, 1929; Quinlan and Mare, 1934; Grant, 1934; Cole and Miller, 1935; Roux, 1936; McKenzie and Terrill, 1937; Underwood and Shier, 1941; Hammond, Jr., 1944; Robinson, 1950, 1951.) By means of laparotomies, McKenzie and Terrill (1937) observed and timed preovulatory growth and

rupture of the follicle. Conspicuous external changes in the follicle were noted only in the last four hours prior to rupture. The cytological changes of the corpus luteum during formation and regression have been detailed by Warbritton (1934).

Changes in the size and number of follicles during the oestrous cycle and in anoestrus have been investigated by several workers. Casida and McKenzie (1932) noted a gradual increase in mean diameter of the follicles, 2 mm. and larger, during the first one-third of the cycle. For the remainder of the cycle the mean diameter was irregular but was fairly well sustained. Some follicles at midcycle were however as large as those just prior to ovulation.

Grant (1934) found that during the interoestrous period, one or more follicles grew to a certain size of 5-7 mm. Those follicles which did not ovulate remained little changed in size during the next interoestrus. When only very small follicles were present they enlarged up to 5-7 mm. in diameter. Kammlade, Jnr., et al. (1952) found that the regression of average follicle diameter on day of the cycle was highly significant.

The number of small follicles present during the interoestrous period was quite variable (Cole and Miller, 1935). McKenzie and Terrill (1937) detected a range from none up to 30 in each ovary. Kammlade, Jnr., et al. (1952) found no relationship between the number of follicles and the stage in the cycle.

E. Oestrogen - Progesterone Control of the Oestrous Cycle

For the ewe and other large animals, information is lacking on the endocrine mechanism associated with reproductive behaviour. This section must therefore include some data derived from small animal studies, but an attempt will be made to relate this with the processes that are thought to operate in the ewe.

The reviews of Hisaw (1947), Dutt (1953 a) and Amoroso (1955) show that the ovarian-pituitary relationship is intimate. Evidence for the cyclic nature of the oestrous cycle suggests a humoral interplay between the ovaries and the anterior pituitary. The secretion of follicle stimulating and luteinizing hormones (FSH and LH, names which describe their function) by the pituitary, are thought to be under separate control. Further, the gonadotrophic potency of the pituitary is modulated by a feed-back mechanism, determined by the level of ovarian hormones (Zuckermann, 1955). Oestrogen is secreted by ovarian tissue under the influence of FSH. With the consequent rise of oestrogen in the blood, it inhibits the release of further FSH. If the level of oestrogen does not rise too high, it stimulates the release of LH. Follicular maturation can occur and subsequently ovulation and corpus luteum formation. Luteotrophic hormone (LTH), from the pituitary, is responsible for the maintenance of the corpus luteum and secretion of progesterone (Astwood, 1941). Ovulation thus occurs as a result of the combined action

of oestrogen and progesterone on the follicle.

It is realized that many other factors exert an influence on the oestrous cycle (Amoroso, 1955). In this section only oestrogen and progesterone will be stressed. The other factors are briefly mentioned.

1. Oestrogen

Hammond and Day (1944) observed with cows, that high doses of oestrogen prevented full growth of follicles. Hohlweg (1934) found that oestrogen stimulated LH and caused ovulation. Hensel et al. (1952) with cattle and Simpson (1952) with ewes could not hasten ovulation with oestrogen injections. These workers suggested some doubt as to the efficacy of oestrogens to cause release of LH in these two species. However, the importance of doses within "physiological limits", to explain some of the apparently conflicting results, has been stressed (Dutt, 1953 a).

The time of injection of oestrogens has been reported by Kidder et al. (1955) as being important in pigs. A large dose, 3 mg. stilboestrol 11 days after heat, prolonged the cycle by approximately six days and caused luteinization of follicles. The same dose given six days after heat produced no effect, but in animals injected 16 days after heat a variable response was obtained.

2. Progesterone

Removal of the corpus luteum from the cow (Williams

and Williams, 1921; Hammond, 1927; and the ewe (McKenzie and Terrill, 1937) resulted in ovulation and heat occurring within two to four days. This suggested that progesterone from the corpus luteum had an inhibitory effect on follicle growth and ovulation. The mechanism causing the effect has been investigated by Makepiece et al. (1936), Astwood and Fevold (1939), Burrows (1939), Biddulph et al. (1940), Dutt and Casida (1948), O'Mary et al. (1950), Ulberg et al. (1951 a, b), Dauzier et al. (1953), Hough et al. (1955) Nishikawa (1955), Nellor & Cole (1957), using small and large animals. The results suggested that progesterone inhibited the release of LH and prevented pre-ovulatory growth and ovulation.

Control of the time of ovulation has been achieved by a large single injection of progesterone in the beef heifer (Nellor and Cole, 1956) and by continuous injections in the cow (Christian and Casida, 1948; Trimberger and Hansel, 1955), and the ewe (Dutt and Casida, 1948; O'Mary et al. 1950; Dutt, 1953 b; Hunter, 1954; Robinson, 1956). Ovulation in a large proportion of the ewes occurred three days after the last injection.

Maintenance of the corpus luteum and inhibition of ovulation in several species has been achieved by hysterectomy (guinea pig; Loeb, 1927; rabbit: Asdell and Hammond, 1933; Chu et al., 1946; rat: Hatcher et al., 1940; ewe and heifer: Wilthbank and Casida, 1956).

Cystic follicles have been noted in ewes (Dutt and

Casida, 1948) and gilts (Ulberg et al., 1951 a) at some levels of progesterone treatment. Dutt (1953 a) postulated that the FSH activity was great but that LH was partially suppressed by progesterone. Thus, ovulation failed and a large overgrown cystic follicle resulted.

The stimulating effect of progesterone on follicular growth in dioestrus rats has been demonstrated (Everett, 1943;

1948). Hansel and Trimberger (1952) found with heifers that the length of oestrus and the time from end of oestrus to ovulation was significantly reduced by progesterone injections. Further, the histological changes in the ovary suggested progesterone was produced before ovulation. It is thus likely that the hormone may play a role in both LH release and ovulation. The findings of Simpson (1952) with ewes are in agreement with those above.

It has been suggested that the granulosa cells of the follicle produce progesterone just prior to ovulation (Pfeiffer, 1950). Progesterone has since been shown to be present in the follicular fluid of the sow (Edgar, 1953) and woman (Zander, 1954). There is also the possibility that ovulation may be the result of the synergistic action of both progesterone and oestrogen within the follicle (Dutt, 1953) a).

3. Oestrogen - Progesterone synergism

Courrier (1950) reviewed interactions between oestrogen and progesterone affecting reproductive phenomena. In the

present study of arborization of cervical mucus and oestrous behaviour have been investigated. Reference has been made to the antagonistic action of the two hormones affecting the properties of cervical mucus. Several workers have shown evidence for a synergistic action of oestrogen and progesterone in mediating oestrus. This has been discussed for the guinea pig, hamster mouse and rat by Dempsey (1952). Thus, sexual receptivity in ovariectomised rodents appeared to respond to the sequential action of oestrogen and progesterone.

Ovulation without oestrus has been noted in the cow and ewe (Grant, 1934; Hammond, 1946). A similar phenomenon is common at the commencement of the breeding season of the ewe (Grant, 1933). Also ovulation has been induced in the anoestrous ewe with pregnant mares' serum (PMS) but heat did not occur. However, oestrus was coincident with a second ovulation induced 16 days later with PMS, (Cole and Miller, 1935; Robinson, 1954 a). These observations have suggested that progesterone from a waning corpus luteum in the presence of a developing follicle may be necessary for oestrus (Hammond Jnr., et al. 1942; Hammond Jnr., 1945; Robinson, 1950).

Asdell et al. (1945, 1949), Alba and Asdell (1946) and Hansel et al. (1949), investigated oestrus and the associated changes in the genital tract of the cow. They found that full development of these changes in the ovariectomised animal was dependent upon a balance between oestrogen and progesterone. Robinson (1952, 1954 b, c) found that progesterone prior to

Oestrogen was necessary for the induction of recurrent oestrus in the ovariectomized ewe. Thus Robinson's findings were in agreement with the concept that progesterone from a waning corpus luteum conditioned the ewe to respond to follicular oestrogen.

F. Quantitative Studies on Oestrogen and Progesterone
Production in Large Animals

1. Oestrous cycle

a. Oestrogens

i. The ewe - Bell et al. (1941) used the criteria of oestrous behaviour, vaginal smears and histological examination of the tract, to study the effect of exogenous oestrogen in castrate ewes. Twenty-one of 22 ovariectomised yearling ewes showed oestrus, characteristic vaginal smears and histological changes, when 1000 rat units of oestradiol benzoate (ODB) were given alone or followed by progesterone. Frank and Appleby (1943), Quin and Van Der Wath (1943) successfully used 1-5 mg. stilboestrol to induce heat in intact anoestrous ewes.

That such large doses were unnecessary for oestrus induction, was shown by Phillips et al. (1946). With ovariectomised ewes relatively low doses of oestrogen (100 ug. and even 25 ug. ODB) gave a high percentage response. When progesterone was given 16-64 hours after ODB, the results showed no evidence for believing progesterone acted

synergistically.

Robinson (1955 a) in a series of trials determined the median effective dose (ED 50) of ODB given alone, or preceded by progesterone, for the induction of oestrus and the associated vaginal changes. The dose response line for oestrous behaviour indicated that 6½ ug. ODB alone or 22 ug. ODB after three days progesterone pretreatment (75 mg) resulted in 50 per cent oestrous response. The corresponding vaginal smear changes, although less satisfactory owing to the difficulty in determining the end point gave values of 2½ ug. ODB when given alone and 1½ ug. ODB after progesterone - ODB treatment. Using these data, Robinson attempted to deduce the quantitative oestrogen production in the intact ewe. It was suggested that since the entire ewe does not show oestrus, except when a waning corpus luteum is present (Robinson, 1954 c), oestrogen production by the graafian follicle was likely to be less than the equivalent of a single injection of 30 ug. ODB. (This value was the dose level below which no ewe responded to ODB alone). Since the dose level and time of administration were not considered optimal, he suggested that the oestrogen produced by the follicle was probably lower than 30 ug.; a value only slightly above that for characteristic vaginal changes was thus indicated. Robinson (1955 b) as a result of work with the anoestrous ewe, concluded that the developing follicle of the Romney Marsh ewe, produced oestrogen, the equivalent of a single injection of 18-20 ug. ODB.

It was also noted that the quantity of ODB necessary for oestrous response could be reduced by increasing the duration

of progesterone pretreatment. Subsequent work (Robinson et al., 1956) showed that the requirement of ODB to induce behavioural oestrus in 90 per cent of ewes, after pretreatment for 12 days, was indistinguishable from that needed for characteristic vaginal changes. The values for oestrus and vaginal responses were 18.9 and 18.6 ug. ODB, respectively. These findings suggested a close approximation in their time-dose relationships to the physiological conditions possibly obtaining in the normal ewe.

Kust and Vogt (1934) and Hayston (1939) by means of bioassay, could not detect oestrogen in the blood or urine of non-pregnant sheep. Bassett et al. (1955) assayed urine using the rat vaginal smear technique. Eleven of 13 urine extracts from ewes during heat, showed a positive response, equivalent to 1.1 - 2.4 ug. oestrone per twenty-four hour urine output. Similar oestrogen values were found during the rest of the cycle. These workers found that oestrogen levels during the breeding season were relatively constant. Oestrous behaviour could thus result from changes in the levels of other hormones relative to oestrogens.

ii. Other large species - Borth and Watteville (1952) have reviewed the published data for oestrogen excretion during the menstrual cycle. In contrast to the ewe oestrogen excretion appeared cyclic. A peak value for oestrone - oestradiol of 20 ug. per 24 hour was noted near ovulation, and another of 25 ug. preceding the onset of menstruation. Minimum values, approximately 4 ug. per 24 hour, occurred from late menstruation

until the tenth day of the cycle. The bioassay limitations have been noted by Harlow et al. (1955). Chemical determinations but with different methods, (Bauld, 1955; Braunsberg et al., 1955; Brown, 1955) show a similar oestrogen excretion pattern to the results obtained with a bioassay although absolute values were slightly higher.

Some information is available concerning oestrogen determination in domestic animals, namely the mare, cow, sow and goat. It was evident from the reviews of Cowie (1948) and Cole (1950) that only small quantities of oestrogen are present in urine and body fluids. Most workers are in agreement that the highest levels occur near oestrus and ovulation.

b. Progestogens*

Emmens (1950) summarized the biological methods for assay of progesterone and found no simple or accurate technique. The chemical estimation of progesterone has been reviewed by Reeside (1954).

1. The ewe - Neher and Zarrow (1954) assayed progestin (progestogen) levels using the Hooker-Forbes (1947) technique. Progestin was found in blood throughout the oestrous cycle. The levels varied in a cyclic manner. A low level, 0.3-2ug./ml. serum at ovulation, was followed by a rapid

*Progestogens - a collective term for substances responsible for progestational changes in the uterus, the most important of which is progesterone (Cowie, 1948).

rise to 6 $\mu\text{g.}/\text{ml.}$ between the eighth and twelfth days. A minimum level of 2 $\mu\text{g.}$ or less was associated with a waning corpus luteum and noted at the onset of the next heat.

Salhanick et al. (1951) and Olsen et al. (1952) showed that other body steroids affected the action of progesterone in the Hooker-Forbes assay. Edgar (1953) suggested that the technique lacked specificity and the substance measured probably was not progesterone alone. Zarrow and Neher (1953) tested the value of the assay and concluded that under normal physiological conditions the technique was valid for untreated blood.

Urinary pregnanediol determinations as a measure of progesterone production have been conducted. Beck (1950) reported negative findings however.

Edgar (1953, 1954), and Edgar and Ronaldson (1957) used a chemical assay technique sensitive to very low concentrations of progesterone (0.1 $\mu\text{g.}/\text{ml.}$ blood). Progesterone was detected in ovarian venous blood but not in peripheral blood. During the first and second days of the cycle the level was less than 0.1 $\mu\text{g.}/\text{ml.}$ of blood. Increasing, detectable amounts were noted from the third and fourth day until the ninth day. An average value of 1.7 $\mu\text{g.}/\text{ml.}$ on day 11 was found. These high values persisted until the sixteenth day when a sudden drop in level followed. Levels for yearling ewes were within the range found in three year-old sheep but large variations

existed between sheep on the same day of the cycle.

Progesterone from follicular fluid has not been demonstrated (Edgar, 1953). However progesterone in blood draining an ovary, containing two mature follicles and no corpus luteum, was detected in one ewe.

11. Other large species - Pregnane - 3 α , 20 α diol and pregnan - 3 α ol-20-one are so far the only proven metabolic end products of progesterone, excreted in the urine (Smith and Smith, 1952). Venning (1937, 1938) and others have utilized these end products to demonstrate progesterone production during the menstrual cycle. The luteal phase showed the greatest output of these metabolites. Watteville (1954) indicated that up to 6 mg. pregnanediol per 24 hours may be excreted during this period. Marrien (1949) reviewed the modifications in the technique of estimation and concluded that the relationship between progesterone production and urinary pregnanediol excretion was not constant. The value of this method for progesterone estimation was thus reduced. However, the more recent techniques involving chromatographic separation of pregnanediol, (Chaney et al., 1952; Stimmel et al., 1952; and Klopfer et al., 1955) suggest that more accurate methods for assessing progesterone secretion rate are now available.

Cole (1950) has mentioned the lack of studies on excretion of progesterone derivatives from domestic animals. Pregnanediol was not detected as an excretion product of the

TABLE 1

Progesterone content of body fluids of non-pregnant animals determined by various methods

Source	Method	Species	Stage of reproduction	Body fluid	Concentration of progesterone (ug./ml.)
Davene de Wit (1938)	Lengthening of Bitterling ovipositor (Davene de Wit, 1938)	Sow	Dioestrus	Follicular fluid	8
		Cow	Dioestrus	Follicular fluid	5
Hooker & Forbes (1947)	Changes in stromal nuclei of mouse endometrium (Hooker & Forbes, 1947)	Sow	Dioestrus	Follicular fluid	4 - 8
Forbes 1949	ditto	Woman	Dioestrus	Plasma	0.3 - 1
Hooker & Forbes (1949)	ditto	Monkey	Dioestrus	Blood	4 - 8
Forbes et al. (1950a)	ditto	4 Women	Ovulation till menstruation	Plasma	1.7 - 5.2 (free)
Forbes et al. (1950b)	ditto	Monkey	Second half of menstrual cycle	Renal arterial plasma	0.8 - 8.9 (free) 0.2 - 2.6 (bound)
Edgar (1953)	Paper chromatography (Bush, 1952) and subsequent ultraviolet absorption (Reynolds & Ginsburg, 1942)	Mare	Non-pregnant	Blood	0.1
		Cow	Mid-cycle	Blood	0.1
		Sow	Non-pregnant	Blood	0.1
		Sow	Recent corpora lutea	Blood	0.4
		2 Sows	Near ovulation	Follicular fluid	3 and 8
			Irregular heat periods	Pathological cysts	1 - 5
Racside & Turner (1955)	ditto	Heifer	Non-pregnant	Plasma	0.1

goat (Boscott, 1952) and the mare and cow (Stevenson, 1947). Cole (1950) has suggested that the negative findings may be owing to the use of methods designed for human urine analysis, and which are possibly not applicable to other species.

Chromatographic methods have been utilized for the estimation of progesterone in blood and body tissues of the human, cow, sow, elephant, and mare (Butt et al., 1951; Edgar, 1953; Zander, 1954; Raesside and Turner, 1955). Values for progesterone found by these methods were markedly lower than the results from the Hooker-Forbes assay (Table 1). The failure by most workers to detect progesterone in peripheral blood was notable since it indicated that only extremely small physiological quantities were present. However, follicular fluid from the human, cow and sow contained a high progesterone content immediately prior to ovulation (Edgar, 1953; and Zander, 1954). Similar results were found with luteal tissue of the elephant and calf (Edgar, 1953).

2. Pregnancy

a. Oestrogens

1. The ewe - Whitten (1943) in a study with two ewes detected oestrogens in urine only during the last few weeks of pregnancy. Beck (1950) assayed oestrogenic extracts of pregnant Merino ewes' urine and faeces using the vaginal smear technique with ovariectomised rats. Oestrogens were barely detectable until the last three or four weeks of

pregnancy and then were present only in small quantities. The daily excretion of oestrogens was greater in faeces than urine Bassett et al. (1955) found considerable variation in the quantity excreted and the time at which detectable amounts appeared in the urine. However, a rise in level toward the end of pregnancy was noted.

11. Other large species - Borth and Watteville (1952) have reviewed oestrogen levels found in human pregnancy urine. Reviews of the literature indicate general agreement concerning oestrogen excretion from the mare, cow, sow, and goat (Cowie, 1948; Cole, 1950). For the human and cow, oestrogen levels increase gradually until parturition but for the mare maximum excretion of oestrogen occurred during the seventh and eighth months with a gradual decline till term. Zondek (1954) noted that urine of pregnant mares was a rich source of oestrogens.

b. Progestogens

i. The ewe - Meher and Zarrow (1954) determined the progestin concentration in blood during pregnancy and parturition. A definite pattern in the levels was indicated. A rise from 1 - 2 μ g. progestin/ml. of serum at oestrus to 6 μ g. was noted during the first 40 - 50 days. This level was maintained until 120 days of pregnancy. A second rise commenced at this period and reached a maximum of 8 - 12 μ g./ml. approximately 20 days prepartum. This level was apparent until about 30 minutes after parturition, when a sudden drop occurred.

Ovariectomy during pregnancy (between days 66 - 114) did

not appear to affect progestin levels. This suggested that pregnancy after day 66 was dependant upon an extra-ovarian source of progestin. This was substantiated when all ewes produced live lambs. Similarly Casida and Warwick (1945) obtained normal gestation in two ewes which had the corpus luteum removed on the 55th and 64th day of pregnancy. It was notable that surgery was performed during the first plateau period of progestin level (Neher and Zarrow 1954) and pregnancy was successful. It was suggested that the placenta takes over the dominant role of progestin production before the 66th day. Casida and Warriek could not however maintain pregnancy in one ewe in which the corpus luteum was removed on the 30th day.

Pregnanediol was not found as an excretion product of the Merino ewe during pregnancy (Beck, 1950).

Edgar (1953) found progesterone (0.5-2.0 μ g./ml. blood), by a chemical technique, in ovarian vein blood. The levels were much lower than those found by Neher and Zarrow (1954) using the Hocker-Forbes bioassay. No progesterone was detected in peripheral blood during pregnancy and the author suggested the hormone to be physiologically active at concentrations less than 0.1 μ g./ml. blood.

ii. Other large species - Corpus luteum ablation and progesterone therapy has been used as an indirect measure of ovarian progesterone secretion. With heifers, Raesside and Turner (1950), and Uren and Raesside (1951) found that 25, 50 and 75 mg. progesterone daily was not sufficient to maintain

pregnancy when the corpus luteum was removed 44-76 days after conception. Similar studies (McDonald et al., 1952) indicated that 100 mg. progesterone would allow pregnancy to continue.

With goats, Meites et al. (1951) found that complete removal of all corpora lutea at 100 or 125 days resulted in abortion, but when only one of two corpora present were ablated, gestation was maintained. Injections of 15 mg. progesterone daily allowed maintenance of pregnancy, but 10 mg. appeared marginal.

Melinkoff (1950) has reported maintenance of pregnancy in women, without progesterone therapy even when ovariectomy was performed as early as the 41st day. Similar results have been noted with monkeys (Hartmann and Corner, 1947).

Watteville (1951) and many others have determined the levels of urinary pregnanediol during the human menstrual cycle. Maximum pregnanediol excretion occurred during the last two months of pregnancy. Values of 50 mg. pregnanediol per 24 hours were noted at this time.

Cole (1950) reviewed pregnanediol excretion from domestic animals during pregnancy. Difficulties associated with methods of extraction and estimation have been noted. Pregnanediol has been detected in the urine of the cow (Marker, 1938), mare (Marker et al., 1937) and rhesus monkey (Boscott, 1952). Glasgow and Mayer (1953) found that the low concentrations of urinary metabolites of progesterone present during early gestation were followed by a progressive increase until shortly before parturition. Further, there was a significant correlation

TABLE 2

Progesterone content of body fluids of pregnant animals determined by various methods

Source	Method	Species	Stage of reproduction	Body fluid	Concentration progesterone (ug./m)
Bloch (1936)	Pregestational response of rabbit uterus (Corner & Allen, 1929)	Women	Pregnant	Serum	2.0
Haskins (1941)	Pregestational response of immature rabbit uterus (McGinty et al., 1939)	1 Woman	Pregnant	Serum	0.13
		19 Women	Pregnant	Serum	0.1
Hoffman & von Lam (1948)	ditto	Woman	Pregnant	Blood	0.1
Haskins (1950)	Ultraviolet adsorption of extracts (Reynolds & Ginsburg, 1942)	Women	Pregnant	Blood	0.1
Butt et al. (1951)	Partition chromatography and subsequent polarographic estimation (Butt et al., 1951)	13 Women	Pregnant	Blood	0.1
		1 Woman	Pregnant (with infective hepatitis)	Blood	0.4
Hooker & Forbes (1949)	Changes in stromal nuclei of mouse endometrium (Hooker & Forbes, 1947)	Women	8 weeks pregnant	Blood	4 - 8
Forbes (1951)	ditto	Monkey	Pregnant	Blood	3
Edgar (1953)	Partition chromatography (Bush, 1952) and subsequent ultraviolet absorption of extracts (Reynolds & Ginsburg, 1942)	Mare	111 days pregnant	Blood	0.1
			63 days pregnant	Blood	0.2
		Cow	8 weeks	Blood	0.1
			7 months	Blood	0.1
		Sow	66 days	Blood	0.4
Pearlman & Thomas (1953)	Countercurrent distribution (Pearlman & Cerceo, 1952) and Ultraviolet spectroscopy (Reynolds & Ginsburg, 1942)	Woman	Parturition	Placental blood	0.4
Salhanick et al. (1954)	Paper chromatography (Bush, 1951) and ultraviolet spectroscopy	Women	3-7 months pregnant	Plasma	0.06
Zander (1954)	ditto	Women	6 months pregnant	Blood	0.08
Raeside & Turner	ditto	Goat	Pregnant	Ovarian venous plasma	1.8, 2
		Goat	Pregnant	Uterine venous plasma	0.1
		4 Goats	Pregnant	Peripheral plasma	0.1
		2 Cows	Pregnant	Peripheral plasma	0.1

between the level of metabolites early in pregnancy and the number of embryos and corpora lutea. No pregnanediol was detected in the urine of the chimpanzee (Elder, 1941) and the goat (Boscott, 1952).

Faecal androgen of the cow has been investigated during various reproductive phases (Turner, 1947, 1948; Gassner, 1952; Miller and Turner, 1955). At least some of these faecal C19 steroids have been identified (Miller et al., 1956) and have been suggested as metabolites of progesterone. No attempt to utilize faecal androgen as an indicator of the level of progesterone (analogous to pregnanediol determinations) has so far been made.

Various methods for assay of progesterone in body fluids have already been noted. Table 2 lists some results obtained from pregnant individuals. The chemical methods of estimation suggest minute quantities in both ovarian and peripheral blood, but only Zander (1954) has detected progesterone in the latter source. The bioassay results show higher levels in peripheral blood. Forbes (1954) suggested that the differences in levels found by the Hooker-Forbes bioassay and the physico-chemical methods was owing to normal physiological variation between donors. Butt et al. (1951), Edgar (1953) and Forbes (1954) alternatively suggest that the bioassay measures progesterone along with other compounds.

III PRELIMINARY INVESTIGATIONS

A. Slaughter material

1. Materials and methods

a. Collection

Genital tracts from ewes slaughtered at Longburn Freezing Works were available during the period 5.x.55 to 13.iii.56. Mostly these were dry ewes although during the October collection, tracts from several in-lamb ewes were examined.

After slaughter the ovaries were collected and placed in buffered formalin (Lamond, 1955). The cervix was cut lengthwise (with a scalpel) to expose the lumen of the tract. Care was taken to avoid cutting prominent blood vessels, since it was thought and later confirmed that blood may influence arborization. Samples of cervical mucus were obtained by scraping the cervical epithelium with a small metal spatula. The mucus was spread on a microscope slide, which was labelled similar to the bottle containing the ovaries of that ewe.

b. Examination

Ovaries were examined 5-14 days after collection. Each ovary was sectioned vertically at 1-2 mm. intervals. Follicles and corpora lutea were measured and counted. Follicles of 5.0 mm. diameter and greater were counted

as large; those less than 5/0 mm. diameter were classed as small.

The slides were air-dried and later examined under low power (x140) using a binocular microscope. All slides were examined on the day of collection except those of 3.xi.55 which were inspected five days later. All slides were again examined several days later as a check. It was apparent that the arborization patterns had tended to fade by the time of this check. Smears were classified as positive or negative according to the descriptions of Bone (1954) and Zondek (1954).

2. Results and discussion

a. Non-pregnant ewes

The follicle counts (Appendix I) show that the ovary is relatively quiescent during deep anoestrus (October, November and December). This is in agreement with the findings of Cole and Miller (1935). However, they did report an increase in follicle number and changes in the vaginal smear, which was characteristic of the oestrous cycle, during early and late anoestrus. The comparative absence of corpora lutea also indicated the ovaries were reduced in activity.

The low incidence of arborization during the anoestrous period is apparent from Table 3.

TABLE 3

Slaughtered ewes - Arborization

Group	Date of Slaughter	Number of ewes	Number with positive smear	<u>Ewes with large follicles</u>	
				Number with positive smear	Number without positive smear
A	5.x.55	10	1	1	6
B	3.xi.55	22	0	0	12
C	6.xii.55	27	1	1	9
D	11.1.56	9	1	1	3
E	8.11.56	20	0	0	15
F	9.11.56	19	5	3	13
G	13.11.56	20	4	2	15

The macroscopic appearance of the follicles and the absence of corpora lutea may suggest a low oestrogen production, as the cause. The results also indicate that most ewes did not show arborization even when the ovaries contained large follicles. This was surprising since some follicles during February and March appeared to be within a few days of ovulation. It was later noted (Section C) that a temperature effect may have operated to influence arborization. This suggests that mucus from some of these ewes when air-dried showed a negative smear, but which under a higher temperature would have produced arborization.

Table 4 shows the state of the ovaries of ewes which produced a positive smear.

TABLE 4

Ovarian follicle counts of ewes showing arborization of cervical mucus

Date	Ewe Number	Follicle Counts		Notes
		Large Follicles	Small Follicles	
5.x.55	6A	1	15	
6.xii.55	12C	1	12	
11.1.56	5D	2	12	One large follicle in both ovaries.
9.11.56	4F	0	33	2 Corpora lutea forming, blood clot still present.
	7F	0	26	2 Corpora lutea forming.
	8F	1	17	
	16F	1	14	
	17F	3	13	
13.111.56	4G	0	16	1 Corpus luteum forming.
	12G	2	54	2 Corpora lutea in one ovary.
	15G	2	20	1 large corpus luteum.
	19G	0	15	2 Corpora lutea forming.

Walker (1943), Goot (1949) and Till (1950) have shown that the anoestrous period for the North Island Romney ewe occurs from mid-August until early March, thus it is likely that ewes 6A, 12C and 5D were in deep anoestrous when arborization was detected. This may suggest that the ovary undergoes some activity during anoestrus. However, the graafian follicle although functional, possibly never attains sufficient size for ovulation to occur. If the presence of cervical mucus arborization in ewes which had one or more large follicles, is dependent upon the output of

oestrogen from the ovary, then it is likely that in ewes where arborization failed, the large follicles had not reached a sufficient level of activity.

Ewes 4F, 7F, 4G, and 19G slaughtered near or during the early breeding season each had forming corpora lutea but no large follicles. This may indicate that the effect of follicular oestrogen persisted for a short period after ovulation. At this time, the inhibitory effect of progesterone on pattern formation may not have been sufficient to counteract the stimulating effect of oestrogen (Zondek, 1954).

For ewes 12G and 15G, arborization may be explained by suggesting that the corpora lutea were in the late luteal phase with possibly reduced progesterone output; while the developing large follicles produced sufficient oestrogen to cause arborization.

b. Pregnant ewes

Three animals were examined. All the resulting smears were negative and thus were in agreement with the results found for the pregnant human and pregnant cow.

B. Live animals

1. Materials and methods

The types of ewes available for study are given

in Table 5. The surgical operations were performed on 16.xi.55.

T A B L E 5

Experimental ewes - previous surgery

Ewe	Tag numbers		Condition
91.49	18.53	25.53	Bilateral ovariectomy
58.48	97.49	161.50	Bilateral ovariectomy
7.50	29.51	229.52	Hysterectomy
35.49	113.49	17.53	Normal: non-pregnant
1	2	3	Normal: 4 months pregnant

Cervical smears were taken daily from all non-pregnant ewes for three weeks during December, 1955. Smears from the pregnant animals were collected each third day over a period of twelve days during the fourth month of pregnancy (July, 1956). The ewe was held in dorsal recumbency by an assistant. A perspex speculum (2 cm. diameter) lubricated with an antiseptic cream, Propamidine,* was inserted into the vagina and the cervix located. An artificial light source was sometimes required but usually daylight was sufficient. Mucus was collected on a 0.6 cm. diameter glass rod, bent slightly at one end to facilitate entry into the lumen of the cervix. This method was found more convenient than collection with either

*May and Baker Ltd., Dagenham, England.

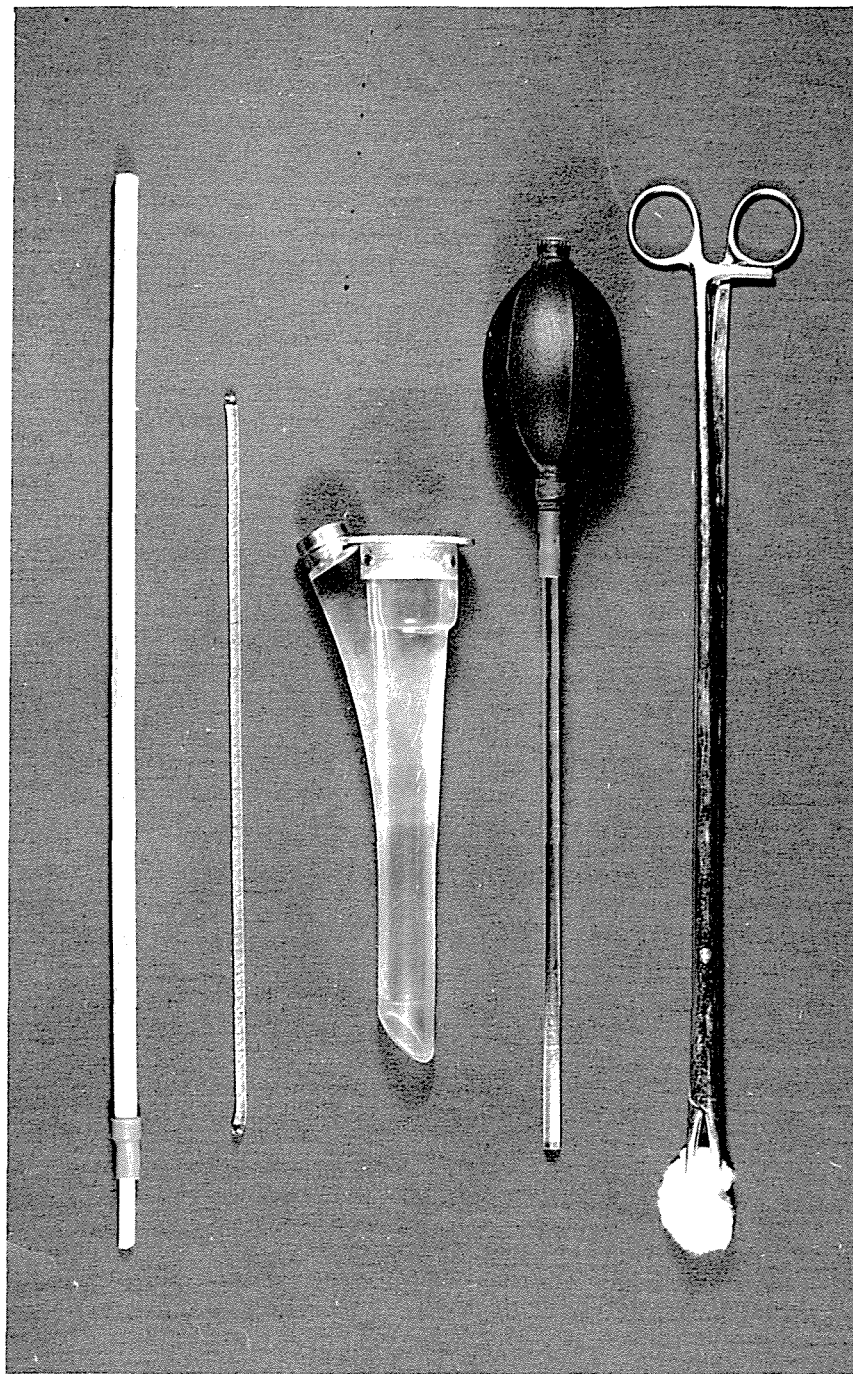
a cotton wool swab, a small metal scraper held on a wooden applicator, or by a glass aspirator. These instruments are shown in Figure 1. In most non-pregnant animals the cervix appeared tightly closed and entry into the canal was difficult. In these cases it was not possible to insert the rod more than approximately a half inch into the cervix. In the pregnant ewes the cervix appeared distended to some extent. The rod was carefully withdrawn and the mucus spread on a microscope slide.

The smears from non-pregnant ewes were air-dried while those from pregnant animals were dried at $37-40^{\circ}\text{C}$ in an oven. All slides were examined under low power on the same day and checked several days later.

2. Results and discussion

A period of three weeks was considered sufficient for mucus collection from the non-pregnant animals, since it allowed any ewes experiencing normal oestrous cycles (14-19 days for the New Zealand Romney: Dry, 1933; Gill, 1933; Walker, 1943; Goot, 1949; Till, 1950; Inkster, 1953) to have at least one opportunity for ovulation. It has been shown previously in other species that arborization normally occurred near the time of ovulation.

All smears were classed as negative. No literature has been reported for species experiencing an anoestrous period, however, the negative cervical mucus from those ewes with ovaries intact was in agreement with the finding



for the slaughter material. The possibility of a temperature effect influencing arborization must be considered, nevertheless recent work (Reeside and McDonald, unpublished data, 1957) has been in agreement with the absence of arborization found in this study. These results for castrate and pregnant ewes were in accordance with the findings already referred to for castrate and pregnant women, and pregnant cows.

C. Temperature

The literature did not indicate temperature to be a factor likely to influence arborization. For other species mucus dried at air temperature was capable of arborization. During these investigations mucus from the ewe also showed the PL reaction when air-dried. Temperature was first noted to be of importance on 17.iv.56 when the genital tract of a ewe in oestrus that day was studied.

1. Experimental procedure

A cervical smear was air-dried and examined two and one half hours after slaughter. The mucus showed no arborization. The slide was left on the microscope stage with the direct source of illumination operating for approximately 15 minutes. When viewed again a PL pattern was seen to be forming. It appeared that the

heat produced by the light beneath the microscope stage promoted arborization. When the slide was left to cool, arborization disappeared. Other smears from this ewe, showed similar changes; these results are given later in Table 6.

Subsequent studies were conducted using mucus from ewes exhibiting oestrus or from oestrogen treated animals.

2. Results and discussion

The findings presented in Table 6 and Appendix II indicated that temperature was a factor affecting the arborization process. This was noted in all the animals examined and not merely in specific ewes. Further it was apparent that atmospheric temperature could affect the process to such an extent that PL formation would not occur. Thus during the winter months, artificial heating of slides was necessary. During summer it is thought that the higher air temperatures prevailing allowed arborization.

In later studies it was necessary to eliminate the effect of atmospheric temperature on the results obtained for cervical mucus arborization. Consequently it was decided to dry all smears in an oven for 10-15 minutes, at 37-40 °C. It is felt from the results obtained that the procedure was justified.

D. Contamination

Zondek (1954) and Bone (1954) emphasised the

TABLE 6

Influence of temperature on cervical mucus arborization*

Date	Slide Number	Treatment to slide	Examination of mucus Air-dried	Heated**	Duration of pattern (hrs)	Smear after further heating***
17.iv.56	1	Air-dried, later heated	Negative	Positive	5	Positive
18.iv.56	2	Air-dried, later heated	Negative	Positive	12	Positive
18.iv.56	3	Heated		Positive	76	Positive
19.iv.56	4	Heated		Positive	74	Positive

* Ewe 80H tugged 8 a.m., 17.iv.56;
slaughtered 2.30 p.m., 17.iv.56.

** Heated on microscope stage for 15 minutes (Slide 1),
or in oven (40 °C) for 10-15 minutes (Slides 2, 3 and 4).

*** Slide reheated 1 day later.

importance of contamination of cervical mucus in influencing arborization and thus causing erroneous results. In the present study contamination by certain substances was also likely. It was thought necessary to check this possibility. The effect of salt solution, perspiration, blood, the anti-septic cream used for lubricating the speculum, and seminal plasma were investigated. The steps taken to avoid contamination are detailed.

1. Experimental procedure

a. Salt solution and perspiration

Cervical mucus obtained from slaughtered and live ewes was spread on slides previously rubbed with human perspiration, or to which a drop of 0.9 per cent sodium chloride solution was added. An uncontaminated smear from each animal was also taken.

b. Blood

Cervical mucus for three smears from each of five oestrous ewes was collected. Before the mucus had dried a drop of blood, obtained from the jugular vein of a vasectomised ram was added. Another smear from each animal acted as the control. The smears were dried at 37-40 °C and examined the same day.

On 13 occasions during the investigations to be reported later, cervical mucus was contaminated with

blood, owing to injury to the cervix wall during sampling. These smears also were dried and examined as described.

c. Lubricant cream

The five ewes above were used as a source of cervical mucus. Propamidine was smeared on the slides (three slides per ewe) prior to adding the mucus. These and the corresponding control slides were dried and examined as before.

d. Seminal plasma

This material was obtained from a vasectomised ram using the artificial vagina detailed by Webster (1939 a, b). Difficulty was experienced in getting the ram to serve most ewes on which the artificial vagina was fitted. Three cervical mucus smears from each of five ewes were collected and a small amount of seminal plasma added to each. Examinations was as before.

The effect of seminal plasma contamination in vivo was also investigated. Smears of vaginal and cervical mucus taken from five oestrous ewes before and after service by a vasectomised ram were examined.

e. Vaginal mucus

The difficulty of getting mucus from within the cervix has been mentioned. It was also found that mucus obtained from the cervix as often contained cellular

material which could have been of vaginal origin. The site of collection of mucus was examined in vivo in five oestrous ewes prior to service. At this time mucus from the anterior portion of the vagina as well as the cervix was studied. Further, the effect of vaginal contamination was examined in vitro. In this case cellular fluid from the vagina was added to the cervical mucus before it dried.

2. Results and discussion

a. Salt solution and perspiration

The marked effects of contamination from this source are shown in Table 7.

TABLE 7

Effect of sodium chloride and perspiration on arborization

Source of mucus	Number of ewes	Number with arborization*	
		After salt solution added	After perspiration added
Slaughtered - normal	15	14	
Live - ovariectomised	3	3	3
Live - ovari hysterectomised	3	3	3
Live			
i. 7 days post oestrus	1	1	
ii. 12 days post oestrus	1	1	
iii. pregnant 4 months	3	3	

*All control slides showed no arborization.

Bone (1954) noted that washing instruments and

slides in soapy water could introduce contamination to the cervical mucus. In this study the following procedure was adopted for cleaning equipment:

glass speculum and glass rod

- i. Cleaned in hot tap water
- ii. Air-dried

microscope slides

- i. cleaned in hot tap water containing a detergent (Teepol)
- ii. rinsed in cold distilled water
- iii. air-dried

Care was also taken to avoid handling the surface of the slide on which was to be spread the mucus.

b. Blood

With one exception all smears contaminated with blood either in vitro or in vivo showed no arborization. This was similar to the results obtained by Zondek (1954) with human cervical mucus. In subsequent studies it was found that any blood contamination of the mucus could easily be seen under the microscope. This was a complicating factor, since it was impossible to classify these smears correctly. It was thus decided to disregard all blood contaminated mucus smears.

c. Lubricant cream

Inhibition of arborization occurred on all slides

examined. The difficulty in observing a fine film of Propamidine cream on the slides, prevented the possibility of excluding any slides so contaminated.

d. Seminal plasma

Cervical mucus to which seminal plasma had been added in vitro, showed no arborization. Zondek (1954) recorded similar results with the human and suggested a "mechanical effect" to be the cause. In later studies, it was necessary to collect mucus from some ewes that had been served by vasectomised rams. The necessity of investigating the effect of seminal plasma on mucus collected after service was apparent. The results for the examination of mucus from the five oestrous ewes are presented in Table 8.

TABLE 8

Arborization of cervical and "vaginal" mucus collected before and after ram service

Ewe number	A r b o r i z a t i o n*			
	Mucus before service		Mucus after service	
	Cervical	Vaginal	Cervical	Vaginal
91.49	6	3	6	1
97.49	6	2	5	2
58.48	6	0	6	0
25.53	6	3	6	1
35.49	6	4	5	0
Total	30	12	28	4

*For each ewe 6 cervical and 6 vaginal smears were collected before and after service.

The probability that the observed differences were

due to chance was calculated in each of the following 2 x 2 contingency tables using Fisher's exact method (Goulden, 1952, pp 372 et seq.)

Mucus before Service				Mucus after service			
	Cervical	Vaginal	Total	Cervical	Vaginal	Total	
+	30	12	42	28	4	32	
-	0	18	18	2	26	28	
Total	30	30	60	30	30	60	

Cervical Mucus				Vaginal Mucus			
	Before Service	After service		Before Service	After Service		
+	30	28	58	12	4	16	
-	0	2	2	18	26	44	
Total	30	30	60	30	30	60	

Cervical mucus v vaginal mucus, before service: $P < .0001$
 Cervical mucus v vaginal mucus, after service: $P < .0001$
 Cervical mucus v cervical mucus, before and
 after service: $p = .2459$
 Vaginal mucus v vaginal mucus, before and
 after service: $p = .0159$

Mucus was collected 3-4 hours after service. This time was considered sufficient to allow the seminal plasma to come in contact with the mucus of the vagina and cervix. Further, this period was comparable to the time of collection

of mucus after service in the studies to be reported presently.

In most cases, before and after service, provided the mucus was collected from the cervical canal arborization was found. Less arborization of vaginal mucus occurred after service than before service. Since this was unlikely to be due to chance, it suggested that seminal plasma and mucus had come in contact in the vagina. Vaginal contamination in vitro caused complete inhibition of arborization. Mucus collected from the cervix showed arborization after service. It appeared likely that the seminal plasma did not penetrate into the cervical canal in/insufficient concentration to inhibit the PL reaction. Thus, for the rest of the investigations, mucus from the cervix even after service by a vasectomised ram was regarded as "uncontaminated" with seminal plasma.

c. Vaginal mucus

The results from Table 8 indicate that the lower incidence of arborization in vaginal mucus compared with cervical mucus was unlikely to be due to chance. Also, arborization of cervical mucus was completely inhibited by vaginal mucus in vitro, provided the cellular material was thoroughly mixed with the cervical secretion prior to drying.

The problem in collecting mucus from the live ewe

was to remove vaginal secretion from the opening to the cervix. A cotton wool swab applied with a pair of forceps was usually satisfactory, except at metoestrus. At this time, contamination was likely.

III FURTHER INVESTIGATIONS

A. Arborization during the oestrous cycle in the ewe

1. Experimental procedure

a. The animals

Twenty New Zealand Romney ewes from the College flocks were available for observation. These were mostly culled for age but some of the younger sheep had been removed for low production. The three hysterectomised ewes previously described were also included in this study, since it was thought at the time that they also would experience cyclic ovarian activity during the breeding season. Observations were initially made on the whole flock, but as some of the ewes were slaughtered for another reproductive study, there was a gradual reduction in the numbers studied. Two vasectomised Romney rams were run with the ewes.

b. Management

The animals were run as one flock during 1956 from 22nd March until 11th July. Access to a grass-legume sward was available, except from the 26th March until 8th April when the animals were kept inside a shed and fed only hay as a precaution against facial eczema (Levy and Smallfield, 1942; McMeekan, 1956). Periodic crutching and footrotting were carried out when necessary. Both rams were raddled freely on the

brisket with coloured tuppung paste daily.

c. Observation of oestrus

The flock was yarded twice daily (7.30 a.m. - 8.30 a.m. and 5 p.m. - 6 p.m.) and checked for tuppung marks. Only ewes showing obvious signs of oestrus were recorded as being on heat. Occasionally a ewe with a light mark was placed in a small yard with a ram and further observations made. No attempt was made to record the intensity and duration of oestrus in any of the ewes.

d. Collection and examination of cervical mucus

Usually one or more cervical mucus smears were taken from the normal ewes once daily (8 a.m. - 9.30 a.m.). Some animals were examined in the afternoon as well (4.30 p.m. - 5.30 p.m.). The hysterectomised ewes were examined at less frequent intervals.

A description of the mucus seen in the vagina and near the cervix os was recorded. The method of collecting the mucus has been described above. The speculum was difficult to insert and mucus hard to obtain, uncontaminated with vaginal material, from two to four days after oestrus. For this reason a number of observations made at this period had to be disregarded.

All cervical mucus smears were examined on the day of collection and at first descriptions recorded. Later

gradings were given to the amount of arborization present. The following system was adopted from Zondek et al. (1955).

- No pattern formation (figure 2)
- + pattern formation faint; individual patterns scattered (figure 3)
- ++ pattern formation faint - prominent individual patterns concentrated (figure 4)
- +++ pattern formation prominent; individual patterns cover all of slide (figure 5).

Until the temperature effect was noted, all slides were air-dried, but subsequently they were dried in an oven (37-40 °C). There is the possibility that temperature may affect the subjective grading system. For ease of presentation of data, the results from the mucus examination are presented as either positive (+) or negative (-). However an attempt to utilize the grading system has been made with the results for the hysterectomised ewes when contamination of mucus was apparent it was noted and the result deleted from the calculations.

e. Examination of ovaries

As some of the normal animals were slaughtered for another study by this Department, the ovaries were available for examination. In addition one hysterectomised ewe was killed. The ovaries were sectioned and the follicles and corpora lutea counted and measured. Sections cut through



Fig. 2. Negative cervical mucus

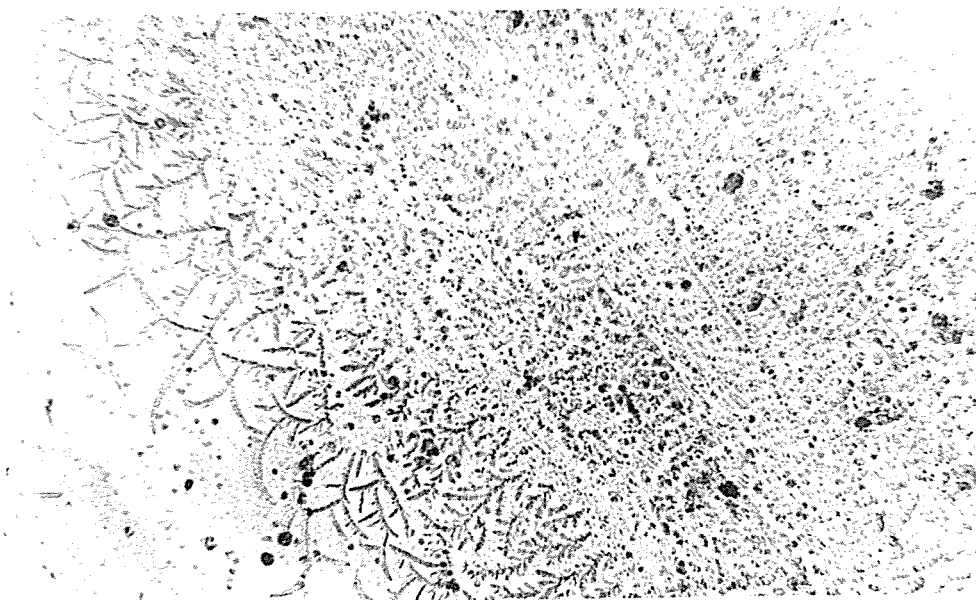


Fig. 3. + Cervical mucus

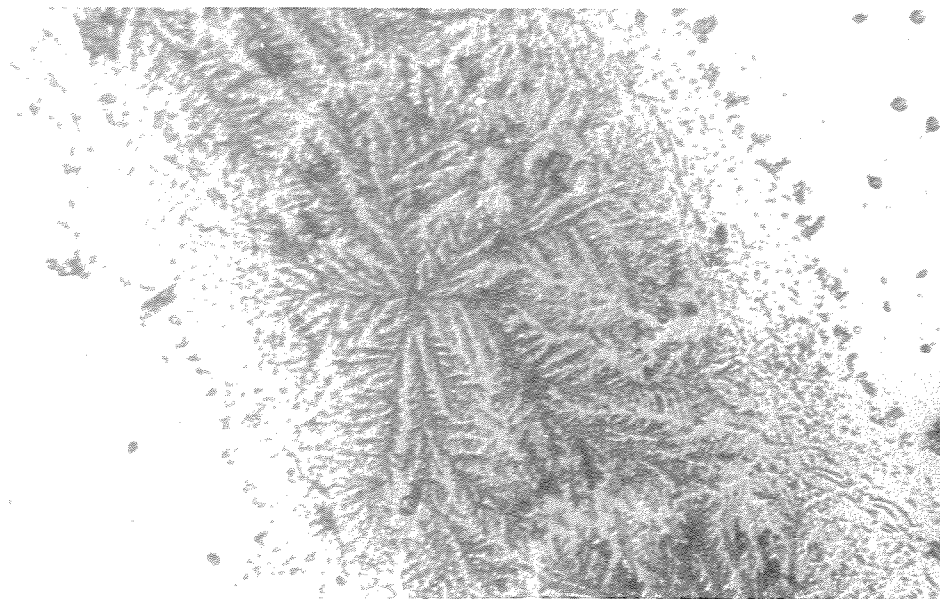


Fig. 4. ++ Cervical mucus



Fig. 5 +++ Cervical mucus

recent corpora lutea were prepared for histological study, using the technique detailed by Lamond (1955).

2. Results and discussion

a. Normal ewes

1. Oestrous cycles - 77.8 per cent of 63 oestrous periods commenced during the night and early morning (6 p.m. - 7.30 a.m.). McKenzie and Terrill (1937) noted the onset of oestrus to be fairly evenly distributed throughout the six hour intervals at which they made their observations. In this study the observations of oestrus were made at unequal intervals during each 24 hours, (the night interval was $14\frac{1}{2}$ hours while the day interval was only $9\frac{1}{2}$ hours). Thus it would appear likely that more ewes should be marked during the night period, if the animals were to come on heat regularly throughout the 24 hours. The findings of this study are somewhat similar to those of Kelley (1937) who found that in Merino ewes 65 per cent of the oestrous periods commenced between 5 p.m. and 7 a.m. They do not agree with the results obtained with Dorset Horn ewes (Kelley, 1937) or with those of Hafez (1952). The latter, with several other breeds including the Romney Marsh, found that up to 64 per cent of the animals came into heat during the day (6 a.m. - 6 p.m.). Inkster (1953) concluded that for New Zealand Romney and Romney-Cheviot halfbred ewes, the peak incidence of onset of oestrus each day occurred during the early part of the daylight hours (7 a.m. - 3 p.m.).

The lack of agreement between various workers suggests that to accurately record the time of onset of oestrus, observations at shorter intervals may be necessary.

The distribution of the lengths of 43 oestrous cycles is presented graphically in figure 6 and numerically in Table 9. Within the normal range of 14-19 days (McKenzie and Terrill, 1937), 86 per cent of the observations occurred. This agrees closely with the findings of Goot (1949), Till (1950) and Inkster (1953). They noted that 93, 93 and 89 per cent respectively, of all oestrous cycles of Romney ewes fell within that range.

The mean length of the 37 normal cycles was 16.0 days, which although less than that found by other workers with the New Zealand Romney ewe, is still close considering the small number of observations. Further, the modal duration of all cycles was 17 days. The next greatest frequency was 16 days which is similar to the results of Till (1950).

It was apparent that the results were fairly similar to those of previous work reported for the Romney ewe. Hence, it was considered that the animals observed in this part of the investigation showed normal reproductive behaviour. Therefore it was felt that these ewes could serve as typical subjects for further reproductive studies involving the use of the cervical mucus smear.

11. Arborization phenomenon - In table 10 and figure 7 & Appn. III are presented the results for the examination of cervical

T A B L E 9

The length of the oestrous cycle

Days	5½	11	14	15	16	16½	17	17½	18	19	19½	20	35	36	38
n	1	1	1	3	8	4	10	5	3	1	2	1	1	1	1
%	2.3	2.3	2.3	6.9	18.6	9.3	23.3	11.6	6.9	2.3	4.6	2.3	2.3	2.3	2.3

FIG 6 THE LENGTH OF THE OESTROUS CYCLE

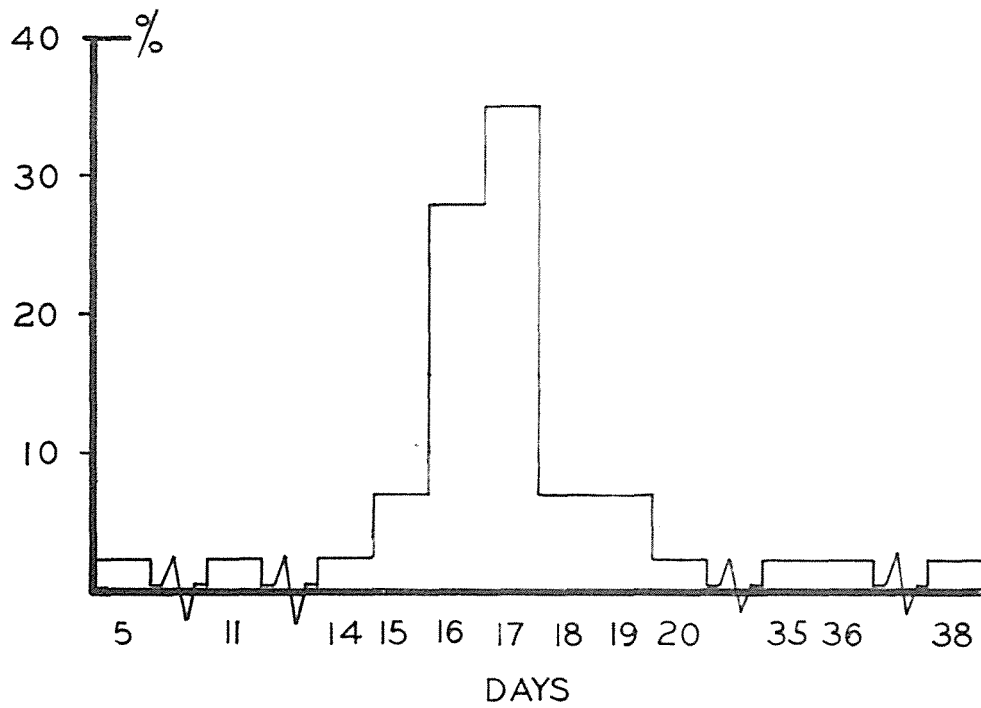
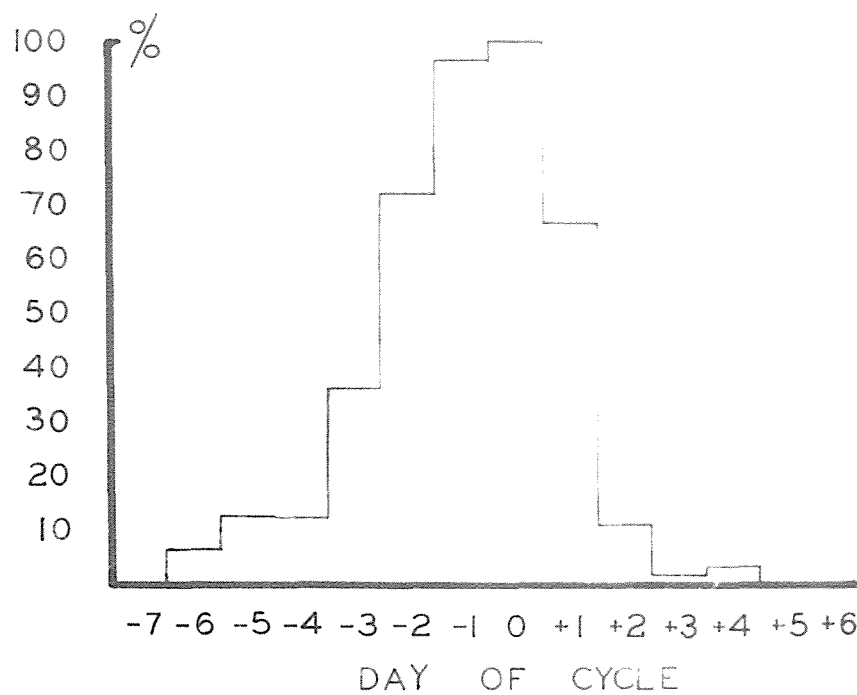


TABLE 10

Duration of arborization — normal oestrous cycles

Day of cycle	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7
n	46	48	49	50	50	53	53	70	65	63	67	65	49	44	40
No +ve	0	3	6	6	18	38	51	70	43	7	1	2	0	0	0
%	0	6.3	12.2	12.0	36.0	71.7	96.2	100.0	66.2	11.1	1.5	3.1	0	0	0

FIG 7 ARBORIZATION DURING OESTRUS CYCLE



mucus smears collected on each day of the oestrous cycle. Day 0 corresponds with the day the ewe was first noticed on heat.

Contamination of cervical mucus by cellular material from the vagina occurred predominantly 2-4 days post oestrus (metoestrus). At this time the vagina appeared extremely dry. It was also difficult to insert the speculum and often impossible to remove most of the cheesy vaginal secretion from near the cervical os. Darlow and Hawkins (1931), Cole and Miller (1931, 1935), McKenzie and Terrill (1937) and Inkster (1953) have also reported this typical metoestrous condition in the vagina. Consequently some of the observations made for arborization during this period were negative but contaminated. These recordings had to be disregarded.

The average duration of arborization for normal cycles was 4.5 days. However, as mucus was collected only once daily on many days more exact measurement was impossible. Further, contamination during metoestrus reduced the likelihood of detecting arborization over a larger period. Garm and Skjerven (1952) found arborization to persist for 14 days in the cow. This occupies a larger proportion of the length of the 24 day cycle of the mature bovine (Asdell, 1946) than does 4.5 days of arborization in the 16.0 day cycle of the ewe. Similarly arborization for 15-17 days was a large proportion of the human menstrual cycle (Holand, 1952).

McKenzie and Terrill (1937) found ovulation to occur 24-36 hours after the commencement of oestrus. This suggested

that PL formation during the follicular stage must last for 3-4 days. Consequently arborization during the early luteal phase must be of short duration. This result may have been owing to the difficulty of obtaining mucus free of vaginal contamination, during metoestrus. The possibility that hormonal influences prevented arborization is also likely.

The relationship of arborization with the time of ovulation indicates that species differences are likely. The findings of Roland (1952) and Urdan and Kurzon (1955) suggest arborization to occur for several days before and after ovulation. Bone (1954) found that PL formation was of greater duration after oestrus than before oestrus.

If the duration of arborization has not been influenced markedly by vaginal contamination during metoestrus then in the ewe, arborization occurs mainly prior to ovulation.

Menstruation in the human clearly divides the period of PL inhibition. During the premenstruum progesterone is the main factor inhibiting arborization, while its absence in the postmenstruum may be due to the low level of oestrogen (Zondek, 1954). In the ewe PL inhibition during the luteal phase cannot be so clearly defined. It may be that during most of the active life of the corpus luteum, progesterone is largely responsible. Edgar (1953, 1954) and, Edgar and Ronaldson (1957) have shown that progesterone is maintained at a high level until the day before oestrus. Negative mucus during the early follicular phase may well be due

to progesterone, and the low level of oestrogen from the developing follicles. However, during the early development of the corpus luteum the progesterone level is low (Edgar, 1953) but also oestrogen in the body may be approaching a low level following the rupture of the follicle (Brown, 1955). In addition endogenous oestrogen may be inactivated quite rapidly. Thus, arborization in the ewe may not persist long after ovulation has occurred and presumably the production of oestrogen markedly decreased.

Seven cycles (15.91 per cent) were outside the normal range of 14-19 days (Table 11).

TABLE 11

Classification of abnormal oestrous cycles

Ewe	Cycle length (days)	Classification (after Hafez, 1952)	Remarks
19	5½	Short single cycle	
17	20	Long single cycle	
23	11	Short single cycle	
27	35	Double multiple cycle	Silent heat
	36½	Double multiple cycle	Silent heat
12	31	Double multiple cycle	Silent heat
10	21+	Incomplete cycle	Silent heat

Roux (1936) considered that abnormal cycles were owing to ovarian dysfunction. McKenzie and Terrill (1937) suggested that cycles 3-6 days and 19-23 days in length were due to failure of ovulation and subsequent luteinization;

these slightly shorter than the normal length were due to early corpus luteum regression. Many other cycles were obviously multiples of the normal cycle and in these ovulation without heat (silent heat) had occurred.

Arborization was detected near oestrus in all abnormal cycles. In the case of the three single cycles, no other information was available to indicate what ovarian dysfunction may have occurred, but the suggestions detailed by McKenzie and Terrill (1937) would apply. The three remaining cycles indicate that silent heats do occur during the breeding season. In each case, arborization was detected over the period when the ewe was expected to exhibit oestrus. The duration of PL formation at this time also indicated ovarian activity similar to the ewe normally in oestrus. Additional information of silent heats occurring was obtained from ewe 10, which was slaughtered 21 days after the last oestrus, and the ovaries collected. These were sectioned; the results are presented in Table 12.

TABLE 12

Macroscopic appearance of ovaries - Ewe 10

Ovary	Corpora lutea	Follicle size		
		5m.m.	5-2 m.m.	2 m.m.
1	1 = 3 m.m.	1 (8 m.m.)	5	8
2	2 = 2 m.m.	-	5	9
	1 = 6 m.m.			

It was obvious macroscopically that the large

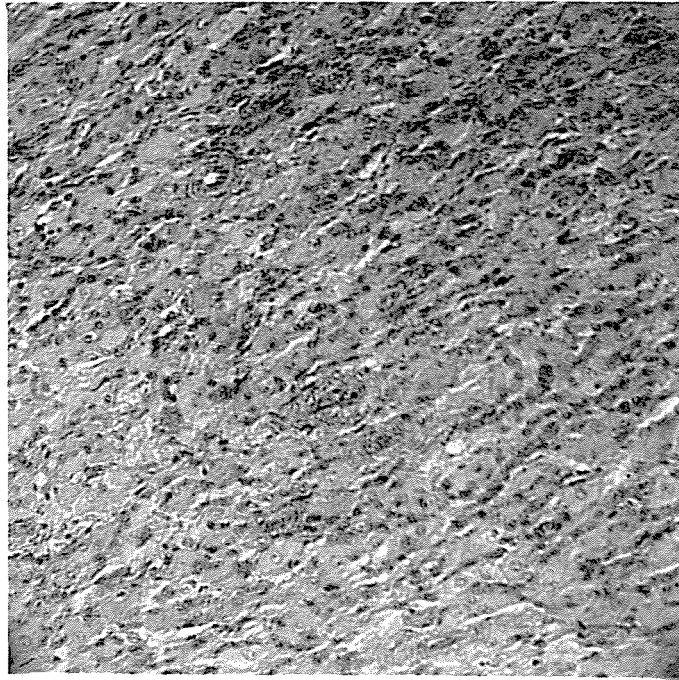


Fig. 8. 4-6 day corpus luteum (x190)

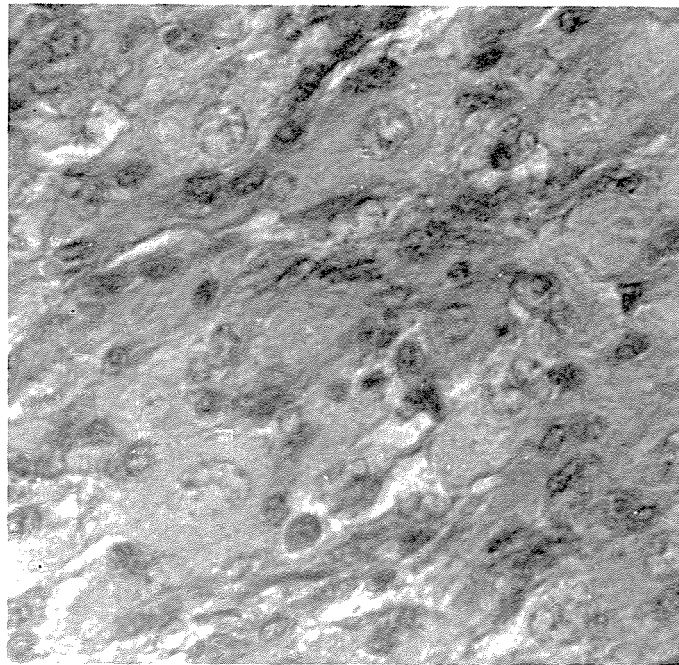


Fig. 9. 4-6 day corpus luteum (x800)



Fig. 10. Regressing corpus luteum (x190)

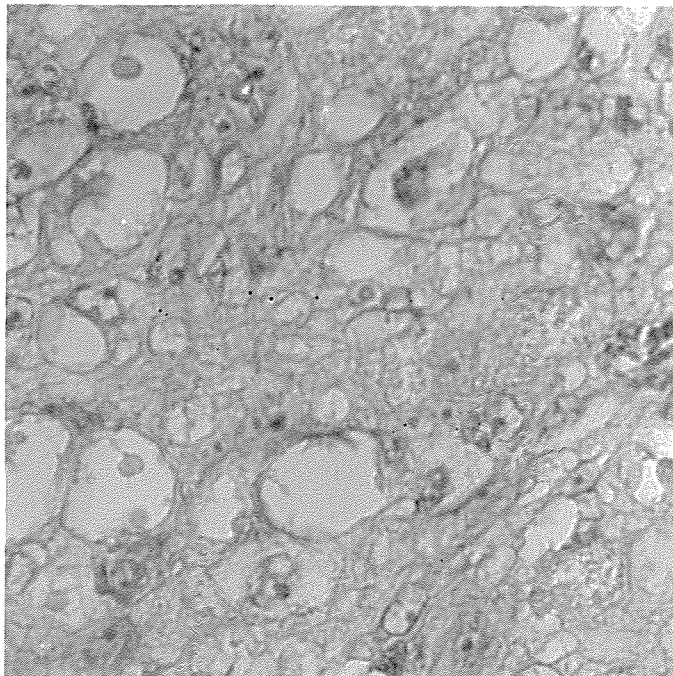


Fig. 11. Regressing corpus luteum (x800)

corpus luteum was the most recent. Histological examination of this corpus luteum (figures 8 and 9) when compared with the work of Warbritton (1934) suggested that ovulation had occurred 4-6 days before slaughter. This was about the time when ovulation, without heat, was indicated by the cervical mucus. It was also approximately 16 days after an overt heat. Examination of the corpus luteum in the other ovary (figure 10 and 11) suggested it was undergoing regression.

b. Hysterectomised ewes

None or little arborization of cervical mucus was shown by the animals during deep anoestrus (December, 1955) and up to the 8th March, 1956. It was apparent that these animals were not undergoing ovarian activity sufficient to promote arborization of cervical mucus.

In an attempt to induce and synchronize oestrus in these animals, 10 m.g. progesterone in arachis oil was injected daily (8th - 22nd March) into the shoulder muscle. Dutt and Casida (1948) and O'Mary et al. (1950) obtained satisfactory results with normal ewes using a similar progesterone level. In the present work three intact ewes similarly treated were tupped within three days of the last injection (Appn. III) but no oestrus was noted in the hysterectomised sheep. It is worth recording that oestrus was not detected in any of the hysterectomised ewes during the breeding season.

No PL formation was detected during the period of

progesterone treatment. This was in agreement with the findings of Zondek (1954) with the human. However, in all animals, arborization was recorded 1-3 days after the end of the treatment period. The duration of the PL reaction was similar in both entire and hysterectomised ewes (2-3 days). Negative mucus occurred after this period.

Wiltbank and Casida (1956) found that hysterectomy caused the prolongation of the life of the corpus luteum. The finding of negative cervical mucus may have indicated a similar phenomenon occurring in the present work. Thus the PL reaction after progesterone treatment suggested ovulation without heat. With the subsequent development of the corpus luteum, negative mucus followed. This persisted for a period considerably longer than that found during a normal cycle. However, positive mucus reappeared sometime between the 23rd May and 26th June, and persisted until the end of these investigations. The results for the examination of cervical mucus are presented in Appendix IV.

Only one ovary was recovered from ewe 229.52, slaughtered on 27th August. A large corpus luteum occupied most of the ovary. Figures 12 and 13 show sections cut through this corpus luteum. Comparisons with luteal tissue of the ewe and other species (Robinson, 1950; Brambell, 1956) showed that the luteal cells were well developed. There were some indications of atrophic changes. Less vascularization and more connective tissue was apparent than that shown in a mature corpus luteum by Robinson (1950). It is suggested

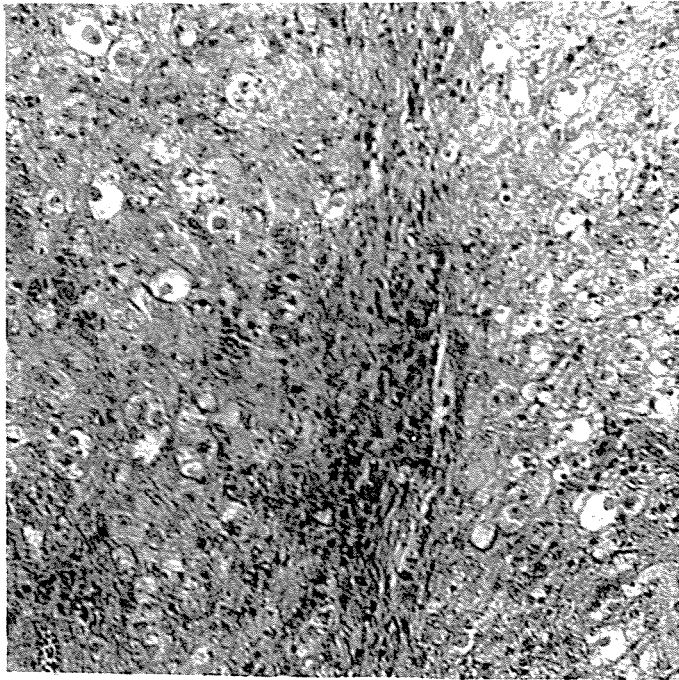


Fig. 12. Corpus luteum - hysterectomised ewe (x190)

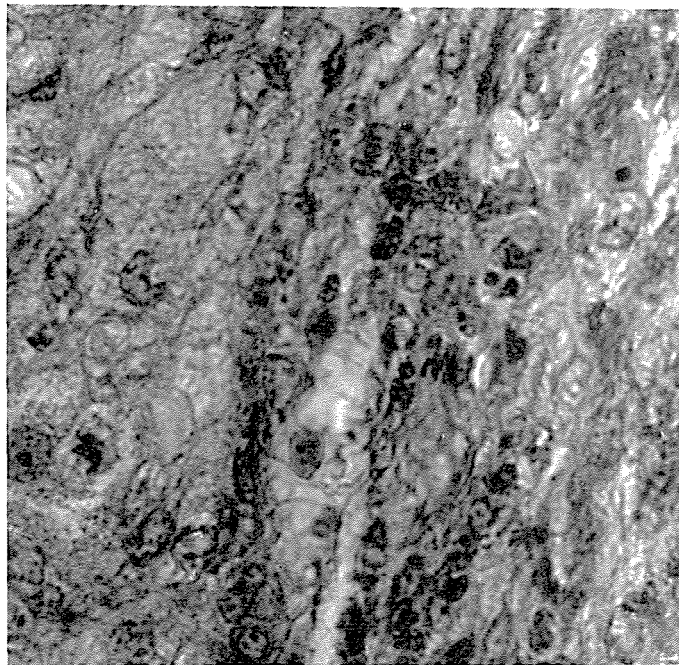


Fig. 13. Corpus luteum - hysterectomised ewe (x800)

that the corpus luteum at slaughter may have undergone at least some regression.

Wiltbank and Casida (1956) showed in hysterectomised animals that corpora lutea were maintained as anatomical structures beyond the time of normal regression and were probably functional but at a different level to those of the intact ewe. The findings of this study may be in agreement with the results and suggestions of Wiltbank and Casida. Thus, the histological changes showed a fully developed but perhaps regressing corpus luteum. Failure to detect subsequent oestrus indicated that the corpus luteum was functional. The negative cervical mucus may have been a reflection that the organ was fully functional when first formed. When arborization was detected some time later it suggested a possible change in the endocrine activity of the corpus luteum had occurred.

B. Injection studies with spayed ewes

1. Experimental procedure

a. The hormones used

i. Progesterone - Crystalline, pure progesterone B.P. (batch number A 1190 and A 1194) obtained from Organon Laboratories Limited, was dissolved in arachis oil and made up to a concentration of 10 mg. per ml. of oil.

ii. Oestradiol benzoate (ODB) - This material (Organon batch number A 1031) was made at concentrations varying

from 5 ug. - 200 ug. per ml. of arachis oil.

b. Times of hormone injections

In the series of trials to be described presently the hormones were injected at the following times:-

i. Progesterone

Twice daily doses: 7.30a.m. - 8.30a.m., and 4.30p.m. - 5.30p.m.
Once daily doses: 7.30a.m. - 8.30a.m.

ii. Oestradiol benzoate

Given alone:	7.30 - 8.30 a.m.
Given simultaneously with progesterone:	7.30 - 8.30 a.m.
Given 24 hours after progesterone :	7.30 - 8.30 a.m.
Given 40 hours after progesterone :	11.30 - 12.00 p.m.

c. Preliminary injection studies

i. The animals - The three ovariectomised ewes and the three ovariectomised ewes previously described were used. Unfortunately one animal (18.53) died during the series of trials, but was replaced by another ewe (299.51) which had been ovariectomised and given a four week period for recovery and reduction of previous hormonal influence from the ovaries.

ii. Grouping - It was desirable to find the approximate minimum doses of oestradiol benzoate necessary for oestrous behaviour and PL formation as quickly as possible. To achieve this, widely differing dose levels were given for first three trials. Three groups of two animals each had

randomly allotted one ovariectomised and one ovariectomised
ewe as follows:-

Group A	97.49	91.49
Group B	161.50	25.53
Group C	58.48	18.53

In the remaining 15 trials only two groups were
used, the groupings being:

Group D	58.48	91.49	161.50 *
Group E	97.49	18.53 ⁺	25.53

* This animal was affected with facial eczema and
was withdrawn from trials 16, 17 and 18. The
ewe was not replaced.

+ This animal died after trial 9 and was replaced
by ewe 299.51 at the commencement of trial 11.

iii. Dose levels - It was decided to conduct the trials
at 14 day intervals similar to Robinson (1954 b, c) since
it was a convenient approximation to the normal length
of the oestrous cycle. At the end of each trial the
results were considered and a decision made as to what
treatment and dose level would be given in the next trial.
To reduce the likelihood of possible effects from the
previous treatment it was decided, after trial 3, to
alternate the groups during the succeeding trials. Thus
group D which received the high dose of ODB in trial 4 was
given the low dose in trial 5, and vice versa.

The treatments and groups are shown in Table 13.

iv. Collection of data - Observations of oestrous

TABLE 12

Treatments — preliminary injection studies with castrate ewes

Trial No.	Group	Date of progesterone treatment	Daily dose of progesterone (mg.)	Total quantity of progesterone (mg.)	Date of ODB treatment	Daily amount of ODB (mg.)
1	A				6.i.	20
	B				"	10
	C				"	5
2	A				13.i.	200
	B				"	100
	C				"	50
3	A	23.i.-25.i	20	60	27.i.	200
	B	"	20	60	"	100
	C	"	20	60	"	50
4	D	6.ii.-8.ii	20	60	10.ii	40
	E	"	20	60	"	20
5	D	20.ii.-22.ii	20	60	24.ii	25
	E	"	20	60	"	35
6	D	5.iii.-7.iii	10	30	8.iii	35
	E	"	10	30	"	25
7	D	19.iii.-21.iii	10	30	22.iii	15
	E	"	10	30	"	30
8	D	2.iv.-4.iv	10	30	5.iv.	25
	E	"	10	30	"	10
9	D	16.iv.-18.iv	10	30	19.iv.	10
	E	"	10	60	"	10
10	D				3.v.	40
	E				"	40
11	D	30.iv.-2.v.	10	30	18.v.	25
	E	15.v.-17.v.	10	30	"	25
12	D	12.v.-17.v.	10	60	1.vi.	25
	E	29.v.-1.vi.	10	40	"	50
13	D	12.vi.-15.vi.	10	40	15.vi.	40
	E	"	10	40	"	20
14	D	26.vi.-29.vi.	10	40	30.vi.	20
	E	"	10	40	"	40

TABLE 13 (Continued)

Trial No.	Group	Date of progesterone treatment	Daily dose of progesterone (mg.)	Total quantity of progesterone (mg.)	Date of ODB treatment	Daily amount of ODB (µg.)
15	D	10.vii.-13.vii.	10	40	13.vii.	60
	E		10	40	"	30
16	D	24.vii.-27.vii.	10	40	27.vii.-30.vii.	30
	E		10	40	"	60
17	D	7.viii.-10.viii.	10	40	10.viii.-13.viii.	20
	E		10	40	"	40
18	D	21.viii.-24.viii.	10	40	24.viii.	20
					25.viii.	30
					26.viii.	40
					27.viii.	50
					28.viii.	60
					24.viii.	20
					25.viii.	20
					26.viii.	20
					27.viii.	20
					28.viii.	20
	E	"	10	40		

behaviour and cervical mucus were made at least daily. A preliminary examination of the vaginal smear was also made. Vaginal mucus was collected using a glass rod and spread on to a microscope slide. The smears were stained with methylene blue and observed under low power ($\times 140$). These were classified according to Robinson (1955 a).

d. Further injection studies

This series of injections was conducted during November and December, 1956. The purpose was to confirm some of the results obtained in the previous studies with spayed ewes. Oestrous, vaginal smear and cervical smear responses to ODB administered 40 hours after progesterone pretreatment were investigated.

1. The animals - Twelve ovariectomised and two ovariectomised animals were obtained for study. Five ewes (58.48, 91.49, 97.49, 299.51, 25.53) already described were included in the number. The remaining nine animals were spayed on 12th October and allowed four weeks for recovery. The animals were run as one flock along with two raddled teaser rams and given similar feeding and management as before.

11. Groupings - Previous experience suggested that the present number of ewes was all that could be handled satisfactorily in this particular section of the study. Further, the results from the preliminary work with spayed ewes suggested that four dose levels of ODB would

be necessary to obtain the desired information of oestrus and PL formation. To obtain sufficient observations it was decided to conduct four trials at 14 day intervals.

The ovariectomised ewes were randomly divided into four equal groups (F G H and J) while the two ovariectomised animals comprised group K (Table 14).

T A B L E 14

Grouping of ewes - November-December trials

Group	Ewe Tag Number		
F	25.53	27.53	32.53
G	29.53	33.53	34.53
H	91.49	26.53	30.53
J	229.57	28.53	31.53
K	58.48	97.49	

iii. Dose levels - The results from the preliminary investigations with castrate ewes suggested that 10 mg. progesterone injected daily for 3 days and followed 40 hours later by 30 μ g. ODB would cause oestrus in most castrate Romney ewes. The corresponding dose of ODB for PL formation was 15 μ g. It was thought that four dose levels of 5, 10, 20 and 40 μ g. ODB would effectively straddle the two endpoints (i.e. oestrous behaviour and PL formation). The results of Robinson (1954 b, 1955 a) and Robinson et al. (1956) indicated that these levels would also be satisfactory for showing the characteristic vaginal smear changes. It was intended to construct dose response lines for the criteria

examined.

Since the possibility arose that one treatment may influence the effect of a subsequent treatment on the physiological response by the ewe, the experiment was conducted using a Latin square design detailed by Cochran and Cox (1955). The layout was as follows:

		<u>Periods</u>			
		I	II	III	IV
		Commencing	Commencing	Commencing	Commencing
		2.xi.56	16.xi.56	30.xi.56	14.xii.56
Doses	40	F	G	H	J
	20	G	J	F	H
	10	H	F	J	G
	5	J	H	G	F

iv. Collection of data - As in the preliminary study, oestrous behaviour, vaginal smears and PL formation were used as criteria for assessing the effects of progesterone and oestrogen.

Mucus was also collected from the nasal passages. The entrance to the nostril was cleaned with cotton wool. Mucus was obtained by inserting into the nasal passage a cotton wool swab held on the end of a pair of forceps. After removal the swab was rolled over a clean glass slide which was later oven dried. The nasal mucus was examined for "crystallisation".

2. Results and discussion

a. Preliminary series of trials

1. ODB alone - (trials 1, 2 and 10). In Table 15 is presented the results for oestrous response and PL formation.

TABLE 15

Oestrus and arborization in ewes treated with ODB alone

Trial	Group	Dose (ug.)	Number Treated	Number showing Oestrus	Number showing PL	Appearance of PL after ODB (Hours)
1.	A	20	2	0	0	-
	B	10	2	0	0	-
	C	5	2	0	0	-
2.	A	200	2	0	1	24-36
	B	100	2	0	1	24
	C	50	2	0	2	24
10.	D	40	3	3	3	24-33

The absence of oestrus in trial 2 was surprising. Robinson (1954 c) with Suffolk x Border Leicester-Merino yearling ewes found that 200 and 40 μ g. ODB gave a 44 and 17 per cent response respectively. There is the possibility that the yearling sheep used by Robinson may have been more sensitive to exogenous hormones than the mature ewes in this study. A breed difference may also be suggested to explain the difference in the results. However Robinson (1956 b) maintains that the endocrine relationships in oestrous

behaviour are similar, both quantitatively and qualitatively, in the two breeds. The small number of animals treated in these trials do not allow definite conclusions to be reached. However later work (Raeside and McDonald, unpublished data, 1957) is in agreement with the findings presented, since, 100 and 200 μ g. ODB did not cause oestrus in ovariectomised ewes at a similar time of the year.

Arborization was detected in trial 2 in ewes on all dose levels. In the early stages of the investigation difficulty was experienced with the technique of collecting uncontaminated mucus. This may explain the absence of arborization in a ewe from each of the 100 and 200 μ g. dose levels. The possibility that temperature could have been responsible for these negative smears is not favoured, since later work indicated that during the warm summer months, artificial heating of slides did not appear so necessary.

The 100 per cent response for oestrous behaviour and PL formation in trial 10 was not expected in view of the previous results. It should be noted that this trial was conducted after seven trials, at 14 day intervals, of progesterone priming and ODB treatment. A nearly similar set of conditions involving progesterone - ODB treatment and ODB alone was utilized by Robinson (1954 b). He concluded that a consistent induction of oestrus depended entirely on whether or not progesterone preceded oestrogen. In this study it is possible that the effects of previous progesterone - ODB treatment may have conditioned the animal to respond to the 40 μ g. ODB, even though it was less than the oestrogen given in the second

trial. To test this hypothesis a further one or two trials at 14 day intervals after trial 10 should have been conducted, using the same animals and the same dose of ODB without progesterone pretreatment. The results from Robinson (1954 b, c) suggest that the outcome would be a decreased oestrous response associated with the development in the ewe of a refractory condition to oestrogen. No prediction can be made for the effect of this set of conditions on PL formation.

Since the first two trials in January and trial 10 in May were conducted under different climatic conditions (January : mean day length, 14.8 hours; May : mean day length, 10.3 hours; Schwass, 1957) there is the likelihood of a seasonal effect influencing oestrous behaviour. Further work (Raeside, unpublished data, 1956) showed that doses of ODB alone up to 200 μ g. were not sufficient to cause oestrus in spayed ewes during September and October. Thus there is the possibility that 200 μ g. ODB alone will not facilitate an oestrous response at the time intact Romney ewes are normally anoestrus. However, 40 μ g. ODB will facilitate oestrus during the breeding season.

11. Three days progesterone followed by ODB (trials 3-10) -
In Table 16 is presented the results for these trials. Fortnightly injections of ODB following three days of progesterone pretreatment gave results for oestrous behaviour similar to those found by Robinson (1954 b, c) and Robinson et al. (1956). In the first three trials (3, 4 and 5) progesterone was given as a twice daily dose involving 20 mg. per day. In subsequent trials (6, 7, 8, 9 and 10) a more

TABLE 16

Oestrous and arborization response to oestradiol benzoate
given 40 hours after 3 days progesterone pretreatment

Trial	Group	Dose ODB (ug.)	Number treated	Number showing oestrus	Number showing PL	Appearance of PL after ODB (Hours)
3	A	200	2	2	2	< 8
	B	100	2	2	2	< 8
	C	50	2	2	2	8-16
4	D	40	3	3	3	16-32
	E	20	3	0	2	32-56
5	D	25	3	1	3	< 13
	E	35	3	3	3	< 13
6	D	35	3	3	3	< 8-16
	E	25	3	1	3	< 8-16
7	D	15	3	1	3	16-32
	E	30	3	2	2	< 8-16
8	D	25	3	3	3	8-16
	E	10	3	0	1	16-32
9	D	10	3	0	1	8-16
10	E	40	2	2	2	< 8-32

convenient single daily injection of 10 mg. was administered. It should not be assumed however that since the results were similar for trial 5 (progesterone once daily), that the latter treatment conditioned the ewe to respond to oestrogen as well as did the twice daily injections. Further, Robinson (1954 a, 1955 b) found that twice daily doses of progesterone given for 3 days produced a greater oestrous response to subsequent oestrogen, than did the same total quantity of progesterone injected as one dose or as three single daily doses. In this present work, the progesterone priming has been considered as consisting of three daily injections regardless of whether they were twice or once daily.

A dose of 10 μ g. ODB was below the threshold for a behavioural response in all ewes treated. An increase to 25 μ g. resulted in six responses from twelve treatments while levels of 35 μ g. and above were sufficient for the maximum response.

The results for arborization of cervical mucus indicated that 10 μ g. ODB would facilitate a response (two responses from six treatments) while 25 μ g. was necessary for maximum effect.

iii. Duration of progesterone pretreatment (trials 9 and 11).

Some data on the effect of length of progesterone priming was available from the above trials (Table 17).

TABLE 17

The Effect of Duration of progesterone pretreatment on
Oestrous and PL response

Trial	Group	Progesterone (days)	ODB (μ g.)	Number Treated	Number showing oestrus	Number showing PL	Appen- ance of PL after ODB (hours)
9	D	3	10	3	0	1	8-24
	E	6	10	3	0	0	-
11	D	3	25	3	1	3	8-24
	E	6	25	3	3	3	8-24

It was apparent that six days progesterone priming gave nearly similar results as those after three days of priming, for both oestrus and PL formation, when ODB was given at the 10 μ g. level. In trial 11, the results for oestrus may have indicated that a six day pretreatment caused a greater response after 25 μ g. ODB. However this may be questioned since in trial 8 all animals receiving 3 days progesterone priming and 25 μ g. ODB were tugged.

Robinson et al. (1956) found that with increasing duration of progesterone pretreatment (at levels between 3-24 mg.) there was an increase in the degree of sensitivity to ODB, when measured by oestrous behaviour.

There was no interaction between duration of priming or daily dose level of progesterone and the vaginal response to ODB.

Information concerning the duration of progesterone pretreatment and cervical mucus arborization is desirable, if the arborization phenomenon is to be used as a criterion for quantitative studies. There is the possibility that with six days priming as against three days, less ODB would be required to give a positive response and thus progesterone and oestrogen would have acted synergistically, as Robinson et al. (1956) showed for oestrous behaviour. The above results did not confirm this contention. However, other workers, notably Zondek and his group, have shown progesterone was antagonistic towards arborization. The possibility arises that this latter effect may have been stronger and completely hid the conditioning action of progesterone. Further data from trial 11 is perhaps in support of this view. Arborization for both groups occurred 8-24 hours after oestrogen administration. Thus, the longer duration of progesterone pretreatment did not markedly advance the time of onset of arborization as was shown for oestrous behaviour by Robinson (1954 c).

An experiment similar to that of Robinson et al. (1956) using various quantities and durations of progesterone priming and followed by dose levels of 5-25 µg. ODB* would probably indicate the effect of daily dose level and duration of progesterone pretreatment on PL formation.

*Subsequent information suggested that 5 µg. would be a satisfactory level for the lower limit of the ODB dose range.

- iv. Simultaneous progesterone-oestradiol benzoate injections, after progesterone pretreatment.
(Trials 12, 13, 14 and 15).

The results of trials conducted to gain some information on the progesterone-oestrogen antagonism affecting PL formation, is presented in Table 18.

TABLE 18

Oestrous and arborization response to simultaneous progesterone - oestradiol benzoate given after three days of progesterone pretreatment

Trial	Group	ODB (μ g.)	Number Treated	Number showing oestrus	Number showing PL	Appearance of PL after ODB (Hours)
12	D	25	3	0	3	8-24
	E	50	3	3	3	8-24
13	D	40	3	0	3	8-24
	E	20	3	0	1	< 24
15	D	60	2	2	2	8-24
	E	30	3	0	3	< 24

This series of trials was different from those conducted previously, since: ODB was given only 24 hours after the third injection of progesterone (not 40 hours as before):

A fourth injection of progesterone was given at the same time as the oestrogen. Thus, progesterone and

ODB were administered simultaneously 24 hours after three days of progesterone pretreatment.

The results for oestrous behaviour indicated that doses greater than 40 μ g. ODB were necessary when progesterone was given at the same time. The corresponding values for the PL response indicated that 25 μ g. ODB was necessary, while 20 μ g. would be perhaps a marginal dose. However it was necessary to obtain some data on the effect of injecting ODB after the 24 hour interval rather than the 40 hour period, thus trial 14 was conducted. These results are shown in Table 19.

TABLE 19

Oestrous and arborization response to Oestradiol benzoate given 24 hours after three days progesterone pretreatment

Trial	Group	ODB (μ g.)	Number Treated	Number showing oestrus	Number showing PL.	Appearance of PL after ODB (Hours)
14	D	20	3	0	3	8-24
	E	40	3	1	3	8-24

The results for oestrous response (trial 14) did not appear to indicate what effect progesterone had when given simultaneously with oestrogen in trials 12, 13 and 15, however the effect on PL formation was more apparent. In trial 13, arborization was barely detected at the 20 μ g.

level, but in trial 14 the absence of the simultaneous progesterone dose allowed a full response.

Reference has already been made to a progesterone-oestrogen antagonism affecting PL formation and which in this case seemed a possible explanation for the results above. No such effect is suggested for oestrous behaviour nor has there been demonstrated the synergistic action shown by Robinson (1954 b, c, 1955 a). In view of the lack of trials similar to trial 14, this perhaps is not surprising.

The absence of oestrus in ewes which received 40 μ g. ODB or less may be due to oestrogen being given simultaneously with progesterone (trials 12, 13 and 15), or only 24 hours after progesterone (trial 14). Previously 25 μ g. ODB gave a good response when given 40 hours after the pretreatment.

It is thus possible that progesterone did not have sufficient time to condition the ewe to respond to doses up to 40 μ g. ODB. The further possibility that the antagonistic action of oestrogen has reduced the conditioning effect of progesterone, must also be considered.

The antagonism between progesterone and oestrogen offers the possibility of determining the progesterone/oestrogen ratio ($\frac{P}{E}$) affecting arborization. Zondek similarly determined this ratio in the human, by this technique. In the present work, progesterone pretreatment was kept constant over three days but the $\frac{P}{E}$ ratio was varied on the fourth day of the injections. The ratio on this day might be considered as being directly responsible for the results observed for

arborization. Doses of ODB 25 μ g. and upwards all produced a 100 per cent response and gave a $\frac{P}{E}$ ratio of:

$$\frac{\text{Progesterone}}{\text{Oestrogen}} = \frac{P}{E} = \frac{10 \text{ mg.}}{25 \text{ } \mu\text{g.}} = \frac{400}{1}$$

There was no dose level at which no responses occurred, but 20 μ g. ODB appeared a marginal level:

$$\frac{P}{E} = \frac{10 \text{ mg.}}{20 \text{ } \mu\text{g.}} = \frac{500}{1}$$

v. Increased duration of oestradiol benzoate injections, after progesterone pretreatment (trials 16, 17 and 18)

The previous series of trials indicated that a progesterone-ODB antagonism affected PL formation. Thus, 10 mg. progesterone inhibited the stimulating effect of approximately 20 μ g. ODB. It seemed desirable to investigate whether similar daily quantities of oestrogen given over a longer period would allow arborization to reappear. Table 20 presents these results.

T A B L E 20

Oestrus and arborization response to progesterone pretreatment, simultaneous progesterone-oestradiol benzoate and further oestradiol benzoate

Trial	Group	Daily Dose ODB (μ g.)	Number Treated	Number showing oestrus	Number showing PL	Appearance of PL after ODB (hours)
16	D	60	2	2	2	< 24
	E	30	3	3	3	< 24
17	E	20	3	0	3	< 24
	D	10	2	0	0	-
18	E	20	3	2	3	< 24

A full response for oestrus and arborization was found at levels of 30 μ g. and 20 μ g. respectively, which was lower than that found in the previous series of trials. This is not surprising since a greater total quantity, as well as a longer duration of oestrogen treatment was given. Regarding the arborization phenomenon, positive mucus was detected 24 hours after the simultaneous progesterone-ODB treatment and before the next oestrogen injection. Thus the subsequent oestrogen injections have had no effect in causing a lowering in the level of ODB necessary for arborization. The same cannot be stated for the behaviour response, since oestrus in the animals in which it occurred was after the second oestrogen injection. Thus, the lower daily dose for oestrus may have been caused by the greater total quantity and/or the larger duration of ODB treatment.

Further data on the progesterone-oestrogen antagonism affecting cervical mucus may be derived from Table 20. A total response for arborization was given with 20 μ g. ODB while no response occurred at the 10 μ g. level. The marginal level of ODB for arborization thus indicates a $\frac{P}{E}$ ratio in the range.

$$\begin{aligned} \frac{P}{E} &= \frac{10 \text{ mg.}}{20 \text{ } \mu\text{g.}} - \frac{10 \text{ mg.}}{10 \text{ } \mu\text{g.}} \\ &= \frac{500}{1} - \frac{1000}{1} \end{aligned}$$

The time at which oestrus was first observed and the duration of oestrus in these trials, was similar to

that noted in the previous studies. Thus oestrus occurred 30-48 hours after the first oestrogen injection and was usually of less than two days duration. Since the continuous oestrogen injections did not prolong the oestrous period, a phenomenon similar to that reported by Aedell et al. (1945) may have occurred. These workers suggested the cow remains in heat for the normal period (one day) and then an "oestrous block" sets in. The duration of arborization was increased by continuous oestrogen injections. (PL formation was found on each day oestrogen was given). Thus it is probable the block occurs in the central nervous system.

b. November-December series of trials

1. Oestrous behaviour - only one ewe was tapped during these investigations. This animal (ewe 29.53) received the 20 μ g. dose during the first trial and was marked after 40 μ g. ODB given in the second trial.

The marked absence of oestrus in this study was surprising, in view of the preliminary investigations. However, the November-December trials were conducted during the period when the Romney ewe is normally anoestrus. The corresponding studies in the preliminary investigations were performed from March until May i.e. during the normal breeding season. Since the dose levels given in both series of investigations were comparable, it is suggested that during the breeding season the castrate ewe is more sensitive to exogenous oestrogen,

than at other times. It is noteworthy that the only ewe tupped received the high dose. The possibility exists that greater levels of ODB would have produced a larger response. Subsequent work (Raeside and McDonald, unpublished data, 1957) suggests that similar injections of progesterone and up to 40 µg. ODB are not sufficient to cause oestrus in January and February. Oestrus was however, detected during early March.

The possibility that the rams during November and December were inactive and would not mount a ewe, even if showing signs of heat, must be considered. However, one ewe was marked. Also observations of the activity of the rams in the field indicated they were active in seeking signs of oestrus.

11. Vaginal smears - It was apparent during the preliminary investigations that characteristic cellular changes occurred in response to injections of hormones. These however appear less striking than those reported in rodents by several authorities. Robinson (1955 a) and Robinson and Moore (1956), with the ewe, found that oestrus behaviour was not always associated with complete cornification; that cornified smears did not appear until several days after oestrus; and that there was considerable variation in the day or days on which cornification appeared. Similar findings were noted with the New Zealand Romney ewe during the preliminary investigations.

In this study the method of assessing positive smears

was approximately that adopted by Robinson (1955 a). Robinson et al. (1956) found the assessment of the vaginal smears was, more satisfactory when using the technique of Robinson and Moore (1956). This latter report however, was not available at the time of the present work.

The qualitative nature of the response places limitations upon the quantitative results from these trials. The findings are presented in Table 21.

T A B L E 21

Vaginal smear responses of ewes injected with oestrogen following progesterone pretreatment

Dose ODB (μ g.)	Number treated per period	Responses per period				Total
		I	II	III	IV	
40	3	3	3	3	3	12
20	3	3	3	2	3	11
10	3	0	1	0	1	2
5	3	0	0	0	0	0

In figure 14 is presented the linear regression of probit on log dose of oestrogen (Finney, 1952). The value of ED 50 for the vaginal criterion is given in Table 22.

iii. Cervical smears - As the November-December trials were conducted to confirm previous findings of the preliminary trials

TABLE 22

Estimates of ED 50 for oestrus, vaginal smear changes and arborisation of cervical mucus

	Oestrus*	Vaginal smear**	Cervical smear***
Upper 95% fiducial limit	27.8	15.9	13.5
ED 50	22.8	12.9	5.5
Lower 95% fiducial limit	16.5	10.9	0.1

$$\begin{aligned}
 Y(\text{oestrus}) &= -3.72 + 6.42 X \\
 Y(\text{vaginal smear}) &= -3.86 + 7.91 X \\
 Y(\text{cervical smear}) &= 3.59 + 1.90 X
 \end{aligned}$$

- * Values calculated from preliminary trial data
- ** Values calculated from November-December trial data
- *** Values calculated from pooled data

the results of both series are presented in Tables 23 and 24.

TABLE 23

Trials 5-10 preliminary series -- Cervical mucus response following three days progesterone pretreatment and oestradiol benzoate

Dose ODB (μ g.)	Number of ewes treated	Number of ewes responding
40	2	2
35	6	6
30	3	2
25	12	11
15	3	3
10	6	2

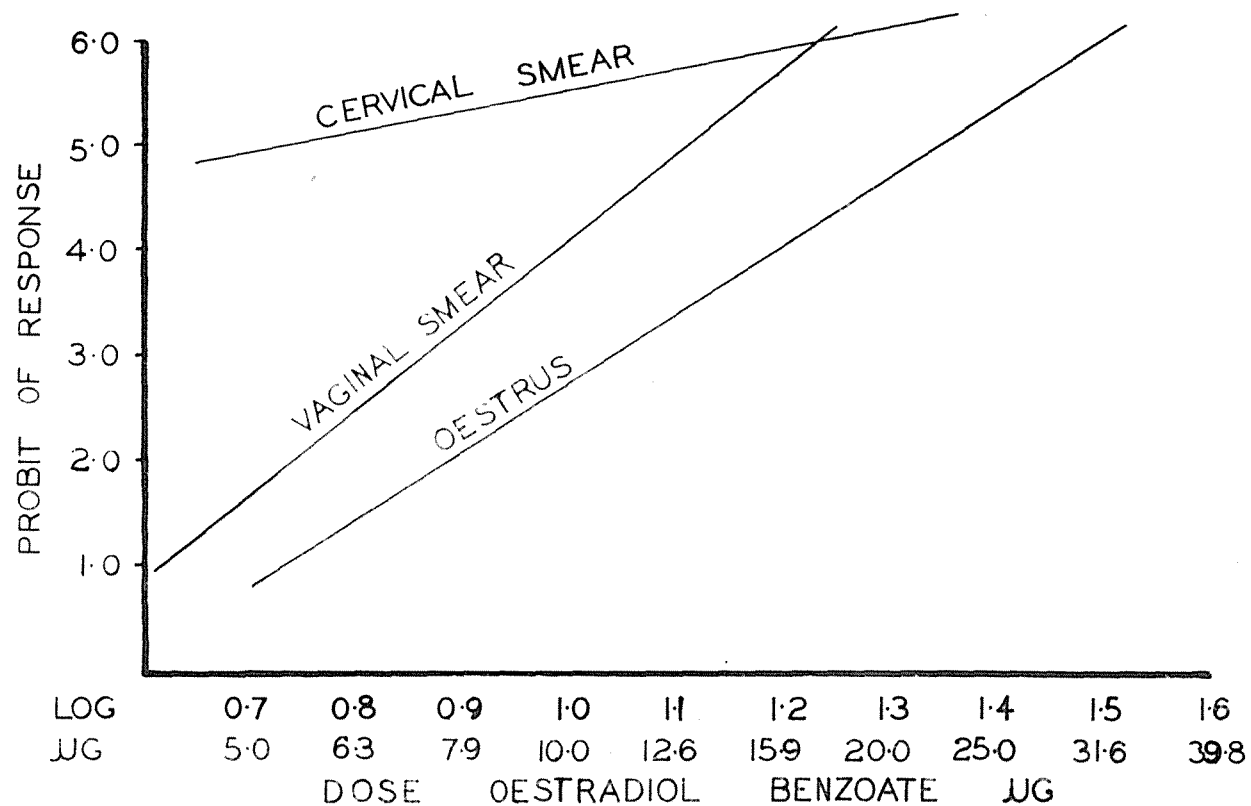
TABLE 24

November-December trials -- Cervical mucus response following three days progesterone pretreatment and oestradiol benzoate

Dose ODB (μ g.)	Number treated per period	Responses per period				Total
		I	II	III	IV	
40	3	3	2	3	3	11
20	3	3	3	2	3	11
10	3	3	3	2	1	9
5	3	1	1	2	2	6

The results were analysed for χ^2 (Finney, 1952, page 71)

FIG 14 DOSE-RESPONSE LINES FOR OESTRUS,
VAGINAL SMEAR AND CERVICAL SMEAR



with respect to homogeneity in the reactivity of cervical mucus following hormone treatment, for the subjects of both series of trials.

Analysis of χ^2 - Preliminary and November-December trials

Source of variation	d.f.	Sums of Squares
Heterogeneity	1	1.2627
Preliminary + Nov.-Dec. data	6	5.8186
Total	7	7.0813

$$\chi^2(1 \text{ d.f.}) \quad p = 0.05 \text{ is } 3.8$$

Since the χ^2 value of 1.26 was not significant all the data were pooled and then used to calculate the linear regressions of probit on log dose of oestradiol benzoate (Figure 14) and the value of ED 50 (Table 22).

Unlike oestrous behaviour, the arborization phenomenon in response to progesterone and ODB does not appear to be affected by any seasonal influence. This is an advantage for quantitative work. Further, this seasonal effect on the behavioural response probably indicates a relationship of the central nervous system with oestrous behaviour, but not with arborization in cervical mucus.

iv. Nasal mucus - This study of nasal mucus was prompted by the report of Henderson (1956) who detected cyclic changes in such mucus during the menstrual cycle in two women.

Two types of nasal secretion were found in the

ovariectomised ewe. A thick, tacky secretion, which did not spread easily on the slide, exhibited some "crystallisation" after injections of 40 and 20 µg. ODB. "Crystallisation" patterns were more readily found in the thin watery mucus. This was noted at all levels of ODB and even in some of the control sheep (Table 25).

TABLE 25

Nasal mucus response following three days progesterone pretreatment and oestradiol benzoate

Dose ODB (µg.)	Number treated per period	Responses* per period				Total
		I	II	III	IV	
40	3	3	3	3	3	12
20	3	3	3	3	3	12
10	3	2	3	2	3	10
5	3	2	2	1	2	7
0	2	2	1	0	1	4

Considerable variation existed in the type of pattern in nasal secretion. In thick mucus, "crystallisation" resembled cervical mucus arborization. However in the watery nasal mucus distinctive patterns were seen (figures 15, 16, 17 and 18). The different types of pattern seen may be due to the

*A positive response was recorded when "crystallisation" was seen regardless of the thickness of the mucus.

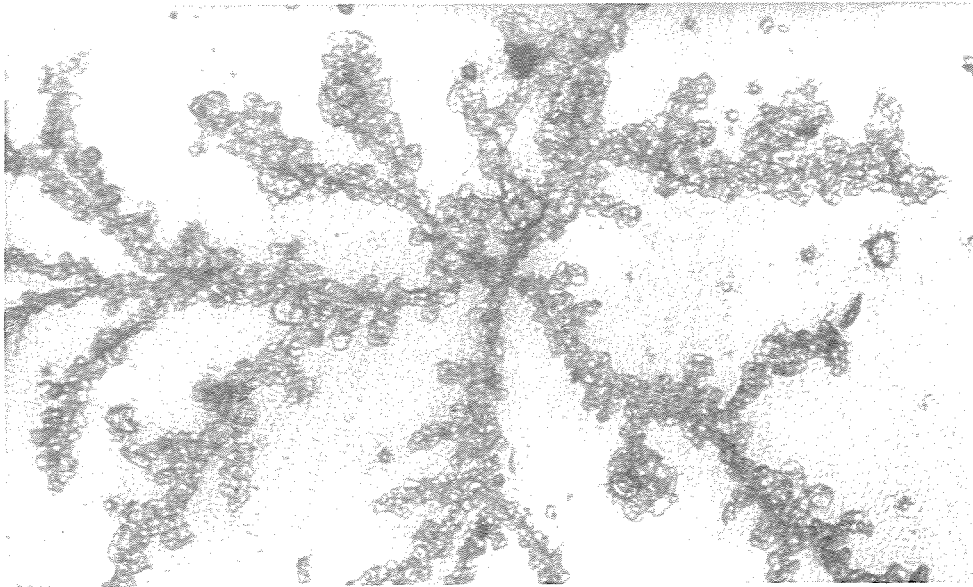


Fig. 15. Nasal mucus arborization - distinctive type (I)

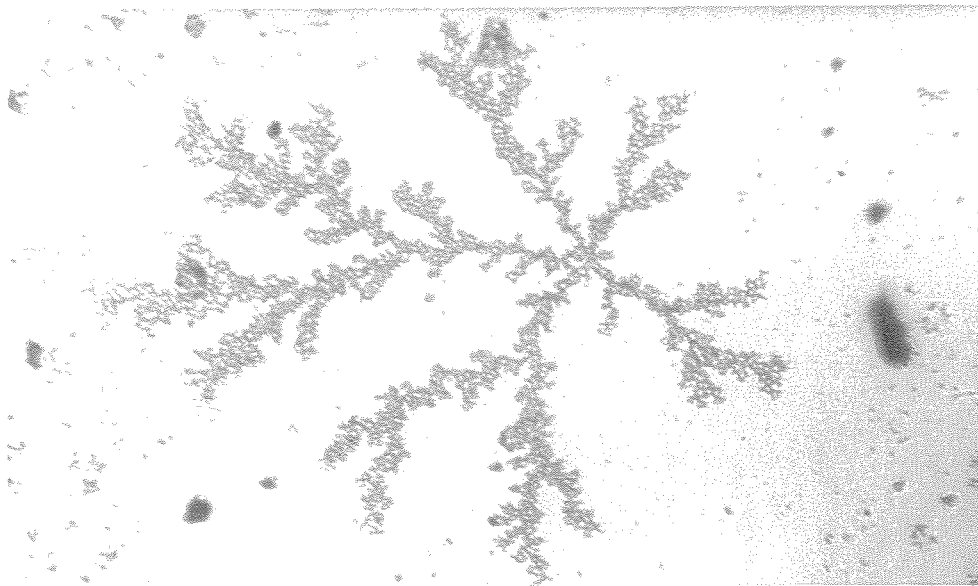


Fig. 16. Nasal mucus arborization - distinctive type (II)

difference in thickness of the mucus since a similar phenomenon has been noted with cervical mucus (Zondek, 1954). However the distinctive "crystallisation" of watery mucus, regardless of the oestrogen treatment given, may indicate the presence of electrolytes not normally found in the thick mucus.

The limitations of this criterion for quantitative studies are apparent. However further investigation of thick nasal mucus would appear worthwhile. One problem is to classify mucus intermediate in thickness between "thick mucus" and "thin watery mucus".

C. Injection Studies with Intact Ewes

1. Experimental procedure

a. Animals

Ten Romney ewes were available for this phase of the study, from 8th May until the end of the breeding season. These animals had previously been observed for oestrus and PL formation during the oestrous cycle. They appeared to be experiencing normal ovarian activity. The grazing available and management given was similar to that for the other ewes in the study. Two raddled teaser rams were also run with the ewes.

b. Doses

As the purpose of this investigation was to find the marginal quantities of ODB in arachis oil, necessary to produce arborization on various days of the cycle, the amount of oestrogen to be injected in one trial was largely determined by the results obtained from the previous trials. Initially the dose levels of ODB were chosen at intervals sufficiently wide to get some idea of the quantity necessary for arborization. Thereafter the dose levels more nearly approached the marginal quantity.

c. Collection of data

As ODB was to be injected on certain particular days of the cycle, the first day of oestrus, as detected by the raddled teaser rams, was taken as the first day of the cycle. In most cases even after the ODB injection during the cycle, the next oestrus occurred at the normal time, thus it was felt that ovarian activity had not been greatly upset by the treatment. Further it might be assumed that the result of an ODB injection during the next cycle would not be influenced by the previous ODB treatment. It may have been preferable to have allowed a cycle without hormone administration, to elapse between cycles with oestrogen injections. Since so few animals were available and the end of the breeding season was close, this added precaution could not be taken. Any ewes which did not exhibit oestrous behaviour at the expected time were not subsequently treated with ODB until they had experienced a cycle of normal duration.

Cervical mucus was collected and examined as previously

described.

2. Results and discussion

Negative cervical mucus was previously found to be present from 3-12 days and occasionally until 14 days post oestrus. Consequently it was assumed that within this period ODB could be given and any resultant PL formation attributed to the exogenous oestrogen. Further, it was the intention to give one ODB injection per cycle. This, with the small number of animals and the limit imposed by the breeding season, made it necessary to restrict the investigation to certain days of the cycle.

The following three periods of the luteal phase were chosen as being representative of stages in the cycle when arborization was normally absent:

Days 3-4 Warbritton (1934) showed the corpus luteum underwent development until the end of the fourth day. For the purposes of this study this period (days 3-4) has been termed the early luteal phase.

Days 6-9 This was chosen as representing the stage when the corpus luteum was fully developed. Warbritton showed the organ remained in this state until the 14th day.

Days 10-12 This period was selected only because these were the last days on which arborization did not normally occur. It would have been desirable to investigate those days nearer the end of the cycle when the corpus luteum was undergoing regression (Warbritton, 1934).

Since arborization usually commenced in normal ewes

after day 14, a response was considered positive when PL formation occurred within 24-36 hours after the oestrogen treatment. Further, the results from injection studies with spayed ewes indicated that if arborization did appear after oestrogen treatment it did so within that time.

The occurrence of arborization after ODB treatment during the cycle is presented in Appendix V and summarized in Table 26.

The difficulty in collecting uncontaminated cervical mucus 2-4 days post oestrus has previously been mentioned. In this study three ewes were examined during this period, with only one showing negative mucus. From experience it was felt that this smear was not contaminated. Hence it was concluded that the oestrogen given (25 μ g. ODB) was not sufficient to promote the PL reaction at this stage of the cycle.

With the relatively few observations made it is difficult to draw conclusions regarding the quantities of ODB necessary to induce PL formation at various stages of the cycle. Consideration of the minimum quantities of ODB necessary for a full response during the three periods shows that 50, 150 and 20 μ g. respectively of exogenous oestrogen was required. This suggests differences in the levels necessary for PL formation. Additional evidence, from the results at the 50 and 100 μ g. levels, shows that these doses were marginal at midcycle whereas a full response was given during the other two periods.

In the early luteal period of the human cycle the oestrogen level is low (Brown, 1955). Also the progesterone

TABLE 26

Arborization of cervical mucus within 24-36 hours of a single oestradiol benzoate injection given during the oestrous cycle

ODB (µg.)	Days after tupping			10-12		
	3-4	6-9	10-12	Number of sheep	Number showing PL	Number showing PL
200	1	1	2	2	2	2
150	2	2	2	2	2	2
100	1	6	2	1	1	1
50	1	3	1	2	2	2
40				2	2	1
25	1					
20				2	2	2
15	1		0		0	
10	1		0	3	0	0
5	2		0	3	0	0

level is lower than that found later in the cycle (Edgar, 1953; 1954). The results may suggest that 50 μ g. ODB at this time is sufficient to alter the progesterone-oestrogen balance and to allow arborization. At midcycle, progesterone is at a high level and consequently much larger doses of ODB are necessary to obtain the PL reaction. During the late luteal stage the progesterone level does not appear to fall until the 16th day. (Edgar, 1954; Edgar and Ronaldson, 1957). However a rising level of oestrogen from the developing follicles may decrease the progesterone/oestrogen ratio. Thus only low quantities of exogenous oestrogen would be necessary to cause arborization at this time.

V DISCUSSION

To understand more fully the role of the female sex hormones in reproduction, quantitative studies to determine the amount of each hormone which act upon the target organs, are desirable. However, the two hormones do not act independently. On some tissues they may be synergistic in action but on others, antagonistic. Also the absolute amount of oestrogen and progesterone present may affect any antagonistic or synergistic phenomenon.

Most chemical and biological assay techniques attempt to measure, by some means outside of the animal, the quantity of hormone within the animal body. One criticism of some of these methods is that they do not allow for possible hormonal interactions. Thus, the results for the assay might not indicate the true activity of either hormone on the target organ. Techniques which utilize criteria obtained from within the animal may therefore have advantages, since they will probably reflect the endocrine balance of the animal at that time. Courrier (1950) in his review noted the antagonism of the female sex hormones on cervical mucus of women and suggested this as a possible technique. Thus the quantity of oestrogen physiologically active during the cycle might be assessed by ascertaining the amount of progesterone necessary to prevent the appearance of characteristic clear, glairy mucus ("glairé filante"). The phenomenon of arborization of cervical mucus might similarly be used.

The sensitivity of cervical mucus to progesterone

and oestrogen was apparent from the investigation with spayed ewes. These results indicated that extremely small quantities of ODB given 40 hours after progesterone produced a response within a day of injection. When however the ODB was given simultaneously with progesterone, larger quantities were required for a response. This suggested that progesterone was antagonistic to this action of oestrogen on arborization. When single injections were given during the luteal phase of the cycle in the intact ewe, larger quantities were required than those found with spayed animals. This was not surprising since progesterone from the corpus luteum would be present. However, the work of Brown (1955) with human subjects has indicated that endogenous oestrogen may also be present. If arborization is dependent upon the interaction of progesterone and oestrogen, then the PL reaction in ewes that received an ODB injection during the luteal phase, would be the result of the interaction between endogenous progesterone and endogenous plus exogenous oestrogen. Although the number of observations at each of the three luteal phases investigated was limited, the results probably indicated differences in the requirement of exogenous oestrogen. The results however do not provide any indication of the separate changes in endogenous progesterone and oestrogen.

The requirement of ODB for the induction of oestrus in the spayed ewe was determined during the normal breeding season. Greater levels, even up to 40 μ g. ODB, failed to produce responses during November and December (one exception). This was suggestive of a seasonal influence affecting the manifestation

of oestrus in the spayed ewe. Several workers have induced oestrus in spayed ewes with large but probably "unphysiological" doses of oestrogen during the normal anoestrous period. There was the possibility that doses above 40 μ g. ODB but still near to "physiological" levels would have caused oestrus in the ewes of this work. Robinson (1955 b) detected a behavioural response in anoestrous Romney ewes treated with 25 μ g. ODB following 75 mg. progesterone given as six equal injections over three days. The greater quantity and more frequent injection of progesterone may have better conditioned the ewe to respond, than lower quantities given as three single daily treatments in this study. Robinson in addition used intact sheep. These may have been producing some oestrogen from the follicles which exist during anoestrus (Kammlade Jnr. et al. 1952). Thus 25 μ g. exogenous oestrogen in addition to endogenous oestrogen may have allowed a postulated threshold for oestrous behaviour to be attained.

Data for the quantity of ODB necessary for oestrus in spayed and intact ewes was obtained during the breeding season.

The likelihood of any seasonal influence being important was thus not critical to the comparison between spayed and intact ewes. The ED 50 value of 23 μ g. ODB for the castrate ewe was similar to the level determined by Robinson (1954 b, c, 1955 a) and Robinson et al. (1956). With the intact ewes in this work oestrus was not detected even after doses of up to 200 μ g. ODB, other than at the time expected in the normal cycle. The synergistic action of the two ovarian hormones

on oestrous behaviour in spayed ewes has been demonstrated (Robinson, 1954 c). However, with the cycling ewes of this study, no synergism was detected if the advancement of the time of oestrus is considered the indicator of the possible synergism.

Repeated doses of oestrogen in spayed ewes produced a refractory condition to oestrogen (Robinson, 1954 c). Perhaps within the cycle follicular oestrogen also produces a refractory state. This possibly is removed by the action of progesterone from the subsequent corpus luteum. Thus exogenous oestrogen given during the period prior to the removal of this refractory state, may be incapable of promoting oestrus.

Doses of 100 and 200 µg. ODB during the late luteal phase inhibited oestrus in three ewes. Possibly ovulation without heat occurred. Robinson (1954 e) suggested that failure to exhibit oestrus was due to an "incomplete conditioning" as a result of the failure of corpus luteum function, or to an insufficiency of endogenous oestrogen. In these three ewes the high dose of oestrogen given a few days prior to the time normally expected for oestrus may have retarded the conditioning action of progesterone.

The ED 50 for the vaginal response to progesterone-ODB treatment was comparable with that presented by Robinson (1954 b, 1955 a) and Robinson et al. (1956). This further supports the contention of Robinson (1955 b) that between Romney and Suffolk cross bred ewes at least, the endocrine

balance is similar quantitatively and qualitatively.

The ease of collection and the absence of the problem of contamination of mucus suggest advantages in using the vaginal smear criterion. Further, the value of such a technique for quantitative assay work has been stressed in small animals (Emmens, 1950) and in the ewe (Robinson and Moore, 1956). However the difficulty of determining a positive response is inherent in this qualitative method, especially in the case of large animals. Arborization of cervical mucus, provided the temperature is not limiting, leaves little doubt as to a positive response. Further this phenomenon appears more sensitive to circulating hormones than does the vaginal response.

Nasal mucus, which was relatively easy to collect, unfortunately did not show consistent results for arborization in response to injected hormones. However the investigation of this criterion was only preliminary - valid conclusions can hardly be drawn. Cyclic changes in the oral epithelium during the menstrual cycle have also been demonstrated (Ziskin and Moulton, 1948). This might suggest that a study of the effects of hormones on the epithelium and secretions of the nasal and oral cavities would be worthwhile in the ewe.

The value of the technique of mucus arborization for assessing ovarian activity has been demonstrated quite by chance with the hysterectomised ewes. Arborization in ewe 229.52 which had a corpus luteum present, may indicate that the organ

was not fully functional and thus was in agreement with the suggestion of Wiltbank and Casida (1956). The use of this technique, for determining such phenomena as maintenance of the corpus luteum after prolactin treatment (Moore and Nalbandov, 1956) and for investigations into the effects of plant oestrogens within the animal, seems possible.

The stimulus of the sudden presence of the ram near the onset of the breeding season has been shown to markedly alter ovarian activity (Underwood et al., (1944); Thompson and Shinckel, (1952); Ritches and Watson, 1954; Shinckel, 1954 a, b;) Raeside, unpublished data, 1957). Detection of first oestrus by the use of male sheep is thus confounded to some extent by this exteroceptive factor. It is likely that mucus arborization may be a means of more accurately determining the onset of the breeding season.

VI SUMMARY AND CONCLUSIONS

1. A preliminary investigation of the phenomenon of arborization of cervical mucus in the New Zealand Romney has been described. Mucus collected from various live and slaughtered ewes in different stages of reproduction was studied.
2. Eighteen fortnightly trials were conducted with six spayed ewes during the normal breeding season. The effects of various combinations of progesterone and oestrogen on the arborization and oestrous responses was investigated. When oestrogen was given 40 hours after progesterone pretreatment, 15 μ g. caused arborization and 25 μ g. lead to oestrus behaviour. Levels of 20-25 μ g. and 50 μ g. oestradiol benzoate were necessary when oestrogen and progesterone were given simultaneously. This has indicated the different requirements of oestrogen for oestrus and arborization responses and also the antagonism of progesterone and oestrogen affecting the two criteria.
3. A further series (November-December), with 14 spayed ewes, was performed to obtain the dose response relationships for progesterone-oestrogen treatment on oestrous behaviour, vaginal smear changes and arborization of cervical mucus. At this time, the oestrous response was negligible. In the earlier

trials however, greater responses were obtained. This indicated a possible seasonal effect.

4. Arborization during the normal oestrous cycle was studied with 20 intact ewes. The phenomenon usually persisted from 3-4 days before, to 1-2 days post oestrus.
5. In thirty-seven oestrous cycles a single injection of oestradiol benzoate was given to determine the minimum quantity necessary for arborization during the luteal phase. On the third day 50 μ g. was successful while on the sixth day 150 μ g. gave a full response and 100 μ g. appeared marginal. From the ninth to the twelfth days 50 μ g. always caused pattern formation as did several injections of 20 μ g.
6. Three hysterectomised ewes were studied. Negative mucus persisted for several months during the breeding season which possibly indicated the life of the corpus luteum had been prolonged. Although none of these ewes have been in oestrus, arborization was detected towards the end of the breeding season and during anoestrus. This, with the histology of one ovary of a ewe suggested the corpus luteum was not fully functional.

7. Silent heat was detected in four ewes by means of the cervical mucus technique. Ovulation was subsequently confirmed in one ewe by examination of the ovaries.
8. Some possible applications of this technique have been discussed. The results presented indicate the sensitivity of arborization, of cervical mucus to oestrogen and progesterone. This has suggested its suitability as a method for studies of reproductive phenomena in the ewe.

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APPENDICES

APPENDIX I

Slaughtered ewes - follicle counts

Group	A		B		C		D		E		F		G	
Size of follicles	L.	S.	L.	S.	L.	S.	L.	S.	L.	S.	L.	S.	L.	S.
	3	7	4	10	1	11	1	27	2	18	2	26	1	14
	0	5	0	14	0	22	0	43	2	19	1	11	1	9
	1	11	1	18	0	19	0	23	2	12	2	30	0	21
	1	11	1	17	0	20	1	28	1	30	0	33	0	16
	1	12	1	14	0	20	2	12	1	18	1	6	2	11
	1	15	1	25	0	23	0	20	0	41	1	21	2	17
	1	19	1	13	1	10	0	12	1	55	0	36	1	41
	0	15	0	26	0	14	1	11	2	21	1	18	2	24
	1	21	1	12	0	14	0	28	1	17	1	30	1	10
	0	1	1	12	1	18			2	19	3	29	2	18
			1	14	1	13			2	18	1	13	0	36
			1	7	1	14			3	11	0	32	2	54
			0	18	0	13			0	26	2	17	1	8
			0	33	0	19			1	29	2	24	1	22
			1	18	1	21			0	15	1	11	2	20
			0	12	1	21			0	12	1	14	0	25
			0	14	1	11			1	13	3	13	2	11
			0	14	0	21			1	17	2	11	2	15
			0	8	0	24			0	26	1	15	0	15
			0	18	1	13			1	19			2	17
			1	18	0	27								
			0	15	0	19								
					0	13								
					0	15								
					0	19								
					0	16								
					1	13								

A P P E N D I X II

Arborization of cervical mucus from ovariectomised,
hysterectomised and intact ewes after heat treatment.

Date	Ewe Number	Treatment to ewe	PL formation	
			Air-dried	Oven-dried
5.v.	97.49	Progesterone-ODB (Trial 10)	+	+++
	25.53		-	+++
20.v.	58.48	Progesterone-ODB (Trial 11)	-	++
	91.49		+	++
	161.50		+	+++
	97.49		-	++
	299.51		-	++
	25.53		-	+++
3.vi.	58.48	Progesterone-ODB (Trial 12)	+	++
	91.49		++	++
	161.50		+	+++
	97.49		+	++
	299.51		-	+++
	25.53		-	+++
17.vi.	58.48	Progesterone-ODB (Trial 13)	+	++
	91.49		-	++
	161.50		-	+++
	97.49		-	+
	299.51		-	-
	25.53		-	-
1.v.	20	Luteal phase	-	-
6.v.	20	Oestrus	+	+++
10.v.	20	Early luteal phase	-	-
1.v.	30	Late luteal phase	-	-
4.v.	30	Oestrus	+	+++
7.v.	30	Early luteal phase	-	-

Date	Ewe Number	Treatment to ewe	PL formation	
			Air-dried	Oven-dried
6.vii.	19	Luteal phase	-	-
11.vii.	19	Oestrus	-	+++
13.vii.	19	Early luteal phase	-	-
3.vii.	29.49	?	-	-
3.vii.	229.49	?	-	++
3.vii.	7.50	?	+	++
5.vii.	29.49	?	-	+
5.vii.	229.49	?	+	++
5.vii.	7.50	?	+	++
12.vii.	29.49	?	-	+
12.vii.	229.49	?	+	++
12.vii.	7.50	?	-	++

Arborization during oestrous cycle

No.	Date	Cycle length	Day of cycle													
			-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6
24	24.iii.a.m.										+	+	-	-	-	-
	8.iv. a.m.	15	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	27.iv. a.m.	19	-	-		-	-	-	-	+	+	+	-	+	-	-
	4.vii.a.m.		-	-	+	+	+	+	+	+	+	-	-	+	-	
26	27.iii.a.m.										+	+	-	-	-	-
	10.iv. a.m.	14	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	23.iv. a.m.	18	-	-	-	-	-	-	+	+	+	-	-	-	-	-
	14.v. p.m.	16½	-	-	-	-	-	+	+	+	+	-	-	-	-	-
17	20.iii.a.m.	*									+	+	-	-	-	-
	9.iv. a.m.	20	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	26.iv. p.m.	17½	-	-				+	+	+	+	-	-	-	-	-
21	30.iii.a.m.										+	+	-	-	-	-
	18.iv. a.m.	19½	-	-	-	-	+	+	+	+						
	4.v. a.m.	16	-	-	-	-	-	+	+	+	+	✓	-	-	-	-
	21.v. a.m.	17	-	-	-	-	-	+	+	+	+	✓	✓	-	-	-
22	30.iii.p.m.										+	-	-	-	-	-
	17.iv. a.m.	17½	-	-	-	-	+	+	+	+	+	-	-	-	-	-
	2.v. a.m.	15	-	-	-	-	+	+	+	+	+	-	-	-	-	-
30	1.iv. a.m.										+	-	-	-	-	-
	18.iv. a.m.	16½	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	4.v. a.m.	16	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	20.v. a.m.	16½	-	-	-	-	+	-	+	+	+	-	-	+	-	-
20	22.iii.a.m.										+	-	-	-	-	-
	6.v. a.m.		-	-	-	+	+	+	+	+	-	-	-	-	-	-
	23.v. a.m.	17	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	10.vi. a.m.	18	-	-	-	-	-	-	+	+	+	-	-	-	-	-
	27.vi. a.m.	17	-	-	+	✓	-	-	+	+	+	-	-	-	-	-
15	25.iii.a.m.										+	+	-	-	-	-
	11.iv. a.m.	17	-	-	-	-	-	+	+	+	+	-	-	-	-	-
1	16.iv. a.m.										+	+	+	+	-	-
	1.v. p.m.	17½	-	-	-	-	-	-	+	+	+	+	-	-	-	-
29	31.iii.p.m.										+	-	-	-	-	-
	16.iv. a.m.	16½	-	-	-	-	-	-	+	+	+	-	-	-	-	-
	2.v. a.m.	16						+	+	B	+	+	-	-	-	-
35	25.iii.a.m.	*									+	+	-	-	-	-
	10.iv. a.m.	16	-	-	-	-	-	-	+	+	+	+	✓	-	-	-
	27.iv. a.m.	17	-	+	+	+	+	+	+	+	+	-	-	-	-	-
113	24.iii.p.m.	*									+	+	-	-	-	-
	1.v. p.m.	38	-	+	+	+	+	+	+	+	+	+	-	-	-	-
19	31.iii.p.m.										+	-	+			
	5.iv. a.m.	5½									+	-	-	-	-	-
	22.iv. a.m.	17	-	-	-	-	-	-	+	+	+	✓	-	-	-	-
	9.v. a.m.	17	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	25.v. a.m.	16	-	-	-	-	-	+	+	B	+	-	✓	-	-	-
	10.vi. a.m.	16	-	-	-	+	+	+	+	+		✓	-	-	-	-
	26.vi. a.m.	16	-	-	+	✓	+	+	+	+	+					
	11.vii.a.m.	16	-	-	-	-	-	+	+	+	+	-	-	-	-	-

A P P E N D I X III (Continued)

Ewe No.	Date Tupped	Cycle length	D a y o f c y c l e													
			-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6
12	1.iv. p.m.									+	-	-	-	-	-	-
	20.iv. a.m.	19 $\frac{1}{2}$ **		-	-	-	-	+	+	+	-	-	-	-	-	-
	21.v. a.m.	31		-	-	-	-	+	+	+	+	-	-	-	-	-
7	24.iii.a.m.									+	-	-	-	-	-	-
	8.iv. a.m.	15	-	-	-	-	-	+	+	+	v	v	-	-	-	-
	26.iv. a.m.	18	-	-	-	-	-	+	+	+	v	v	-	-	-	-
27	30.iii.p.m.									+	-	-	-	-	-	-
		**	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	3.v. p.m.	35 **	-	-	-	-	+	+	+	+	-	-	-	-	-	-
		**	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	10.vi. a.m.	36 $\frac{1}{2}$	-	-	-	-	+	+	+	+	+	-	+	-	-	-
15	25.iii.a.m.									+	+	v	-	-	-	-
	11.iv. a.m.	17	-	-	-	-	+	+	+	+	+	-	-	-	-	-
23	1.iv. p.m.									+	+	-	-	-	-	-
	19.iv. a.m.	17 $\frac{1}{2}$								+	+	v	-	-	-	-
	30.iv. a.m.	11								+	+	-	-	-	-	-
	17.v. a.m.	17	-	-	-	-	+	+	+	+	+	-	-	-	-	-
10	31.iii.a.m.						-	+	+	+	-	-	-	-	-	-
	15.iv. a.m.	16	-	-	-	-	-	-	+	+	-	-	-	-	-	-
		**					+	+	+	+	+	+	-	-	-	-

v = cellular material, probably of vaginal origin.

B = blood contamination of cervical mucus.

* = ewe tupped after 14 days progesterone treatment.

** = silent heat thought to have occurred.

APPENDIX IV

Arborization of cervical mucus in ewes after hysterectomy.

The symbols used are as follows:-

- + = pattern formation faint,
individual patterns scattered
- ++ = pattern formation faint -
prominent, individual patterns
concentrated
- +++ = pattern formation prominent
individual patterns cover all
of smear
- = no pattern formation
- A = a typical mucus pattern.

Date of Examination	Eve tag Number	Notes
3.x11.55	29.49 229.49 7.50	
4		
5		
8		
10		
7.1.56		
8		
10		
14		
16		
17		
18		
25		
26		
27		
11.11.56		
12		

Date of Examination Ewe tag Number Notes
29.49 229.49 7.50

13

14

15

8.11.56

10 mg. progesterone injection

9

10

11

12

13

14

15

16

17

18

19

20

21

Date of Examination	Hwe tag Number		Notes
	29.49	229.49	7.50
23	++	++	++
24	++	++	++++
25	+++	+	+++ 29.49 possibly showed faint oestrus
26	++	++	++
27	++	++	++
28	++	++	++
5.IV.56	++	++	++
6	++	++	++
7	++	++	++
8	++	++	++
10	++	++	++
12	++	++	++
27	++	++	++
29	++	++	++
30	++	++	++
20.V.56	++	++	++
21	++		++

Date of Examination	Ewe tag Number	Notes
22	29.49	229.49 7.50
23	29.49	229.49 7.50
26.vi.56	29.49	229.49 7.50
27	29.49	229.49 7.50
28	29.49	229.49 7.50
29	29.49	229.49 7.50
2.vii.56	29.49	229.49 7.50
3	29.49	229.49 7.50
4	29.49	229.49 7.50
5	29.49	229.49 7.50
9	29.49	229.49 7.50
10	29.49	229.49 7.50
12	29.49	229.49 7.50
16	29.49	229.49 7.50
19	29.49	229.49 7.50
20	29.49	229.49 7.50
23	29.49	229.49 7.50

Date of Examination	Ewe tag Number		Notes
	29.49	229.49	7.50
6.vii.56	++	++	++
10	+	+	+++
14	++	++	+++
16			++
20			++
27		++	++
Ewe 229.49 slaughtered: Ovaries recovered			
5.ix.56	+		+
15	++		+
27	+		++
3.x.56	+		++
12.xi.56	+		+
18	..		+
19	+		+
20	++		+
3.xi.56	+++		+++
4	++		+++
18	++		-

Laparotomy on 229.49: Ovaries not located

Date of Examination	Ewe tag Number	Notes
19	29.49	29.49 Mid Ventral Laparotomy: Ovaries not located.
22	229.49	
10.1.57	229.49	
5.11.57	229.49	
15	229.49	

A P P E N D I X IV

Arborization in entire ewes after ODB injections

Ewe No.	Dose ODB. (ug.)	Day injected after tupping	P L	Appearance of PL after ODB (hours)	Cycle length (days)
24	200	11	+	8 - 24	*
17	200	11½	+	< 8	*
26	100	10	+	< 8	*
7	50	12	+	< 8	19
35	50	11	+	16 - 24	19½
29	40	11½	-	-	16½
113	40	12	+	24 - 32	15½
22	20	11	+	16 - 24	17
1	20	11	+	16 - 24	17
1	10	11½	-	-	15½
22	10	10	-	-	17
29	10	11	-	-	16½
1	5	10½	-	-	15
35	5	11½	-	-	17
113	5	11½	-	-	18
1	200	6	+	< 8	*
26	150	6	+	8 - 24	16
35	150	6	+	8 - 24	*
17	100	6	-	-	15½
17	100	7	+	8 - 24	**
24	100	6	-	-	16½
26	100	6	+	8 - 24	16
113	100	7	-	-	17
113	100	7½	-	-	*
1	50	6	+	8 - 24	16
24	50	6	-	-	17½
26	50	6	-	-	16
22	100	3	+	8 - 24	17
35	50	3	+	8 - 24	16½
24	25	4	-	-	16½

* Cycle length outside normal range.

** Ewe slaughtered before expected time of oestrus.