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# CERVICAL MUCUS ARBORIZATION

## ITS USE IN ASSESSING OVARIAN ACTIVITY IN THE EWE

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MANY OF THE GROSS physiological processes affecting reproduction in animals have already been elucidated. The availability of the ovarian hormones in relatively pure form has further allowed confirmation of their role. That there is general agreement and confidence in the findings of much of this work is suggested by the increasing application of steroids to animal production. However, it is apparent that, for a thorough understanding of the basic mechanisms controlling reproduction, it will be necessary to study in detail the quantitative changes in hormone levels and their associated effects in the animal.

Progress in the investigation of quantitative changes is dependent upon the use of suitable techniques of study. Many criteria have been utilized to study ovarian activity and ovarian hormone levels. Unlike some large species, palpation of the ovaries per rectum is not possible with the ewe. Oestrous behaviour and visual observation after laparotomy thus remain the more direct methods for assessment of ovarian activity. Biological assay of body fluids and excretory products has been successful, but is time-consuming. To avoid the attendant problems of the bioassay, chemical methods are being developed but as yet have met with only limited success. In addition biological endpoints obtained from within the animal have been used. For example, the characteristic vaginal smear changes in the ewe have recently been found satisfactory (Robinson and Moore, 1956).

In this paper some results of investigations of ovarian activity in ewes utilizing certain changes in the cervical mucus are reported.

### Review of Literature

Papanicolaou (1945) noted that, when the cervical mucus from women was spread to dry on a microscope slide, a characteristic crystallization occurred. Several workers have

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described the crystals as fern-like or palm-like and the process as arborization. The phenomenon was noticed several days before and after ovulation, but during the remainder of the menstrual cycle a cellular smear consisting mainly of epithelial cells and leucocytes was seen. Similarly, arborization in the cow occurred from approximately 3 days prior to oestrus until 9 to 11 days post-oestrus (Garm and Skjerven, 1952; Bone, 1954).

Investigations into the relationship between arborization and ovarian activity showed the phenomenon to occur naturally under the influence of oestrogen, while the hormone of the corpus luteum, progesterone, inhibited the reaction. Thus, ovariectomized women and postmenopausal subjects have shown arborization after oestrogen therapy, but the patterns disappeared soon after the injections ceased.

The mechanism of arborization has been studied by Zondek (1954). He found arborization to occur when a protein complex was exposed to electrolytes and the mixture completely dried. This is a non-specific process since nasal mucus and many other mucous secretions of the body also produce patterns. However, Zondek suggests that these other mucous secretions produce crystallization at all times, whereas cervical mucus produces arborization only at certain periods, depending upon the presence of oestrogenic hormone. Later work by Henderson (1956) and Andreoli and Porta (1957a,b) suggests that nasal mucus and saliva are also under the influence of hormones of the sexual cycle.

In summary, it may be said that during the oestrous cycle arborization occurs before, at and after ovulation, and is caused by oestrogenic hormone, allowing the release of electrolytes from the cervix. The cellular smear during the remainder of the cycle is caused by (a) the inhibiting action of progesterone, or (b) the lack of oestrogenic hormone. In pregnancy a different set of conditions exists. The cells of the cervix appear to lose their ability to secrete electrolytes and arborization is not characteristic.

### **Materials and Methods**

In this paper some results of observations made on New Zealand Romney ewes run at Massey Agricultural College during 1956 and 1957 are presented. The following animals were studied:

- (a) Ewes experiencing normal oestrous cycles.
- (b) Ewes experiencing oestrous cycles during which an injection of oestradiol benzoate (ODB) had been given.
- (c) Pregnant ewes.

Two raddled vasectomized rams were run continually with the cycling ewes which were checked twice daily for tuppung marks. For collection of mucus all ewes were held in dorsal recumbency by an assistant.\* Mucus was collected using a perspex speculum and glass rod and spread on glass slides. Smears after drying were examined within 2 to 4 hours at a low magnification with reduced lighting. The slides were classified as either positive or negative according to the presence or absence of arborization (fern structures). Figures 1 to 6 show typical examples encountered. It was apparent that considerable variation existed both in type of pattern, as well as in the intensity of pattern formation. Further, in some cases only a small amount of fern structure appeared and some doubt existed in classifying these smears. In this paper, only definite fern formation was scored as positive. Smears mainly of cellular material with slight signs of atypical material were regarded as negative.

The ewes which were injected with oestrogen (ODB in peanut oil) were given only a single injection in any one cycle. Oestrogen was given only in the period 3 to 10 days post-oestrus.

## Results

### CYCLING EWES

Examination of smears taken daily during 63 oestrous cycles showed arborization to be present mainly near the time of heat and presumably ovulation. These cycles were all within the normal range of 14 to 19 days as suggested by McKenzie and Terrill (1937) and others. The occurrence of arborization during the cycle is shown in Fig. 7.

A further 5 oestrous cycles were approximately twice the normal length. This suggested the possibility that ovulation without heat (silent heat) had occurred. Silent heat was presumed in these ewes since arborization occurred on days similar to those found for animals undergoing a 14 to 19 day cycle. In addition one ewe was slaughtered several days after the presumed silent heat and ovulation confirmed.

### OESTROGEN INJECTED EWES

The above results for the normal oestrous cycle showed that arborization was absent during the period from approximately the third until the fourteenth day of the cycle. At this time the corpus luteum is present (Warbritton, 1934). During this period also progesterone is at a high level (Edgar and

\*In subsequent work, mucus has been recovered from ewes restrained in a standing position.



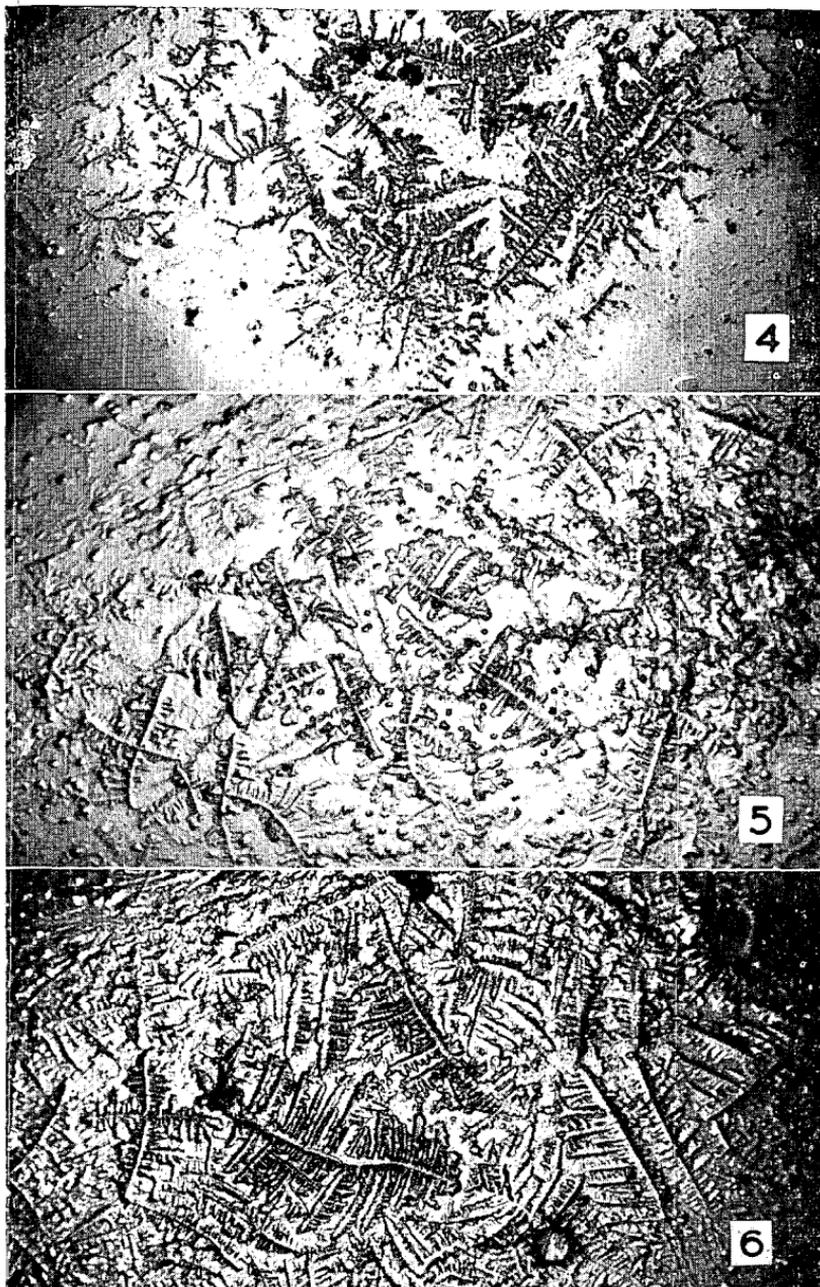


Fig. 1: Negative mucus, arborization absent ( $\times 125$ ).

Figs. 2 to 6: Positive mucus, arborization present in increasing amounts—  
Fig. 2 least development, Fig. 6 greatest development ( $\times 125$ ).

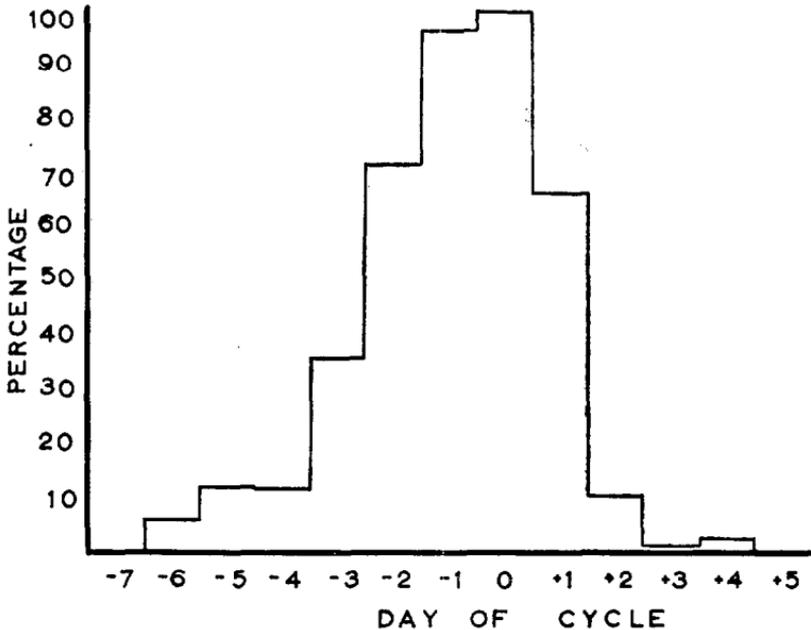


Fig. 7: Arborization during oestrous cycle.

Ronaldson, 1957). Thus it was of considerable interest to determine what quantities of injected oestrogen would be necessary to counteract the fern-leaf inhibiting action of progesterone from the corpus luteum. Thus ODB as a single injection was given during the luteal period.

The minimum quantities of oestrogen which produced arborization in all animals treated is shown in Fig. 8.

Although the number of oestrous cycles was limited, there did appear to be differences in the quantity of ODB required to produce arborization on various days of the cycle. At mid-cycle, 150  $\mu\text{g}$  ODB has always given a total response whereas 100  $\mu\text{g}$  ODB appears marginal. Both these quantities are greater than that required during the early and late luteal phases.

#### PREGNANT EWES

In the pregnant woman, arborization has been characteristically absent. As an example, Zondek (1954) was not able to promote arborization even when doses of up to 270 mg stilboestrol and 90 mg oestrone over nine days were administered. In sheep, previous work (McDonald and Raeside, 1956; McDonald and Raeside, unpublished data) indicated an absence of fern formation during pregnancy. This fact, together with

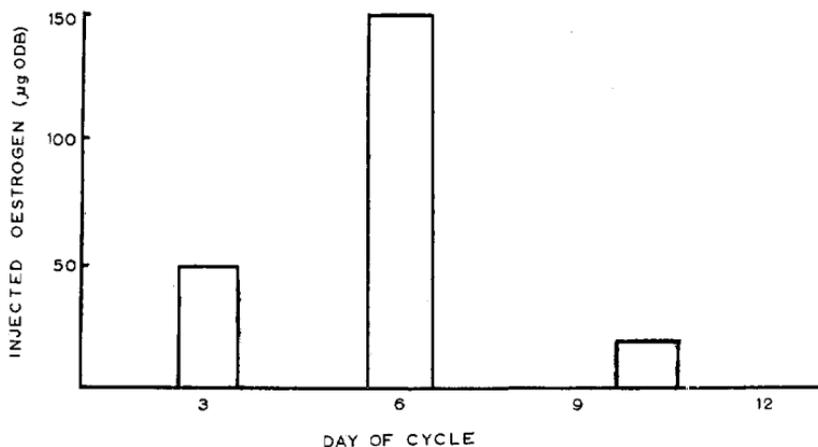


Fig. 8: Levels of ODB promoting arborization during the luteal phase.

some knowledge of the requirement of oestrogen necessary to produce arborization against a fully functional corpus luteum of the oestrous cycle, suggested the possibility of a pregnancy diagnosis along the following lines.

The test animal is given a single injection of ODB of at least the quantity necessary to produce fern patterns at the height of luteal activity. During the following day the cervical mucus is examined. Work with entire and ovariectomized ewes (Raeside and McDonald, unpublished) indicated that, if arborization was to occur, it did so within 24 hours of oestrogen treatment. If arborization is produced, the ewe may be diagnosed as non-pregnant. In this case, the oestrogen probably counteracted the progesterone from the corpus luteum of the cycle. When no crystallization occurs, the animal may be regarded as pregnant. In this case, the cervix is not stimulated by the injected oestrogen and arborization fails.

Seventy-two ewes, mainly full-mouth, were studied. These had all been tupped 2 to 5 weeks previously. Each was given 200 µg ODB in 1 ml of peanut oil. Smears were taken before and after injection. The results of this pregnancy diagnosis are given in Table 1.

The large number of false positive diagnoses is important. Thus eighteen animals showed arborization after oestrogen treatment, yet lambed. Nine of these eighteen ewes also exhibited some crystallization the day before the injection. This is difficult to account for, since the corpus luteum of pregnancy is likely to be active at this time. However, the possibility that a hormonal imbalance existed at this time may be suggested. The simple solution that contamination of the mucus with

TABLE 1: PREGNANCY DIAGNOSIS IN ROMNEY EWES 2 TO 5 WEEKS AFTER MATING.

Diagnosis	Pregnant	Non-pregnant
Number correct	50	4
Number incorrect	18	0
Percentage correct	73.5	100

electrolyte took place prior to examination under the microscope is considered unlikely.

The remaining nine ewes that showed 'ferns' after oestrogen treatment suggest the possibility that up to 2 to 5 weeks of gestation the cervix may still be responsive to injected oestrogen and thus release electrolytes into the mucus. The time of gestation at which the cervix is no longer responsive to oestrogen will be further investigated, since it determines at what time the test can be undertaken. In this respect, the accuracy of diagnosis is likely to be influenced by the stage of gestation.

### Discussion

It must be stressed that the results presented are of a preliminary nature because only a small number of animals have yet been investigated. However, the results are fairly consistent and some degree of confidence can be placed in them.

A problem encountered in this work has been the difficulty of collecting cervical mucus free of vaginal material, which is mainly of a cellular nature. Unfortunately, with the ewe it is not normally possible to collect mucus from well within the cervix owing to the tightly constricted lumen of the organ. This limitation does not apply to the same extent in the human and cow where cervical mucus can be got relatively free of vaginal material. Bone (1954) has shown that vaginal mucus from cows, as distinct from cervical mucus, gave a high incidence of false reactions and similar findings have been noted in this work.

The basis of this investigation was to ascertain whether the arborization phenomenon could be used in studying ovarian activity in sheep, particularly during the oestrous cycle. The results have shown the presence of fern-like crystals near the time of ovulation and their absence during the luteal phase. Further, in five cycles silent heat has been detected but accurate timing of ovulation could not be made. As well in this paper, the use of cervical mucus as the basis of a pregnancy diagnosis has been developed.

Bone (1954) and Scott Blair and Glover (1957) have reported attempts to diagnose pregnancy in cattle utilizing the

presence of 'ferns' to represent the non-pregnant state and cellular mucus to indicate pregnant cattle. However, this method does not take account of the fact that from 9 days post-oestrus until 3 days before oestrus, the mucus is normally negative and consequently any non-pregnant cows with functional corpora lutea will be falsely diagnosed as 'pregnant'. In the ewe, arborization is also absent near midcycle but may be induced by an injection of 150  $\mu\text{g}$  ODB. In this pregnancy diagnosis, a slightly higher level (200  $\mu\text{g}$ ) of ODB was used. The lambing results suggest that this higher level did not cause hormonal upsets and possible abortions.

The time in gestation at which diagnosis can be accurately made is important. Bone did note that, where accurate records were kept, pregnancy could be indicated as early as ten days after conception. The ewes in the above tests were examined 2 to 5 weeks after mating. As well, tests were carried out on a few ewes in the second, third and fourth months of pregnancy and somewhat better results were obtained.

### Acknowledgments

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### DISCUSSION

Q.: Arborization during the normal cycle shows a scatter at oestrus as do other biological tests and unfortunately therefore can be only partially successful at defining the time of ovulation. I was interested in the finding that lower amounts of oestrogen were sufficient to induce arborization between days 9 and 12 of the cycle since our chemical determination

indicated that the level of endogenous progesterone was maintained at a high level until day 16 or 17, when it fell rapidly.

I wonder if the false positives in some ewes which were pregnant could be due to the fact that between ewes there are very considerable differences in the level of ovarian blood progesterone. Animals having a lower level of progesterone in the blood might then respond to the injection of oestrogen and thus provide the 'false positive'. Further, we have found that until approximately day 35 the level of progesterone is low but rises after that time. It was of interest therefore to note that the authors had obtained better results with their pregnancy diagnoses after the 5th week.

There is little doubt that there is an effect of the oestrous cycle on the discharge of nasal mucus. Have the authors looked for arborization in nasal mucus?

A: : We have examined nasal mucus collected from ovariectomized ewes and found that arborization could be induced following oestradiol benzoate treatment. However, the mucus from some of the castrate ewes even before the injection showed some signs of pattern formation. The patterns in this case were not the typical type seen after the oestrogen treatment. If, however, ovariectomized ewes do normally show arborization then this would suggest that nasal mucus is not specific to circulating hormones. This situation has been suggested for the human by Zondek (1954).

DR. JOHN HAMMOND: : It seems that the method described by the authors offers a method of detecting the 'still heats' which precede the breeding season. The method of pregnancy diagnosis described here bears some resemblance to a Japanese method where oestrogen is injected at the 15th day of pregnancy (at a level necessary to produce heat in spayed ewes).

Q: : *On the question of the level of oestradiol benzoate, for the pregnancy diagnosis, would not more consistent results be obtained by the use of two injections at a lower level rather than a single injection?*

A: : The results presented in this paper for the pregnancy diagnosis represent our first attempt using a single dose of ODB. Two doses, spaced a few days apart, may well give better results.

Q: : *What are these electrolytes which are responsible for the crystallization patterns, how specific is their effect and how does their level of secretion vary during the cycle?*

A: : Zondek (1954) has reported on the substances which are involved in arborization. A number of salt solutions like sodium chloride, potassium chloride and potassium bromide, can promote the reaction, but several other solutions, including barium chloride, calcium chloride, sodium bromide, potassium nitrate and sodium iodide, failed. It was shown also that electrolytes were essential to the arborization process and many mucous secretions which did not crystallize when dried could be activated to form patterns by mixing the dried mucus with dilute salt solutions. There appears to be little work reported on the level of electrolyte secretion during the cycle.

Q: : *In your earlier work did you not find that the appearance of the fern pattern was temperature conditioned; could this effect have contributed (in reverse) to the failure to diagnose a proportion of pregnancies?*

A: : Yes, we did find that slight heating of slides before examination was often necessary to ensure obtaining dried mucus. Thus as a routine procedure all slides are now heated. In the work reported in this paper any 'temperature effect' was not a limiting factor.