INVESTIGATIONS ON THE VARIATIONS IN THE CRYSTAL PATTERNS OF CERVICAL AND NASAL MUCUS DURING THE ESTROUS CYCLE IN EWES

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INTRODUCTION

Investigations on cervical mucus during the last twenty years and more recently on nasal mucus have provided a number of interesting observations to determine the role of these glandular secretions in assessing the hormonal activity of the ovary. It has been noted that the cervical mucus undergoes cyclic changes in its physicochemical properties during the recurring follicular and luteal phases of the menstrual and estrous cycles in several species. These changes have been known to be much more noticeable particularly during heat, or at about the time of ovulation in women. The importance of a fluid condition of cervical mucus to provide a congenial environment for the preservation and ascent of the spermatozoa to the uterine cavity soon became evident.

Fundamental differences in many physical properties of cervical mucus have been well documented in the literature. One such property of cervical mucus to form crystal patterns was first discovered by Papanicolaou in 1946, who observed relative changes in the degree and form of crystallization. These changes became intensive at the follicular phase and reduced or absent in the luteal phase of menstrual cycle, with a close relationship between this phenomenon and the rhythmic endocrine activity of the ovary. These observations, thus, opened a new vista in the further study of evaluating the follicular activity as well as other events of reproduction. Although, the significance of this phenomenon in animal reproduction has been recognized, research dealing with it in evaluating the stages of estrous cycle of sheep has been very limited. A striking similarity, however, could be established in the results obtained in human, bovine and other species of animals. The success with these species has encouraged investigations to utilize them as a basis for devising certain useful methods of detecting estrus and predicting other events in the reproductive cycle of females.

The present study has been undertaken to investigate whether the morphological variations in crystal patterns of cervical mucus are in any way related to the various stages of estrous cycle in ewes. An attempt is also made to study if the similar patterns could be detected in the nasal mucus.

REVIEW OF LITERATURE

Cervicovaginal mucus has been known in the past to undergo cyclic changes during the various phases of estrous or menstrual cycle, becoming more pronounced in amount and other characters at the time of heat, or during the ovulatory phase of menstrual cycle. The changes primarily noted in amount, viscosity, elasticity, and spinnbarkeit (thread forming tendency) have now been regarded as a function of hormonal activity of the ovary. The rhythmic endocrine activity of the ovary is known to govern the secretion of mucus in the genital tract. Recently, various criteria have been devised to measure these physical properties of cervical mucus and the results obtained are not always encouraging, largely because of the relative paucity and seemingly indifferent nature of the mucus.

Periodic discharge of a large amount of thin, viscous and translucent mucus from the external genitalia of cows has long been known to be associated with the estrus behaviour in cattle. This activity of the mucus secretion was thus considered as one of the most reliable and easily recognizable signs of heat in cows. Polge (1960) elucidated the species differences in the quality and quantity of cervical secretions of cattle, sheep, horses and pigs and found that the cyclic changes in the mucus were more pronounced in cows, least obvious in sows and intermediate in mares and ewes.

In the earliest attempts to apply the vaginal smear technique in a more comprehensive analysis of the menstrual and reproductive functions in females, studies were conducted to relate the cellular contents in the cervicovaginal mucus with the phases of estrous cycle in ewes. Cole and Miller (1931, 1935) investigated the cyclic changes in the mucus collected with a small dental spatula inserted 3 to 4 inches into the vagina. Early estrus was characterized by a profuse flow of transparent watery mucus; metestrus by a copious dry cheesy mass consisting chiefly of large squamous epithelial cells; and diestrus by a scant smear consisting of epithelial cells and a variable number of leucocytes. During early estrus a profuse inflow of voluminous and transparent mucus originating from the cervix was invariably present in the vagina by the fourth hour of heat in the ewes. The secretory activity of the epithelial cells lining the cervical canal was found to be more predominant during proestrus and early estrus. Collecting the samples daily from the external os of the cervix, Grant (1934) found a detectable quantity of the mucus several hours before the beginning of heat and the maximum deviations in the appearance of the mucus ranged from 24 hours before to 8 hours after heat. However, the maximum accummulation of mucus in the anterior part of the vagina rarely occurred before the well-advanced stage of estrus in ewes. A copious flow of clear, watery mucus during estrus resulted from the liquefaction of the tenacious and stringy mucus which collects in the cervical canal during the diestrus period. The mucus secretion in the genital tract of ewes was thus regarded to be the phenomenon of proestrus since the detectable quantity of mucus in the cervical canal was noted several hours before the onset of estrus. While noting the similar type of cyclic changes in the mucus secretion, Radford and Watson (1955) observed the mucus with cheesy consistency appearing within 3 days after the cessation of estrus in 25 Merino ewes included in their experiment.

In a group of 40 non-pregnant ewes, McKenzie and Terrill (1937) noticed a continuous secretory activity of the columnar epithelium of the cervix. In certain cases a tendency to build up the mucus in the cells during the luteal phase of estrous cycle was observed. During late luteal and proestrual stages, the mucus was eliminated from the cervix and a thin, fluid mucus appeared in the vagina during estrus period in ewes.

The increased amount of mucus secretion at the time of heat was found to be due to the incorporation of water in the mucus present in the cervical canal. Woodman and Hammond (1925) studied the cyclic activity of mucus secretion in cows and also analysed the mucus for water content during the various phases of estrous cycle. The percentage of water in the pre-estrual samples ranged from 83.7% to 86.8%; whereas, in post-estrual mucus the values ranged from 85.2%

to 89.2%. In illustrating the increased quantity of the mucus during estrus, these research workers pointed out that the thick and tenacious mucus present in the cervix prior to the beginning of heat swells considerably to its original volume due to incorporation of water which causes it to form a slimy liquid which flows into the vagina at the time of heat.

The comparable changes in the mucus secretion of women have been observed during the ovulatory phase of menstrual cycle. Large quantities of clear, transparent and relatively acellular mucus have been observed by Seguy and Vimeux (1933) between tenth and fifteenth day of menstrual cycle.

Viergiver and Pommerenke (1944) made qualitative and quantitative appraisals of the cyclic changes in the cervical mucus secretions of women by weighing the amount of mucus secreted. They also measured the viscosity by drawing the mucus in a small glass capillary tube of about 0.4 mm. diameter. In a total of 151 observations made, the increased quantity of mucus was observed from 8th through 18th day; whereas, the viscosity of the mucus was lowest during the 8th through 16th day of the menstrual cycle. In subsequent studies, Pommerenke (1946) noted a peak of mucus secretion before the 14th day of the normal menstrual cycle of 28-day duration. This peak of mucus secretion was correspondingly accompanied by the maximum decrease in the viscosity and the samples were found to contain 98% water as compared to 94% in the mucus collected during post- and preovulatory phases of menstrual cycle.

Seguy and Simonnet (1933) related the mid-cycle changes in the cervical mucus to the elimination of the greatest amount of estrogen in the urine and this was seen to be associated with ovulation as confirmed by the inspection of the ovaries after laparotomy. Bennet, Jr. (1942) assayed the estrogen excreted in the urine of 80 menopausal women and found a very small amount of urinary estrogen in 324 samples examined. A scanty, viscous cellular mucus was recovered from these women. Following the treatment with estrogenic compounds, the secretion of a clear, abundant, and alkaline acellular mucus was obtained.

In 1938, Sjövall reproduced the cyclic changes in the cervical mucus of castrated guinea pigs by the use of estrogen and progesterone. It was postulated that the mucosal cycle in the cervix is regulated by the cyclic activity of estrogen secreted by the ovaries. Abarbanel (1946) provided substantial evidence of the ovarian hormonal control by producing a clear, glistening watery mucus within 48 to 72 hours after a treatment with various preparations of estrogen in 25 ovariectomized and hysterectomized women. Treatment with 5 to 10 mg. of progesterone caused a remarkable reduction in the volume of the cervical mucus which became more viscous and impermeable to spermatozoa. Pommerenke and Viergiver (1946) used various doses of alpha-estradiol benzoate to reproduce the changes in the mucus characteristic of mid-menstrual cycle in the castrated women. It was noticed that the increase in amount of mucus secreted by the cervix is dependent on the quantity of the hormone administered. The cervical mucus characteristic of estrus was produced by Glover (1959) in ovariectomized cows by the subcutaneous administration of hexoestrol in the doses ranging from 0.1 mg. to 2.0 mg. Despite the hormonal treatment at a constant rate, the decrease in mucus secretion and the increase in the consistency became apparent from about the 4th day after the treatment.

Robinson and Moore (1956) studied the effect of ovarian hormones on the thickening of mucus normally noticed after the heat period in normal ewes. They employed 3 groups of spayed ewes in their experiment. Group 1 consisted of untreated ovariectomized ewes. In group 2 the cycles were induced by 2 injections of 25 mg. estradiol benzoate (ODB) given at 16 days interval. In addition to estradiol benzoate (ODB), ewes in group 3 received two daily injections of 3 mg. progesterone for 12 days, commencing 3 days after the estradiol benzoate treatment and terminating 2 days prior to the subsequent injection of ODB on the 16 day. The ewes of group 4 served as controls and were experiencing normal estrous cycles in response to endogenous hormones. It was found that the untreated spayed ewes always had scanty mucus while those ewes treated with hormones showed a copious flow of mucus on the day corresponding to estrus. The viscosity of mucus in the normal intact ewes became apparent 1 day after estrus; whereas, a comparable degree of thickening in the ODB-progesterone (group 3) treated ewes appeared a day later than that noted in the normal ewes. The overiectomized ewes treated with estrogen alone did not exhibit the comparable viscosity during the metestous and early diestrus period. It was suggested that the progesterone is largely responsible for the rapid thickening of mucus observed the day after estrus; whereas, the rhythmic flow of mucus on the day of heat in ewes was attributed to the influence of estrogen.

Since cervical mucus has been found to exhibit cyclic variations in the appearance and other physical properties, efforts were made to devise an objective method of diagnosing estrus in cattle. Scott Blair, et al. (1941) designed an instrument (the Oestroscope) to measure the physical (rheological) property of the cervical secretion known as flow-elasticity. The instrument consisted of a capillary glass tube of 11 cm. in length and 2 mm. in diameter was connected to the nozzle of an ordinary syringe and an open side-tube was fixed at right-angles between the shank and the 10 cm. mark. The flow-elasticity was measured by drawing the homogenized mucus into the capillary tube while the side tube was closed with a thumb. After the maniscus reached the 10 cm. mark, the mucus was then slowly forced out until the maniscus reached the 7 cm. mark and then by suddenly withdrawing the thumb from the side-tube, the recoil of the mucus along the capillary tube was recorded in millimeters. In 41 samples from cows in, or near, heat the mean values of elastic-recoil in mm. were 14.7±9.6 and for the mucus obtained from 143 cows in mid-cycle the values were 2.4±1.4 mm. They proposed that the values of elastic-recoil more than 5 millimeters should be considered as indicative of cows in or very near, heat.

Clift (1945) measured two rheological properties of human cervical mucus, namely flow-elasticity and spinnbarkeit, the phenomenon showed by liquids to be drawn into threads. The spinnbarkeit is measured by drawing away a coverslip placed on a blob of mucus and recording the length of the thread thus formed. The mid-cycle samples examined for flow-elasticity and spinnbarkeit gave an average value of 30 mm. and 13 cm., respectively. In the pre- and post-menstrual samples the elastic-recoil ranged from 8 to 10 cm. while the phenomenon of spinnbarkeit was absent.

Cohen _et al._ (1952) made observations on 16 women in order to demonstrate the relationship of spinnbarkeit to several other cyclic phenomena occuring during menstrual cycle, such as basal body temperatures, vaginal smears, quantity of cervical mucus secreted and longevity of spermatozoa in the mucus. At the time of maximum production of cervical mucus, the spinnbarkeit reached a length of 10 to 20 cm. A close correlation between maximum spinnbarkeit and optimal cornification in the vaginal smear was noticed. Similarly, the longevity of spermatozoa paralleled the degree of spinnbarkeit. The artificial insemination performed on the days when the spinnbarkeit was maximum resulted in successful conception in the women included in this study. Speck and Halter (1956) used spinnbarkeit test on 38 patients to obtain an estimate of time of ovulation. Fourteen women with a value of 12 centimeters, or better, conceived following natural service. The researchers suggested the spinnbarkeit test to be more accurate in pinpointing the ovulation than the basal body temperatures. Using non-return to service as the criterion of successful insemination, Panigrahi (1964) found that out of 40 cows, 34 (85%) animals inseminated at the time when spinnbarkeit ranged from 5 to 12 centimeters did not return for the second insemination. In the appraisal made on various techniques of determining ovulation, Cohen and Hankin (1960) suggested the maximal spinnbarkeit as the best indicator of optimal fertility.

Since the properties of cervical mucus change significantly at the time of estrus or the ovulatory phase of the menstrual cycle, studies were undertaken to relate these changes with penetrability, and survivability of spermatozoa in the mucus samples obtained at different phases of the sexual cycle. Lamar <u>et al</u>. (1940) correlated the penetrability of human spermatozoa with the various properties of cervical mucus obtained from 58 normally cycling women. The micro-tubes and a barrier marker air bubble was used to measure the rate of progression of spermatozoa. The samples obtained during the period commencing from 9th through 19th day showed penetrability to spermatozoa. The maximum rate of penetration on the day 14-15 was related with the peak in freedom from leucocytes, pH (except during menstrual flow), amount of mucus secreted, and greatest longevity of sperm in the mucus. Mucus samples collected during 20th day to the next menstruation presented a complete barrier to the entry of spermatozoa. Harvey (1954) observed the satisfactory penetration of spermatozoa between the 9th and 20th day after menstruation, with a significant peak occurring at the 15th and 16th day before the next menses. In vivo studies, Bergman (1953) determined the relationship of passage of spermatozoa with the physicochemical properties of cervical mucus in postcoital tests performed on 31 women. It was found that the spermigration was completely inhibited in the cervical mucus having 5.5 percent or more dry matter content. The ovulatory phase characterized by watery and clear or translucent mucus was found to provide optimal conditions for the penetration of spermatozoa in the uterine cavity.

The behaviour of spermatozoa in bovine cervical mucus at varying stages of estrus was studied by Herman and Horton (1948). The viscosity of cervical mucus was found to be lowest during the first 6 hours of heat; whereas, the maximum penetration of spermatozoa occurred in the mucus collected during the first 6-10 hours of heat. Subsequently, Roark and Herman (1950) noticed the average maximum penetration rate in bovine mucus collected at 10 to 13 hours after start of the estrus period. Sperm motility servival time was maximal in the mucus collected during full and late estrus. These workers found a direct relationship of the spermatozoa penetrability and the survivability with the surface tension and flow-elasticity of the mucus, and an inverse correlation with leucocyte concentration and the pH of bovine mucus. The duration of motility of spermatozoa under relatively anaerobic conditions at 37°C. was found to be longer in cervicovaginal mucus and uterine fluid from cows in or near estrus than at other stages of estrous cycle (Olds, 1956; Olds and VanDemark, 1957).

Bacic (1962) studied the relationship between the quality of estrual mucus and the rate of conception in cows. The conception rate was 79.83% for cows with clear mucus and was reduced by 11.50% for those with increasing amounts of cheesy material in the mucus. The conception rate was 10.6-46.4% in cows having traces of mucus in the vagina.

From the research concerned with sheep, cattle and human females, it is possible to deduce that the cyclic variation in the various physical properties of cervical mucus are under the sole influence of the ovarian hormones. The prominent change in the mucus at the time of heat or during the ovulatory phase of menstrual cycle provides an optimal environment for the spermigration for effective conception. Since the cervical mucus is not a truly viscous fluid, there are many handicaps to an accurate estimate of the various parameters used to measure the changes in its physical properties. It is, therefore, imperative to devise some measure which would be adequate to diagnose the phases of estrous cycle more precisely.

The Phenomenon of Arborization in Relation to Menstrual Cycle. The discovery of a crystallization phenomenon, first described in the cervical mucus of women, added new impetus to more comprehensive studies to utilize it as an aid in animal reproduction, in timing insemination, detecting silent heat, and predicting the onset of estrus.

By fixing human vaginal, endocervical or endometrial smears in silver nitrate, Papanicolaou (1945) first noticed a characteristic brown staining secretion (argyrophil secretion) becoming more abundant at the peak of follicular activity. This secretion was found to be reduced in quantity after ovulation. In reproductive disorders associated with reduced ovarian activity, such as amenorrhea and menopause, the argyrophil secretion was absent. After estrogen administration in such patients, the increased amount of secretion was produced. These observations thus established a fundamental relationship of the mucus secretion with the function of ovarian hormone in females. He, therefore, ascribed a specific value to these findings in the study of the normal cycle and the evaluation of follicular activity and estrogenic actions.

Subsequently, Papanicolaou (1946) first demonstrated the occurrence of crystallization in the cervical mucus of women and its relationship with the phases of menstrual cycle. He found that the cervical mucus collected particularly at the peak of follicular activity, or at about the time of ovulation, when spread on a clean glass slide and allowed to dry, produced a characteristic pattern of heavily indented leafy projections. At other times of the cycle, these crystals appeared in round droplets of various sizes in single or bifercated rods or in starlike formations. The formation of these crystals was demonstrable only in the dry smears which were not fixed in a fixative consisting of 95% alcohol and ether. He believed that the variability noted in the form and structure of the crystals is determined by the corresponding changes in the viscosity of mucus.

In more comprehensive and critical studies, Rydberg (1948) related the changes in arborization with the phases of normal menstrual cycle, and with reproductive disorders associated with endocrinal disfunction of ovary. He noticed the occurrence of massive and extensive crystallization at about the time of ovulation when the cervical mucus was less viscous but limpid and very stringy. In describing this type of mucus, he used the term "glair filante." In the days immediately before menstruation and during the periods of the cycle when the mucus was not transparent and stringy, the crystal formation was thinner, smaller and irregular in shape, occupying only a limited parts of the preparation. These cyclic variations in the crystallization of the mucus led him to believe that this phenomenon depended on the hormonal activity of the ovary. This was further confirmed by producing the typical crystal pattern in women with reduced ovarian function after estrogenic therapy. Rydbery, (1948) indentified the substance responsible for the crystal formation. It was found that the crystals were composed of sodium chloride, with mucin necessary for the formation of the characteristic patterns. The explanation of the brown staining mucus (argyrophil secretion) obtained by Papanicolaou (1945) after treating it with silver nitrate was established to be due to the precipitation of brown colored silver chloride.

In a study involving 20 women with a known normal ovulatory cycles, Roland (1952) observed the presence of "fern-like" crystals from the 5th or 7th day of cycle to the 20th or 22nd day. During the remaining period, only the cellular type of patterns were noticed. In another group of 30 women experiencing anovulatory menstrual cycles, the "fern-like" crystal structures persisted throughout the cycle. Mucus samples obtained from 40 menopausal women, some of which were castrated with radiation, did not exhibit the crystal patterns in their mucus smears. When the 10 women selected from this group were injected with 2 to 3 doses of alpha estradiol benzoate, the phenomenon of crystallization appeared several days later. While discussing the possible influence of estrogen and progesterone on the sodium chloride and mucin metabolism, Roland (1952) suggested the use of a grading system based on the quantity and type of "fern" as a means of determining the relative level of estrogen in the body.

Nouel and Aguero (1953) studied 64 women with regular cycles and showed the presence of crystals from the 8th through 15th day. In a group of 51 women with irregular menstrual cycles, the phenomenon was not restricted to a particular phase but was seen to be scattered throughout the menstrual cycle.

Campos de Paz and Costa Lama (1953) compared the degree of crystallization with the cellular content, amount of mucus and the spinnbarkeit (threadability) in a group of 6 normal women. The degree of crystallization which was of less intensity (atypical) soon after menstruation was found to increase subsequently to its peak activity during the middle of the cycle. The height of crystallization at mid-cycle was accompanied by minimum cellular content and the maximum threadability. These mid-cycle changes in the mucus were then progressively followed by the decrease in the degree of crystallization with a corresponding increase in the cellular content and the minimum threadability, gradually culminating in the disappearance of arborization at the end of the cycle. Twenty-one menopausal women did not exhibit the arborization in the mucus smears. When 9 women were injected with 1 mg. of estradiol benzoate, the phenomenon of crystallization appeared in 5 to 11 days after the cessation of the treatment. The administration of progesterone was found to be ineffective in initiating the response in the 4 menopausal women.

Sylvian and Borno (1956) suggested the use of this technique for the diagnosis of an excess or deficiency of ovarian hormones. In their studies, the degree of crystallization was graded from 0 to 100 percent and a normal curve was drawn for each cycle. In a group of 90 women studied, the curve which began at 0 percent immediately after menses, reached the peak value of 100 percent during 1 to 4 days prior to ovulation. In another 49 patients with menstrual disorders, 3 main types of abnormal curves were noticed: The monophasic curves around 100% which indicated an excess of estrogen; low curves which were suggestive of a serious impairment of ovarion function; and irregular curves which denoted a hormonal imbalance.

Urdan and Kurzon (1955) observed the initial crystallization to be delicate and light in degree, progressively becoming intense and heavy with the increasing follicular activity reaching its peak at the time of ovulation. Within 24 hours following ovulation, crystallization became less intense and showed mixed patterns which consisted of disintegrated branching arms surrounded by an amorphous mass of cylindrical cells. Within the following 3 days, the cellular matter replaced the crystal patterns completely. On the basis of these observations it was claimed that the functioning of the corpus luteum could be recognized by observing the replacement of fern patterns by the cellular material in the smears.

The existence of several varieties of typical as well as atypical fern patterns during the normal cycle of 28 days duration was revealed by Roland (1962). He found the presence of typical crystal patterns on the days extending from the 12th to 16th day, when cellular matter was absent in the mucus smear. The atypical fern patterns seen during the 6th to 9th day were accompanied by cellular elements. The quality and quantity of fern found on days 10 to 12 and again on days 19 to 22 were similar. While interpreting these changes, Roland (1962) believed that in the early days of the cycle the decreased amount of estrogen, and in the luteal phase, the gradual increase in progesterone concentration determined the extent of typical and atypical arborization in the cervical mucus.

Several studies have indicated a positive relationship of arborization with the endocrine activity of ovary. Raffaele (1953), De La Feunte and Galvez (1954) and others have reported that the phenomenon of crystallization is the reflection of the estrogenic function of ovary. Yenez *et al*. (1953) showed that the intensity of arborization is directly proportional to the estrogenic activity of ovary. The crystals may disappear as a consequence of decreased estrogenic activity or due to the increase in progesterone activity. In their series of experiments, they found that the mucus smears reflected more accurately the hormonal activity than did the other methods of evaluation such as basal body temperatures or vaginal smears.

Finding the relationship between the crystallization of cervical mucus and the estrogenic activity of the ovary, Zondek (1954, 1957) devised a new method of determining the quantitative production of ovarian hormones in the body. The unit of measurement was designated as Cervical Mucus Unit (CMU). He found that a single injection of 1 mg. of estrone; 0.3 mg. of estradiol; 0.2 mg. of estradiol benzoate given in an oily solution was capable of producing arborization in the cervical mucus of castrated women. Thus, 1 mg. of estrone was shown to be equivalent to 1 CMU. It was also demonstrated that the stimulatory action of 1 mg. of estrone on crystallization could be counteracted by a 15 mg. injection of progesterone. With the help of CMU and the ratio of progesterone:estrone (P:E = 15:1), it was suggested that the quantitative production of ovarian hormones in the body could be determined.

The phenomenon of arborization has been investigated as a method of timing ovulation, but the results obtained are not very encouraging. Forman (1956) proposed the method of crystallization to be of accurate diagnostic value in timing ovulation in women. He suggested that in order to pinpoint the time of ovulation, the daily examination of cervical mucus in the preovulatory phase of menstrual cycle may be carried out. On the basis of a relationship between the typical drop in basal body temperature (BBT) and the ovulation in women, he stated that the quantitative peak of arborization which precedes the drop in BBT may be suggestive of ovulation. Zondek (1957) did not consider the palm leaf crystallization of cervical mucus as a test for the detection of the exact day of ovulation. He considered that this method could be utilized for the determination of the functional activity of the corpus luteum. According to Campos de Paz and Costa Lama (1953), the crystallization phenomenon is not a reliable test for the diagnosis of ovulation when performed on a single day during the luteal phase of the cycle. They proposed that the test may be carried out on different days of the cycle so as to compare the maximum crystallization during ovulatory phase to constitute a test for ovulation in women. Roland (1962) indicated the examination of two separate smears, one at midcycle and other before the menstruation in which the presence of true fern in the first and its absence in the second smear would be indicative of whether the ovulation had occurred in a given cycle. He does not consider this test to be of any significance in pinpointing the exact day of ovulation. In anovulatory menstrual cycles, the persistence of true fern patterns, particularly during the latter half of the cycle, would denote the continuous influence of estrogen secretion unopposed by progesterone.

Arronet (1957) evaluated the arborization as a method of timing ovulation in a selected group of 7 women of 27 to 38 years of age. While noticing the peak of crystallization somewhere around the middle of the cycle, he found that this peak coincided more precisely with the height of estrogen elimination in urine. He concluded that the intensity or any type of crystals and their duration would be indicative of estrogen activity of ovary and consequently the arborization method could not constitute a proof for timing the ovulation.

Arborization in Relation to Estrous Cycle in Animals. The predominance of crystallizing activity of the cervical mucus during the ovulatory phase of menstrual cycle and its functional relationship with the endocrine activity of ovary in women established a new approach for the analysis and the study of the phases of estrous cycle in farm animals. The investigations so far carried out are mainly concerned with bovine reproductive cycle.

The relationship of the crystallization phenomenon in estrous cycle was first studied by Campos de Paz and Costa Lama (1953) in cows, donkeys, mares and in a sow. Out of 14 cows examined on the first day of estrus, only 12 cows showed the typical crystal patterns. On the second day of heat, out of 9 cows, 8 animals showed the characteristic patterns. During diestrus, 7 of the 10 cows did not exhibit the crystallization. In the remaining 3 animals, 2 cows gave atypical patterns, while the only cow that showed the typical patterns was found to possess a cystic ovary. The results obtained in other species of animals remained inconclusive since the typical and atypical crystal patterns in mares and female donkeys appeared with great irregularity, and in one sow examined they did not find the type of crystallization normally seen in other animals at the time of heat. The utility of this test was suggested for the determination of the exact time for performing artificial insemination to obtain better results.

Garm and Skjerven (1953) noticed the appearance of atypical fernlike patterns on the 19th day and the abundance of typical crystallization on the 22nd day after the onset of behavioral estrus in cows. The crystal patterns were found to last for about 5 to 6 days subsequent to heat. During the peak of luteal activity, only traces or small amounts of atypical patterns were observed. These results were interpreted to indicate the role of ovary in governing the changes in the crystallization. The massive arborization was considered to reflect the increased follicular activity and its absence as the index of a functionally potent corpus luteum. These research workers also emphasized the importance of this technique for the detection of "silent heat" in cows. Coluzzi and Battistacci (1954) suggested from their studies in 10 Perugia cows that the characteristic fernlike pattern formation in the cervical mucus, as were first described in humans, could be used for the accurate diagnosis of estrus in cows.

Gamcik and Sevcik (1962) observed progressive changes in the degree of arborization from 2 to 3 days prior to heat until about the time of ovulation when it reached the height of its activity. The crystallization pattern similar to that observed before estrus was again seen 3 to 5 days after estrus. The cows with ovarian cysts showed the crystal patterns regardless of the heat period. This test of crystallization was considered suitable for the diagnosis of phases of estrous cycle and other abnormalities associated with it.

Bane and Rajakoski (1961) examined the mucus smears daily during the period in which estrus was expected, and every second day during the remainder of the cycle, from 9 heifers of the Swedish red and white breeds. The intensity of arborization in the air-dried mucus was coded as 0, +, ++ and +++ depending upon the extent of arboreal patterns covering the smears prepared. The maximum values (+++) characterized by the presence of arborized patterns throughout the smear were noticed at about the time of heat and were encountered from day 21 to day 3 of the next cycle. Heavy arborization (+++) on the first day of heat was seen in 94% of the specimens studied; on the other hand, no definite arborization was noticed in 85% of the cervical mucus samples examined between the 7th and 15th day of the cycle. Variation in degree of arborization between days of the cycle was highly significant ($P<0.001$). The authors suggested that the examination of dried cervical mucus could be used as a simple biological test for the study of variation in the endocrine activity of the ovaries.

Quayam and Venkatasami (1964) reported the results on fern patterns studied in 118 female buffaloes (Bos bubalis) brought for artificial insemination. In 4 other animals the cervical mucus was collected daily during one complete estrous cycle and the mucus smears were scored from 0 to

100 % on the basis of intensity of arborization. An average value of 95% was reached at the time of estrus. During metestrus and diestrus the values recorded were 35% and 20%, respectively. An average score of 5% was observed on the 12th and 13th day corresponding with the height of corpus luteum activity. In cows possessing cystic ovaries, the values went up to 80% whereas the score ranged from 0 to 20% in buffaloes with persistent corpora lutea in the ovaries.

Bone (1954) studied the crystallization patterns in the cervical mucus obtained from 20 living cows and additional information regarding the functional activity of the ovary and the state of pregnancy was obtained from 163 slaughtered cows. The 20 living heifers examined for arborization exhibited fernlike patterns from a period of about 3 days before to 9 days after estrus. The peak of corpus luteum activity was accompanied by the absence of crystallization. These observations indicated that estrogen stimulates the crystal formation whereas progesterone inhibits it. Additional evidence on this point was obtained from the examination of the ovaries of slaughtered cows. Of the slaughtered animals, 15 non-pregnant cows which did not exhibit the crystal patterns were found to possess corpus luteum attached to their ovaries.

The main purpose in the studies of Ghannam and Sorensen (Jr.) (1967) was to use the cervical mucus arborization technique for the diagnosis of early pregnancy in cows; however, some observations on the occurrence of crystallization in relation to estrus and/or ovulation were also made. They classified the crystal patterns into 4 main categories, such as, very marked patterns, marked patterns, mixed patterns and negative crystallization. The very marked patterns characterized by long ferns covering the entire smear, appeared 3 days before estrus (average 1.6 days) and continued about 7 days after estrus (average 3.0 days). The marked patterns comprised of short, curved, feather-like patterns present in the major portion of the smear, appeared before and after the very marked patterns. With relation to estrus, these marked patterns were seen 5 days before (average 3.4 days) and continued 8 days after estrus (average 4.7 days). During the luteal phase of each cycle, the pattern fluctuated between the smears showing some fern-like crystals accompanied by areas lacking crystallization, (mixed patterns) and the negative arborization with the former type predominating in the smears. The animal which had silent heat periods showed normal patterns typical of estrus. These workers found wide fluctuations in the shapes of the crystals and experienced difficulty in detecting ovulation through this technique.

Alliston et al. (1958) utilized the method of arborization for the diagnosis of approaching estrus in 10 virgin beef heifers. The mucus samples were collected from each animal at approximately 12 hour intervals from the 16th day after the preceding estrus until such time as estrus and/or ovulation had occurred. The arboreal patterns formed in the mucus smears were assigned numerical values on the basis of the following arbitrary categories: 1.---no patterns; 2.---patterns around bubbles only; 3.---typical patterns but covering less than one half of the total slide area; 4.---typical patterns but covering more than one half of the total slide area, but not complete; 5.---typical patterns over complete area of slide, both short and long ferns present; 6.---typical patterns over complete slide area, only long ferns present. These workers noticed the first indication of the fernlike patterns 84 hours prior to estrus. The average value then gradually increased to a maximum of 4.4 at the time of estrus and then declined prior to the average time of ovulation. Since the regression of the smear value on follicle size was non-linear, the data were subjected to multiple regression analysis and the deviations from linear regression were found to be highly significant ($P<0.01$). It was thus concluded that the extent of crystallization is not controlled by follicular size only.

Abusineina (1962) classified the fernlike crystals into 3 main pattern types in the cervical mucus collected daily for 80 days from 2 non-pregnant cows. In one cow the effect of estrogen injection and the additional implantation of stilbestrol (1 mg.) under the skin on the pattern type was also studied. The crystallization patterns were classified in the following types.

Type A: Crystal forms consisting of long, thin, wavy or curved stems, with clear well-developed venation and tiny subvenation; occurring in about 95% of the film. This pattern was detected when the mucus was translucent, acellular, elastic and easily obtainable from the cervix.

Type B: This pattern was nearest to fern-shape, with clear and well-developed venation and subvenation, occupying only 50% of the smear. This form was noticed when the mucus was semi-clear, elastic and easily obtainable.

Type C: Microscopically, the configuration was variable and classed as atypical. The stems were short, with or without irregular venation and subvenation. Sometimes, stellate, cruciform and linear patterns were noticed which were irregularly scattered over 2 to 5% of the film.

The type A crystallization was noticed once every 21 to 23 days and was found to be on the day nearest to ovulation as confirmed by rectal examination of ovaries. The type B crystal patterns occurred 3 to 5 days prior to ovulation and again 4 to 5 days afterwards. During the remaining part of estrous cycle, type C crystallization was noticed 4 to 5 days and the negative arborization persisted for 7 to 9 days. The cow implanted with stilbestrol showed the pattern B which changed to type A one day after the treatment. It was inferred that the characteristic changes in pattern types were governed by the level of estrogen in the blood. The peak of estrogen level was thought to be notified by the presence of type A pattern whereas the crystal types B and C appeared when the estrogen level was low. The absence of crystals were shown to be associated with the height of progesterone activity. It was suggested that these cyclic changes indicate stages of estrous cycle and also the actual day of ovulation in cows.

Howes *et al.* (1960) compared the phenomenon of arborization for detecting ovulation in cows with other methods such as vaginal temperature and sexual behaviour. These methods were evaluated in terms of findings by rectal examination for new corpora lutea in the ovary. Of the 3 methods evaluated, the arborization test was found to be the best one for detecting ovulation in cattle. However, in the authors' opinion, the 81% accuracy achieved was not considered adequate for reliable use. In a study involving 12 East Friesian ewes, Stephan (1962) observed a close relationship between the intensity of arborization and the rate of conception.

In 1956, McDonald and Raeside first recorded the presence of crystallization on the day of heat in all ewes studied. In some cases, ovulation unaccompanied by behavioral estrus (silent estrus) was also detected by the use of arborization technique. Ovariectomized ewes or those in late pregnancy and anestrus did not exhibit the patterns. Subsequently, Raeside and McDonald (1959) examined 52 estrous cycles in 20 Romney ewes. The phenomenon of crystallization was found to occur 3 days before to 2 days after estrus with an average duration of 4.5 days. Of 13 ewes examined during anestrus, the crystallization was neither striking nor did it occur at regular intervals.

Daniel et al. (1960) made a qualitative approach to arborization in cervical mucus of 8 Columbia ewes. They described 4 characteristic pattern types such as amorphous, rosette, arbor and fern during the estrous cycle. In the period immediately preceding the normal autumn estrous cycles, the rosette and arbor patterns appeared in a 17-day cyclic manner interspaced by the amorphous pattern type. The proestrus period was generally characterized by the rosette and/or arbor patterns; the fern pattern appeared at the beginning of estrus and was again replaced at mid-estrus by the rosette and/or arbor pattern. Occasionally the fern pattern appeared at the end of estrus, followed again by the rosette and/or arbor patterns. The amorphous type appeared very abruptly at the cessation of estrus and continued through metestrum and diestrum occupying about 16.5 to 14.5 days of the usual 17 day cycle.

Betteridge and Raeside (1962) studied 11 sows for arborization in relation to the actual day of behavioral estrus. The mean duration of crystallization was found to be 3.4 days and 6.4 days in trial 1 and trial 2, respectively. Arborization was most frequently noticed 2 days before estrus and was often absent from the cervical mucus of sows actually in heat. More than 70% positive crystallization was found in thin mucus. Arborization comparable to that seen in mature sows was not observed in any of the 4 gilts examined before puberty.

In order to reveal the important role played by ovarian hormones in the initiation of crystallization, Moberg (1959) ovariectomized 3 cows and found the absence of arborization in their mucus. When these cows were injected on 3 consecutive days with 5000 to 50,000 units of estradiol benzoate, the crystal patterns typical of estrus appeared. Subsequent treatment for 3 days with 40 to 60 mg. of progesterone resulted in the formations of atypical crystals or no crystals at all. Berry and Sarvey (1958) found that ewes treated with 12 - 15 mg. stilbestrol in combination with 35 Armour Units of gonadotrophins (FSH) most often resulted in negative smears; however, occasionally atypical patterns were noticed. Some of the ewes which gave typical patterns reverted to a negative response 48 hours after the injection. In spayed ewes, progesterone (10 mg./day) given for 3 consecutive days did not cause arborization. However, when this treatment was followed by estradiol benzoate injection, the crystallization appeared within 36 hours and in some instances even 8 hours after the injection (McDonald and Raeside, 1959).

Arborization in the Nasal Mucus. Only a limited amount of published research has been concerned with the cyclic variation in the crystallization of nasal mucus in women, but no such publication seems to have appeared in literature for farm animals. Results reported in human subjects are not in complete agreement over the cyclic activity in the crystallization phenomenon of nasal mucus. Ullery, et al. (1959) noticed that the nasal smears show cyclic changes similar but not identical to those induced in cervical mucus of women. Ruiz and Paul (1954) reported the occurrence of crystallization in nasal mucus, tears and saliva in 121 patients involved in their study. They found that all these body secretions produce typical patterns of arborization but these patterns did not exhibit the clear cut variation during the menstrual cycle that was typical of the cervical mucus.

Gonzalez (1954) divided 148 subjects into 7 groups depending upon their reproductive status and found a close relationship between the crystallization occurring in nasal and cervical secretions of women. Nouel and Agero (1953) performed the test simultaneously in cervical and nasal mucus of 206 women and found that only 36 (17.6%) women showed a correlation between the 2 smears. Henderson (1956) found comparable changes in both cervical and nasal mucus secretions of 2 subjects. This observation led him to believe that columnar epithelium of nose is subjected to the hormonal influence.

In 1958, Davis and Abou-Shabanah made an extensive study in 125 women with normal menstrual cycles and examined over 2,000 smears to demonstrate, morphologically, the manner in which the crystals in nasal and cervical smears compared. During the early estrogenic phase the cervical smears exhibited mainly the dendritic form of arborization and correspondingly the nasal mucus contained similar arborization but of advanced type. The late estrogenic phase (day 13 of menstrual cycle) was characterized by the presence of typical fern patterns in both smears. During the early secretory phase (day 17 of the cycle) the quantity of arborization was less in degree, and morphologically the patterns were similar in both smears. In 30% of the cases, arborization was superimposed on the underlying organic material in cervical mucus during the late luteal phase. The nasal mucus at this period showed practically the same changes, but were less similarly displayed.

Chemical Basis of Crystallization. The discovery of crystal formation in the cervical mucus of women and farm animals led the researchers to isolate and identify the chemical substance/s responsible for the occurrence of crystallization in the mucus.

Rydberg reported that his co-worker Linderstram-Lang analyzed two samples of clear, copious, transparent and stringy mucus of women. In a composite sample of 365.3 mg. of mucus, the following results were obtained.

Dry matter	1.62%
Organic matter	0.77%
Total ash.	0.85%
Chlorides computed as NaCl	97.0% of the total ash

The role of sodium chloride in crystal formation was further supported by adding 0.9% sodium chloride to egg albumin and saliva which on drying to a film resulted in the formation of palm leaf crystal patterns which were strikingly similar to those observed in cervical mucus. It was assumed that the crystals consist of sodium chloride, and the characteristic pattern formation results due to mucin present in the mucus.

Zondek (1954, 1957) did not consider the presence of mucin as an essential component for the crystal formation, since he noticed that tears which do not contain mucin also crystallize on drying. He also found that not only cervical mucus but also other body fluids such as follicular fluid, cerebrospinal fluid and the secretions in ovarian cysts produced crystallization on drying. Neither the ions of sodium or chlorides were found to be specific for the reaction, since the potassium bromide added to ovalbumin or serum albumin caused the palm leaf structures to appear in the smears. The presence of electrolytes was, however, found to be indispensable for crystallization because on dializing the mucus the crystallization failed to occur unless the electrolytes were added to it. Zondek (1956, 1954, 1957) did not consider the phenomenon of crystallization as a specific process, but thought it to be of special significance because of its dependence on the cyclic endocrinal activity of ovary.

Using X-ray defraction technique, McDonald and Sharman (1959) found 97% or more of sodium chloride and insignificant amount of other crystalline components in the cervical mucus. The predominant form of crystalline substance was found to be sodium chloride but little amounts of potassium chloride were also present. It was thus revealed that crystals in the mucus were composed of a mixture of sodium and potassium chloride in addition to small amounts of organic matter.

Significant variations in the inorganic constituents reaching to their maximum concentration at the time of ovulation were noted by Igarashi (1954a) in women. He found that cervical mucus contained sodium, potassium, magnesium and copper; however, the greatest variation was noticed in the excretion of sodium by the cervical glands. He concluded that the phenomenon of crystallization does not depend on percentage of sodium in the total amount of the mucus but on its dry contents. Furthermore, he (Igarashi, 1954b) did not regard sodium as a transudate but as a true secretion.

Herzberg et al. (1964) demonstrated a cyclic variation in the percentage of sodium chloride in the dry cervical mucus. A sharp rise to a maximum of 40-70% was noticed at the time of ovulation as compared with 2-20% during the other phases of the cycle. This maximum salt content was not observed in the cervical mucus of infertile women. They also correlated the peak level of sodium chloride with the sharp increase in water excretion by the cervix, and concluded that the variation in sodium chloride in the mucus is due to the variation in the amount of water excreted. Thus, with the total organic material remaining constant, the sodium chloride in the dry mucus was shown to vary linearly with the amount of water excreted by the cervix. Furthermore, they proposed that the sharp rise in the sodium chloride in the mucus may be used for exact dating of the ovulation in women.

McSweeney and Sbarra (1964) developed a "Spot Test" to measure the cyclic variations in the chloride ion concentration of the cervical mucus. They found that in early proliferative phase of menstrual cycle the concentration of the ions was 0.1% increasing to a maximum of 0.9% at the time of ovulation. A sudden drop to a level of 0.5% was, however, noticed soon after ovulation. Along with cervical mucus, a "Spot Test" was also employed to measure the chloride ion concentration in nasal mucus. The cyclic variations were noted to be identical in each secretion; however, the chloride concentration was considerably less in the nasal mucus. Observations on the castrated and menopausal women revealed the insignificant amounts of chloride ions in their cervical mucus. However, within one week following estrogen administration to these women, an appreciable, sometimes quite large, amount of chlorides appeared in their cervical mucus. The failure of arborization to take place in the cervical mucus of castrated or menopausal women or those suffering from amenorrhea, or during the pre- and postmenstrual period of normal women has been shown by Zondek (1957) to be due to the absence of electrolytes. These mucus samples were mixed in dried state with highly dilute salt solutions (potassium chloride, sodium chloride and potassium bromide) and allowed them to dry, produced the typical arborization.

Several interesting observations were made on the biochemical aspect of arborization by Abou-Shabanah and Plotz (1957) to demonstrate how the phenomenon depends upon electroylyte concentration. They used 6 sodium salts (chloride, iodide, carbonate, bromide, hydroxide, and nitrate), 3 potassium salts (chloride, hydroxide, and nitrate) and also the chlorides of calcium, zinc, barium and magnesium, silver nitrate and copper sulphate. From a 5% salt solution, 17 further dilutions were made down to 0.1%. The measured quantities of salt solutions were transferred onto a clean glass slide and the smears thus prepared were dried and examined under the microscope. In addition, aqueous solutions (1%) were prepared from the powdered form of a variety of protein substances and saccharides. Admixture of a salt and a protein or a salt and a saccharide were made and the slides were prepared for examinations of crystallization.

In this study it was noticed that the crystal patterns from aqueous solutions differed basically at each dilution. Fern--or palm leaf-like reaction appeared in salt concentrations ranging from 3.5 to 1.5% and again at lower concentrations of 0.5 and 0.25%. The arborization reaction obtained at concentrations above 1% was comparable to a typical regular and fully formed fern, while the atypical, particularly the beaded or pearly patterns, were formed when the salt concentration was below 1%. The chemical ferns were found to differ in appearance with the different salt solutions. Sodium chloride in 1.25% concentration gave a pattern of long poles carrying perpendicular side branches, while 1.5% potassium chloride showed a pattern of arborization like bent needles, and 1% sodium carbonate gave a pattern of palm leaves--like branching. It was further shown that the same salt would give different patterns in different concentrations. Barium chloride in 1.5% concentration showed pinelike branching patterns; whereas, at 0.6% the same salt gave featherlike patterns. Studies on crystallization patterns of salts when admixed with 1% protein solutions and disaccharide solutions, produced palm leaf or fern reaction only when the resultant strength of the salt solution was at a level where the salt alone would form the patterns.

These workers further studied the nasal mucus to emphasize the predominant role of inorganic salts in the formation of crystal patterns in the mucus. A composite sample of nasal mucus obtained in the first half of menstrual cycle from 6 women and the smear prepared showed the fern patterns. This sample of mucus when dehydrated and the dried material again brought into solution gave a fern pattern. The only difference noted was that the composite fresh mucus showed bushlike patterns while the dehydrated mucus brought in solution gave a more fern-like pattern. The composite mucus sample was ashed and all the organic matter was eliminated, and the residual ash was then dissolved in 1 c.c.

distilled water and the smear prepared from this material gave the fern reaction.

In essence, the results of this experiment pointed out the indispensable role of salts in the crystallizing ability of mucus and condemned the belief that mucin is necessary for the reaction of arborization. The authors also revealed that the organic substances (protein and/or saccharides) influence the pattern only insofar as thickness, length, or density, or in short the "crystal habit" are concerned. But these organic substances do not influence the type of crystal skeleton or internal structure.

De Vuyst _et al_. (1961) analyzed the cervical mucus to determine the cyclic variations in certain constituents of the mucus such as water, organic matter, ash, sodium and potassium, and presented microscopic interpretations of these changes with the formation of fern leaf patterns in the mucus of cows. In correlating the presence of fern leaf patterns with the chemical constituents of the cervical mucus, it was found that the appearance of fern patterns at the time of heat coincided with lowest concentration of dry matter, organic matter and ash content in the fresh mucus; however, on dry matter basis, cervical mucus contained more ash and less organic material than at any other time of the estrous cycle. The dry matter portion of the mucus was found to contain mucoproteins and mineral salts. Thus, the appearance of fernlike crystallization in the estrual mucus of cows was related to the presence of water, mineral salts and mucoproteins. The presence of dense or coarse branching crystals during diestrus was found to be associated with the highest concentration of organic cellular matter and decreased water content.

When the process of crystal formation was observed under the electron microscope, it was observed that the larger crystals, which were found to be protein molecules, conglomerated on the particles of lesser density. It was stated that the rapid evaporation of water from the thin smear results in some crystallization of salts. However, part of solution which was retained through surface tension at larger protein molecules played the role of crystallizer. The increased concentration of salt resulting from the evaporation of water was indicated to provoke extensive crystallization of protein molecules in typical fern leaf formations. It was thus established that in order to get fern formation, three factors, mucoproteins, sodium and potassium chlorides and the increased water content in the cervical mucus, are required.

In a comprehensive review published by Ullery et al. (1959) on the concepts of arborization in the cervical and nasal mucus, it was concluded that the phenomenon of crystallization depends on the concentration of electrolytes in the cervical mucus and the pattern of arborization would vary by the differences in salt concentration. Since water and electrolyte balance in the mucus is influence by female gonadal hormones, the examination of mucus smears for arborization would reflect the endocrine activity of the ovary in females. The reason for the nasal mucus not presenting marked cyclic changes has probably been due to the permeability of nasal mucosa preventing the extreme variations from occurring as markedly as in the cervical canal. In spite of the relatively narrow range of electrolyte variations in the nasal mucus, it has been proposed that the nasal smear can be used as a valuable guide to hormonal activity at all phases of the reproductive cycle.

From the research concerned with the phenomenon of arborization in the cervical mucus of humans as well as farm animals, it appears that the extensive and prolific crystallization is associated with the period of heat in female animals and the ovulatory phase of the menstrual cycle in women. Several studies have also related these changes with the time of ovulation but the criteria used to detect ovulation are variable. In women the extensive arborization indicating ovulation has been judged from the drop in basal body temperature and/or the examination of vaginal smears; whereas, in farm animals it has been assessed through the rectal palpation of ovaries for newly formed corpora lutea. However, the direct relationship between arborization and ovulation as evidenced by examination of ovaries after laparotomy has not so far been established.

The inhibitory influence of progesterone on the crystal formation has been demonstrated by a direct correlation between the peak of corpus luteum activity and the absence or reduced amounts of crystallization in the cervical smears. These and similar observations have definitely revealed the ovarian hormones as the principal factors responsible for governing the intensity of arborization in the mucus. Whether these hormones bring about their effect on crystallization by initiating alterations in the physical properties of mucus such as viscosity, elasticity and increased water content, or through some changes in the chemical composition of the mucus has not yet been fully elucidate. The complex biochemical structure of the mucus has further limited the progress. It is likely that phenomenon of crystallization is associated not only with the chemical changes in the cervical mucus but also with the alterations in other properties such as viscosity, elasticity and the freedom from cellular contents. The increased water content in the cervical mucus obtained at the time of heat and ovulatory phase of menstrual cycle may also be playing significant role in initiating a change in the physicochemical properties of the mucus.

The cyclic variation in the nasal mucus crystallization is a controversial point. The experimental proof of such variation occurring in farm animals is lacking. Whether these changes in the arborization of nasal mucus in animals are really cyclic and depend on hormonal balance, or on certain other conditions not necessarily related to the ovarian activity, are not fully confirmed.

MATERIALS AND METHODS

Five Dorset X Rambouillet crossbred ewes approximately 4 years of age were used in this experiment. These were maintained in a pen in the arena of the Animal Husbandry Department of Oklahoma State University at Stillwater from August 11, 1966 to September 28, 1966. They were then removed to a pen at the old horse-barn till the experiment terminated on October 14, 1966. The animals were fed a daily ration of about 2 pounds legume hay and were watered by means of buckets filled with fresh water every day.

In order to detect estrus in ewes, a vasectomized ram fitted with marking harness was maintained constantly with the ewes. The animals were frequently checked for their behavioral responses and also for color marks on the rump indicating the ram had mounted the ewe. These color marks were wiped off each time the ewe was marked to determine the frequency with which the ram mounted the ewe. A different colored chalk was placed in the marking harness just prior to the start of a new estrous cycle.

Procedure for Collection of Cervical Mucus Samples. Collections of mucus samples were made while the ewe was held in a wooden stanchion. The external genitalia were thoroughly cleaned with a cloth, and a plastic speculum, lubricated with a neutral lubricant (K-Y Sterile Lubricant, Johnson and Johnson, New Brunswick, New Jersey), was inserted full length into the vagina and then withdrawn slowly until the cervical opening was visible. An ordinary pencil flashlight served as a source of light for observation. Vaginal mucus or any whitish, pus-like material around the os uteri was wiped away by means of a cotton swab to avoid contamination of the sample of mucus obtained from endocervix. Cervical mucus free of contamination was necessary to study the phenomenon of arborization since Raeside and McDonald (1959) indicated an inhibitory action on arborization of blood, vaginal mucus, lubricant cream and seminal plasma.

A disposable plastic insemination tube fitted with a plastic bulb was placed through the speculum at the os uteri and then rotated gently to obtain the sample of mucus. A suction force was applied with the bulb and the insemination tube was withdrawn. Care was taken to prevent contamination with any mucus or cellular matter inside the vagina. Each time a sample was obtained, the entire quantity of mucus from the cervical canal was aspirated so as to have the clear cut changes in arborization at each subsequent collection time. A clean tube was used for each collection of a sample of mucus.

Procedure for Collection of Nasal Mucus. Before the sample of nasal mucus was obtained, the ewe's head was firmly secured and the external nasal openings were cleaned with a cotton cloth. A cotton swab fitted to a stick was then inserted gently inside the nasal passage and the mucus sticking around the nasal septum was obtained. Occasionally, the ewe would sneeze and the mucus thus blown out was used for preparing slides. Nasal mucus was frequently contaminated with small particles of dust and small fragments of grass leaves.

Slide Preparation. Immediately after the mucus was obtained, it was blown out on a clean microscope glass slide. A subjective appraisal of amount, turbidity, viscosity and cellular content in the mucus was recorded. The mucus was then spread evenly in a thin layer on the slide. While preparing a smear, the slide on which the mucus was blown out was held in the left hand on a horizontal plane. In the right hand, another clean glass slide which was used as an applicator, was placed slantingly with an angle of 35° to 45° on the blob of the mucus. The applicator in the right hand was then rolled gently in a manner used for preparing a blood smear, thus spreading an even and a thin layer of mucus on the slide held in the left hand. Precautions were taken to prepare a thin, even smear with uniform thickness. It was necessary to observe this care since Zondek (1957) pointed out that the thickness of the smear influences the pattern of crystallization. In the case of the thick and highly viscous and cellular mucus generally appearing after the end of estrus, it was found difficult to prepare a smear of even thickness.

Slides were then air dried at room temperature. At times, when the temperature was low or humidity was high, slides were air dried on the top of the microscope lamp or they were kept in the vicinity of the lamp for effective drying. This procedure was adopted since low atmospheric temperature (Raeside and McDonald, 1959) and high humidity (De Vuyst et al., 1961) interferes with arborization. After the slides were dried, these were examined immediately under a microscope with a magnification of 100 to 160 X with a reflected light. On several occasions, the drying process and the crystal formation was observed under the microscope.

Photographs presented in this work are from the slides which were preserved in a desicator for 24 to 48 hours.

The procedure adopted for preparing the smears of nasal mucus was to squeeze the mucus from the cotton swab on to the edge of the slide and then spreading it as described for cervical mucus. It was found necessary because the smearing with cotton swab as an applicator did not produce a film of even thickness. Other methods, such as drying of slides and their examination was practically the same as that of cervical mucus.

Periods of Collecting Cervical and Nasal Mucus. In this study, mucus samples were collected daily from all the ewes and the data on 14 estrous cycles were obtained. From the day, the cervical mucus would exhibit recognizable degree of crystallization sampling was continued at every four hour intervals till such time as the mucus showed reduced, or absence of, activity as regards crystal formation. In order to study the parallel changes in the nasal mucus, the samples were also collected concurrently from nose. Sometimes, the mucus collection was made at shorter intervals of 2 to 3 hours to study the variations in crystal patterns. Generally, the sampling of 4 hourly interval began before estrus, extended through estrus and terminated during post estrual stage. In subsequent days, the mucus was collected every day in a regular fashion.

Presentation of Data. Graphic presentations were used to depict the changes in the occurrence of crystallization pattern types during the various periods of estrous cycle in relation to first day of estrus (0-day) as evidenced by the marking of an ewe by the ram. The first day of estrus was divided into 6 divisions of 4 hours each to illustrate the sequential changes in the occurrence of various crystal patterns after the heat was established. The period consisting of one day before (-1 day) and one day after (+1 day) the first day (0-day) of heat was subdivided into two halves of 12 hours each to indicate the appearance of particular type of crystal during pre- and post-estrual stages of the estrous cycle.

The Standard Error of means were calculated according to procedure set forth by Steele and Torrie (1960).

RESULTS AND DISCUSSION

Crystallization Pattern Types. During the course of this study, various forms of arboreal patterns were observed in the cervical mucus obtained at different phases of estrous cycle in ewes. In general, the pattern was a fern-like configuration comprised of a central stem with venations and subvenations arising perpendicularly. In some cases, branches may offshoot from the main stems and these branches may again divide giving rise to sub-branches which in turn would possess regular venations and subvenations as shown in Figure 1. In describing the different varieties of crystallization, the general outline proposed by Abusineina (1962) in the cervical mucus of cows was adopted. On the basis of configuration and the appearance of crystal structures under microscope, the patterns of arborization were categorized in four main types.

Type I: The arborization patterns classified under this type are shown in Figures 2 and 3. Microscopically, this variety of crystallization consists of a thick main stem with or without venation and subvenation. The venations, if present, are club shaped or stumpy in appearance and are located on the main stem leaving wider gaps in between. This type of crystals does not have a definite organization of the different components, such as well-defined

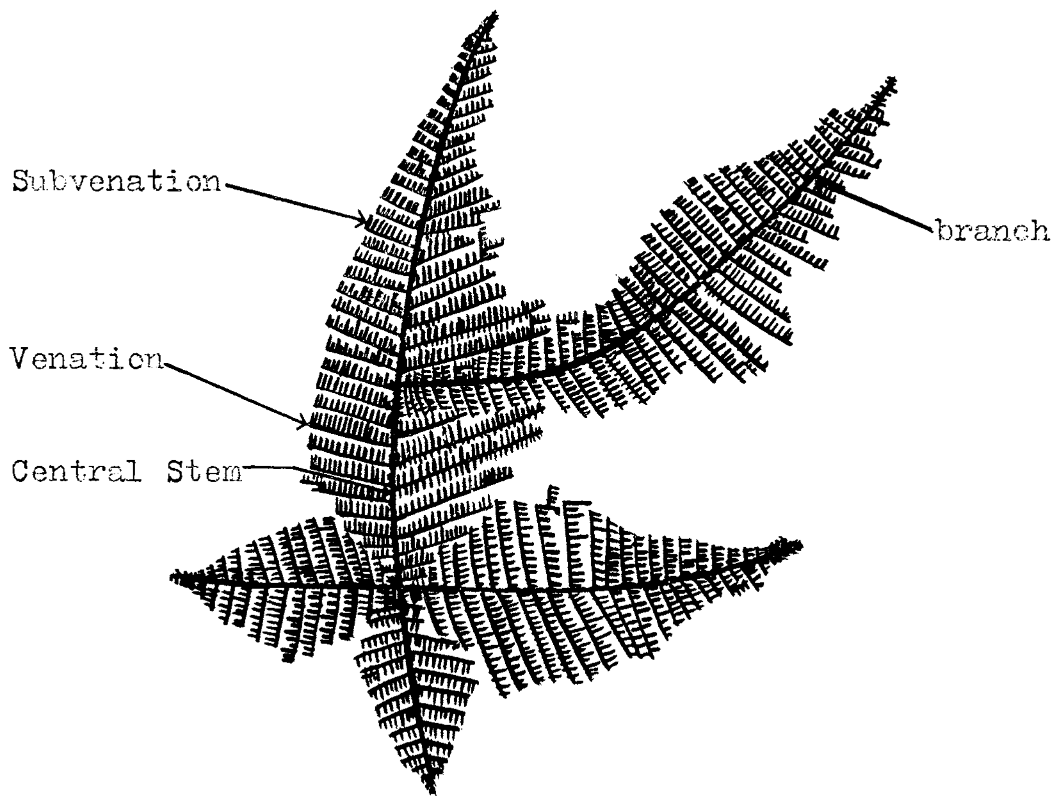

Figure 1. Diagram Showing the General Fernlike Configuration.

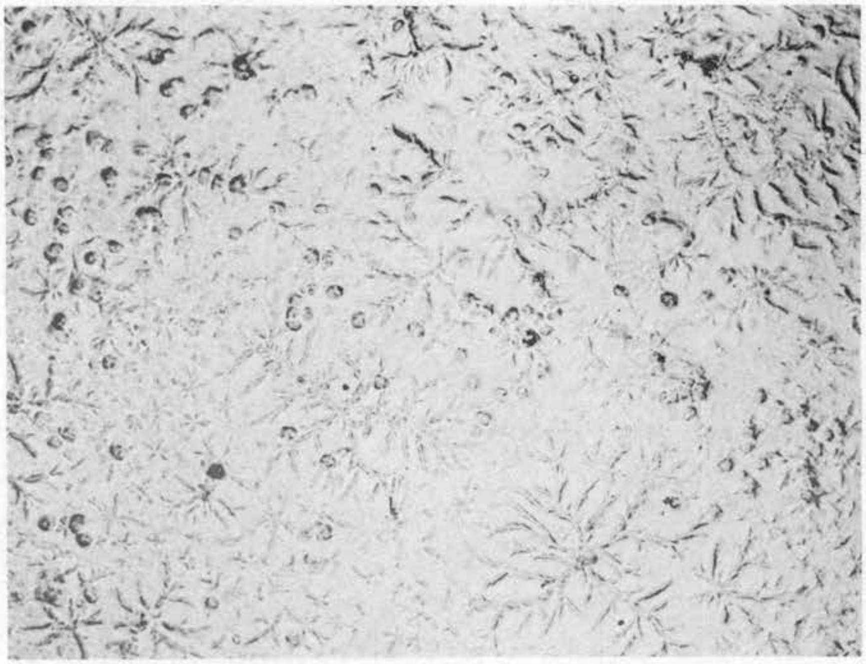

Figure 2. Type I Pattern: Observed During the Late Diestrus Phase of the Estrous Cycle

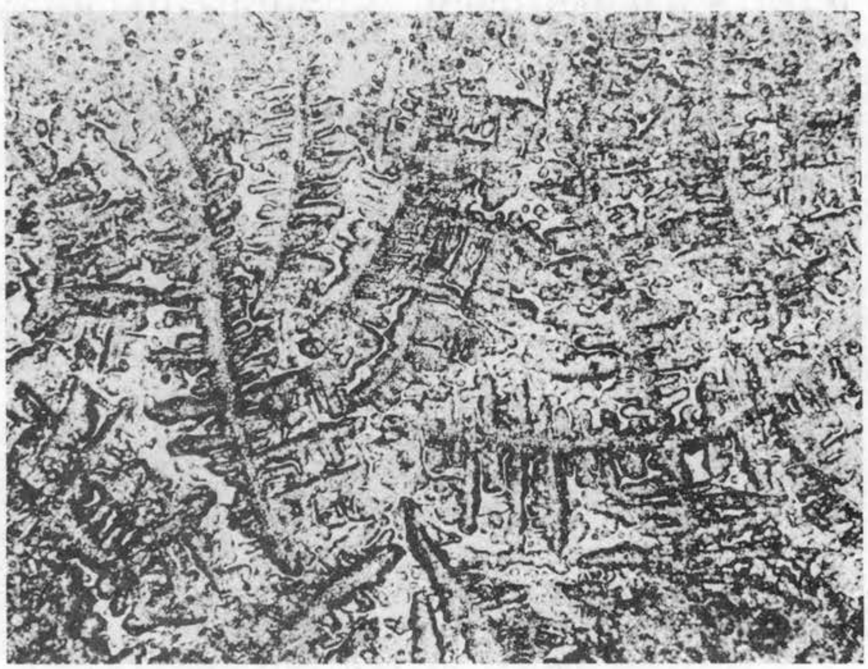

Figure 3. Type I Pattern: Observed 23 Hours After the Onset of Estrus

regular stems or definite venation and subvenation. In addition to the crystals shown in Figures 2 and 3, the crystals of stellate, cruciform, dendritic or linear forms, sometimes present, are seen scattered irregularly occupying only a limited part of the mucus smear. This pattern type is present in opaque, highly cellular mucus and, thus, the crystals are seen superimposed on the mucoid material generally surrounded by the cellular material in the dried film.

Type II: Crystal patterns included in this category are characterized by long, relatively thin, and wavy or curved stems with very fine and distinct venation and subvenation emerging perpendicularly from the main stem and closely located on the main axis. (Figure 4). Other varieties included were those with very long, wavy or cruved stems possessing extensive branches and sub-branches which spread profusely throughout the smear (Figures 5 through 8). Papanicolaou (1946) described these patterns as "the form of anastomosing branches with heavily indented leafy projections." The centrally located crystals in the mucus smear have restricted branching system and assume a compact configuration as shown in Figure 7. The form of crystallization occurring at the edge of the smear differs slightly in that the main stem and also the branches and sub-branches are delicate and light in degree (Figure 8). This type of arborization exists in almost every part of the smear. Under a microscope, these crystals appear smooth, relatively

Figure 4. Type II Pattern: Seen Within 2
Hours After the Onset of Estrus

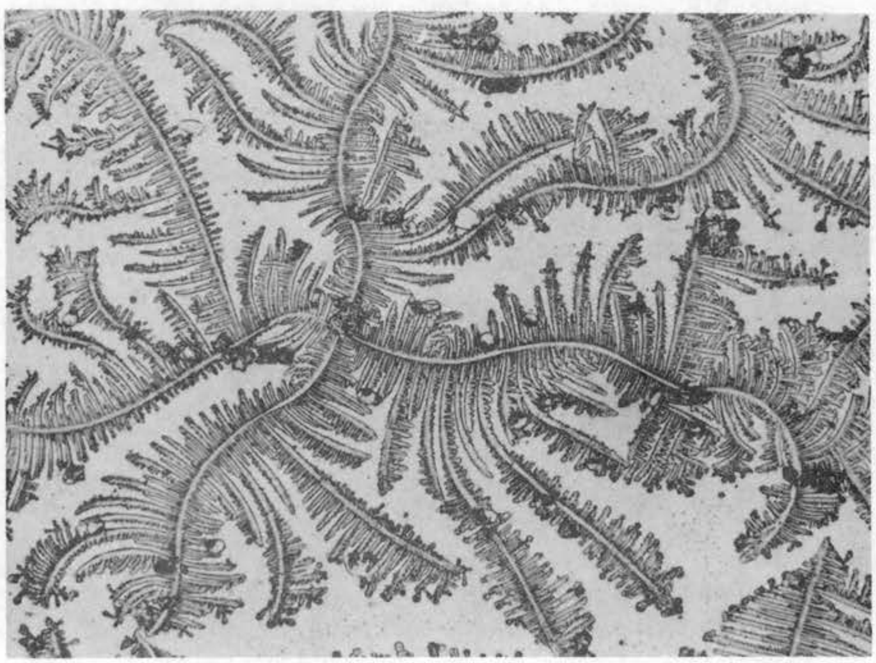

Figure 5. Type II Pattern: Seen 3 Hours
After the Onset of Estrus

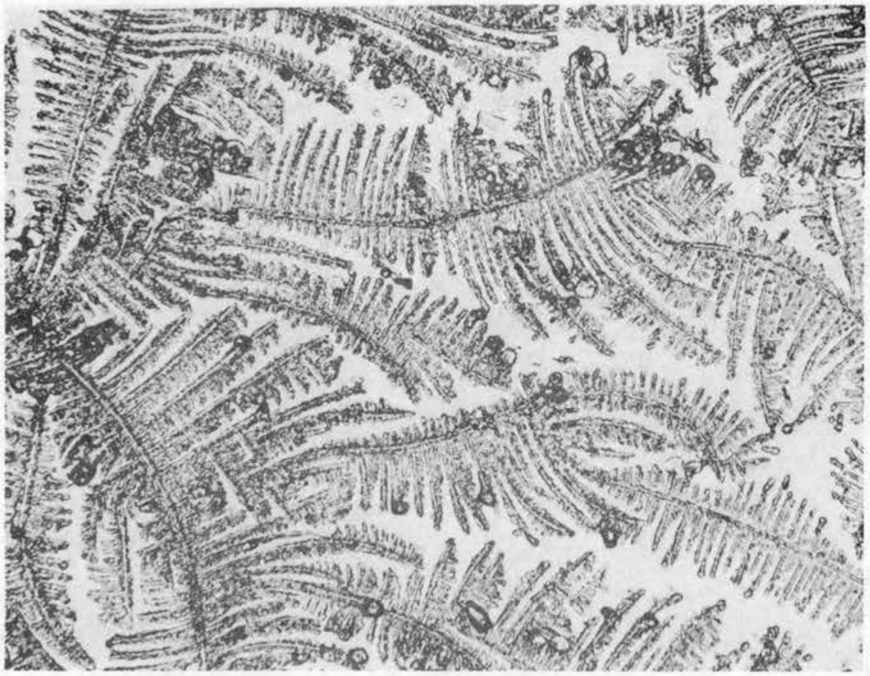

Figure 6. Type II Pattern: Noticed 5 Hours After the Onset of Estrus

Figure 7. Type II Pattern: Seen in the Center of the Smear of the Same Slide as Figure 6

Figure 8. Type II Pattern: Observed 9 Hours After the Onset of Estrus. Configuration at the Edge of the Smear

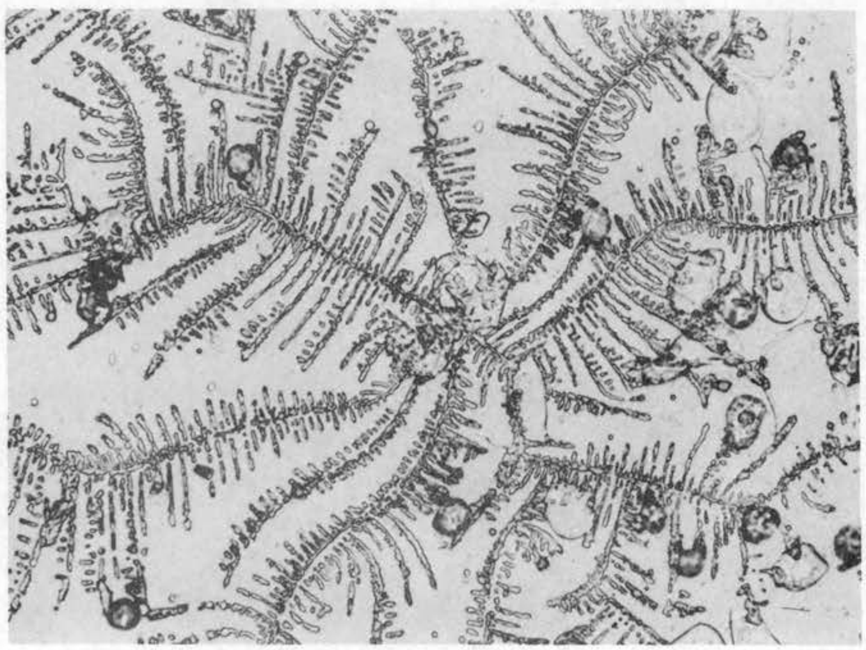

Figure 9. Type II Pattern: Disintegrated Form of Crystallization

transparent and are seen superimposed on a clear glassy mucoid material in the film. These types of crystals are invariably present when the mucus is copious, glairy, acellular and viscous or elastic in consistency.

Type III: Microscopically, this pattern is fern shaped in appearance. The stems are thick, either straight or slightly curved, and have thick venations with subvenations giving either a serrated or dented appearance as shown in Figures 10 and 11. These crystal types are somewhat crude and rough in their microscopic appearance depending upon the amount of cellular material present in the preparation. The mucus cells, sometimes agglomerate on the crystal configurations and thereby obliterate the clear and distinct appearance of crystal morphology (Figure 12). The crystallization of this type appears in the mucus which is semi-transparent or translucent in appearance, copious to moderate in quantity, and viscous or elastic in consistency.

Type IV: The configuration of this crystal type more nearly resembles a palm leaf than a fern in appearance. The stems are invariably straight, although sometimes slightly cruved, and are of medium thickness. The venations are situated close to each other on the main stem. The subvenations present on either side of the venations give a leaf-like appearance depending upon the cellular and/or organic material present in the mucus. The various crystal patterns included under this group are illustrated in Figures 13 through 16. The distinct and clear appearance of different

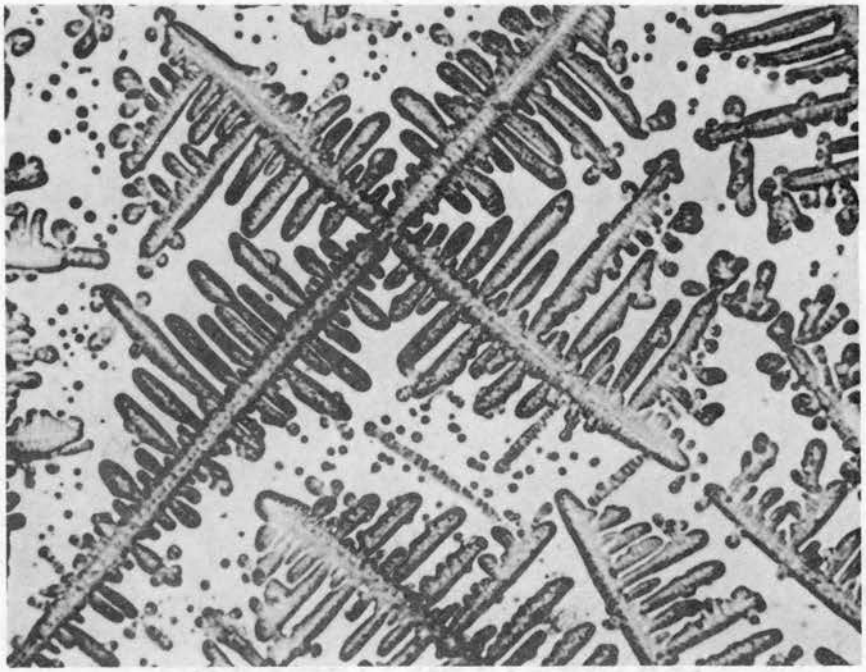

Figure 10. Type III Pattern: Observed 5 Hours Before the Onset of Estrus

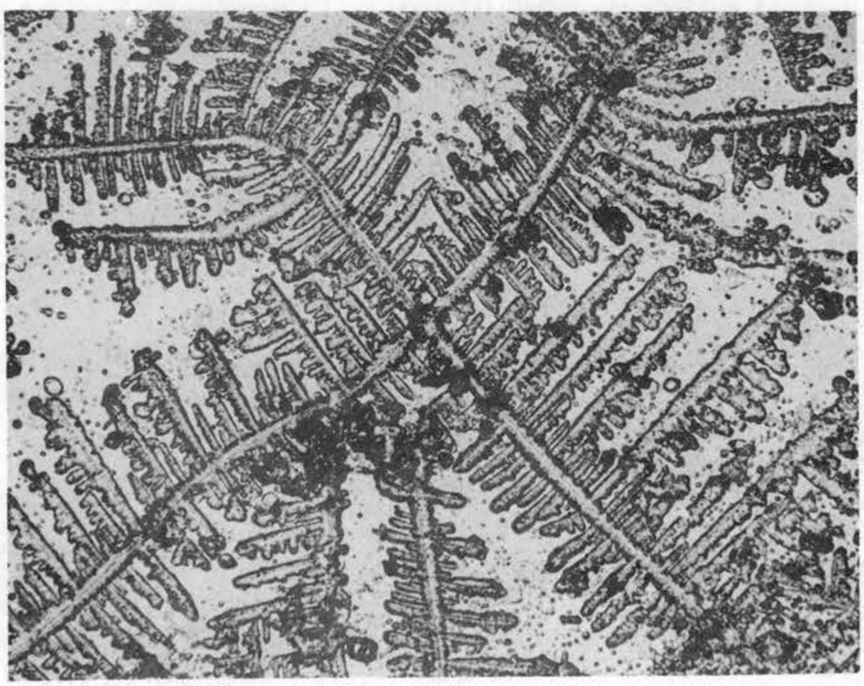

Figure 11. Type III Pattern: Seen 15 Hours After the Onset of Estrus

Figure 12. Type III Pattern: Note the Deposition of the Cellular Material on the Crystals

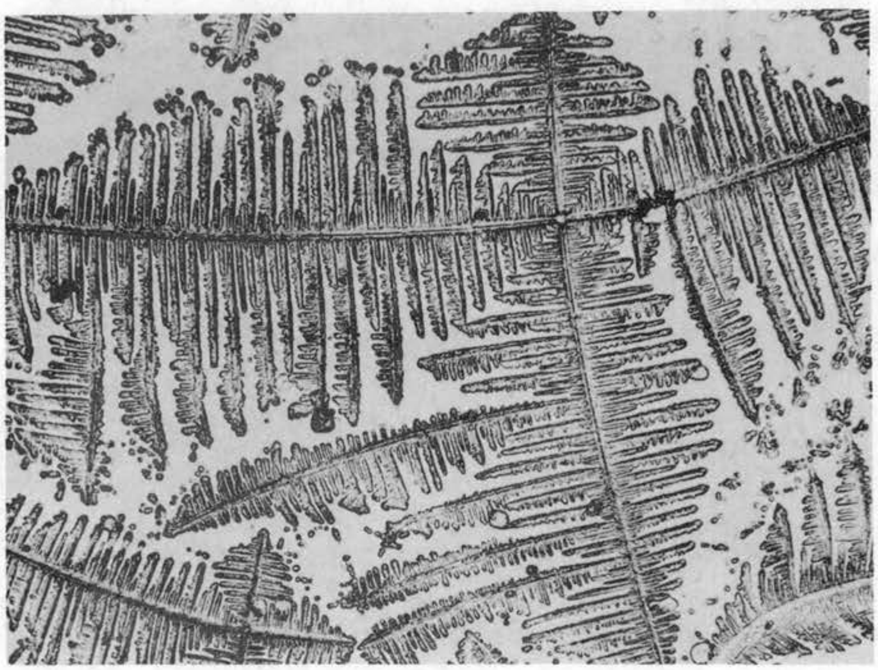

Figure 13. Type IV Pattern: Seen 7 Hours After the Onset of Estrus

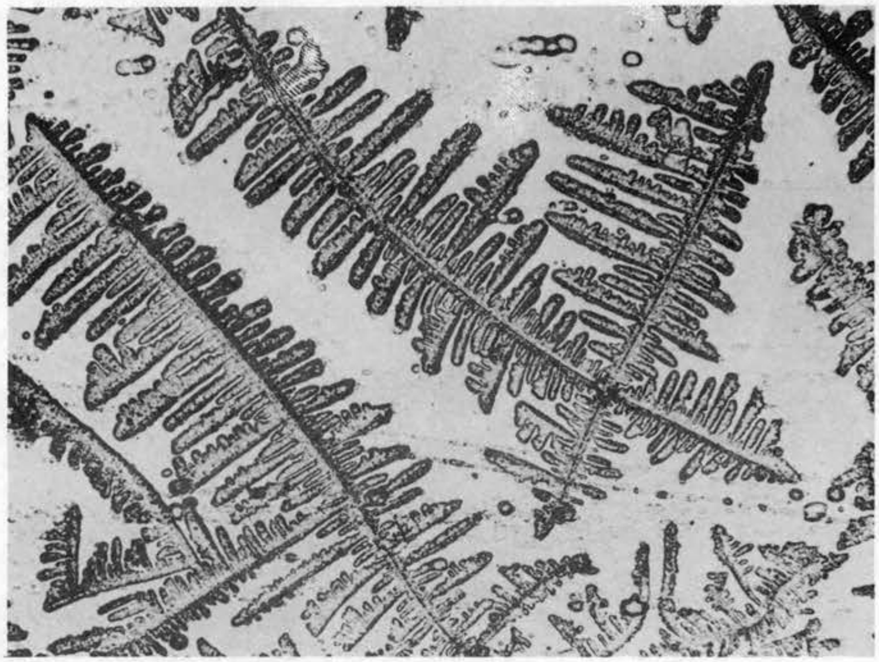

Figure 14. Type IV Pattern: Noticed 18 Hours After the Onset of Estrus in the Clear Portion of the Smear

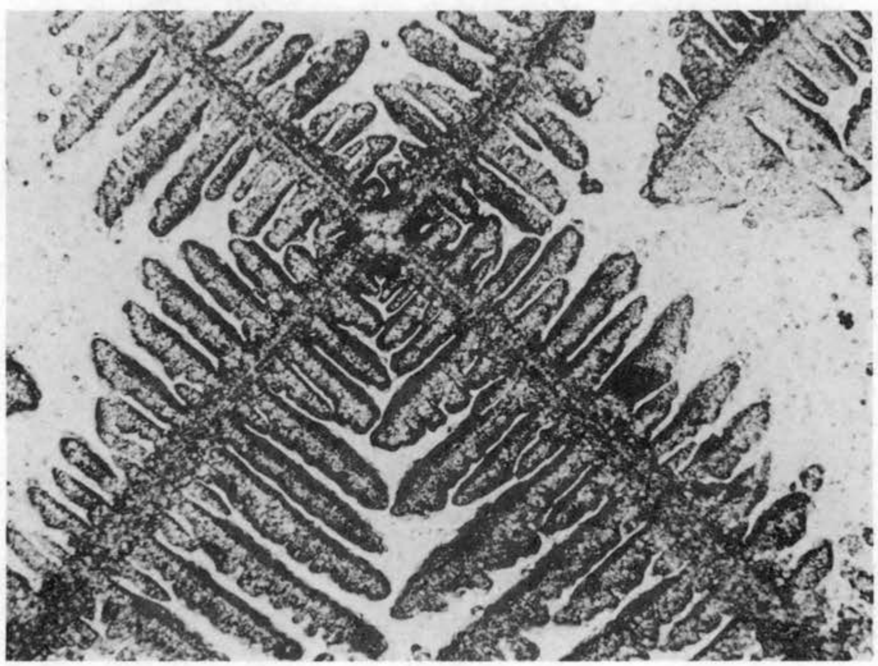

Figure 15. Type IV Pattern: Observed on the Same Slide as Figure 13. Note the Organic Material

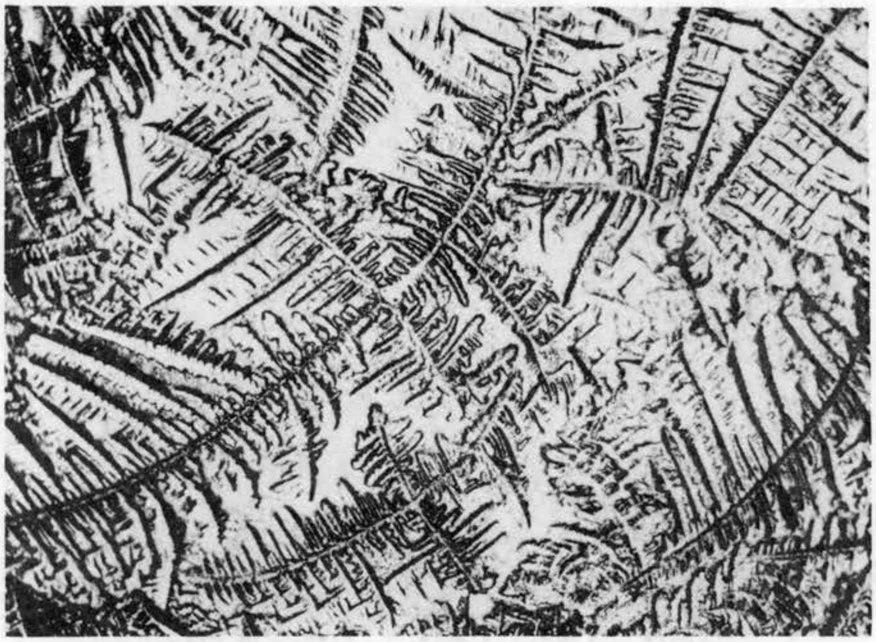

Figure 16. Type IV Pattern: Observed 21 Hours After the Onset of Estrus

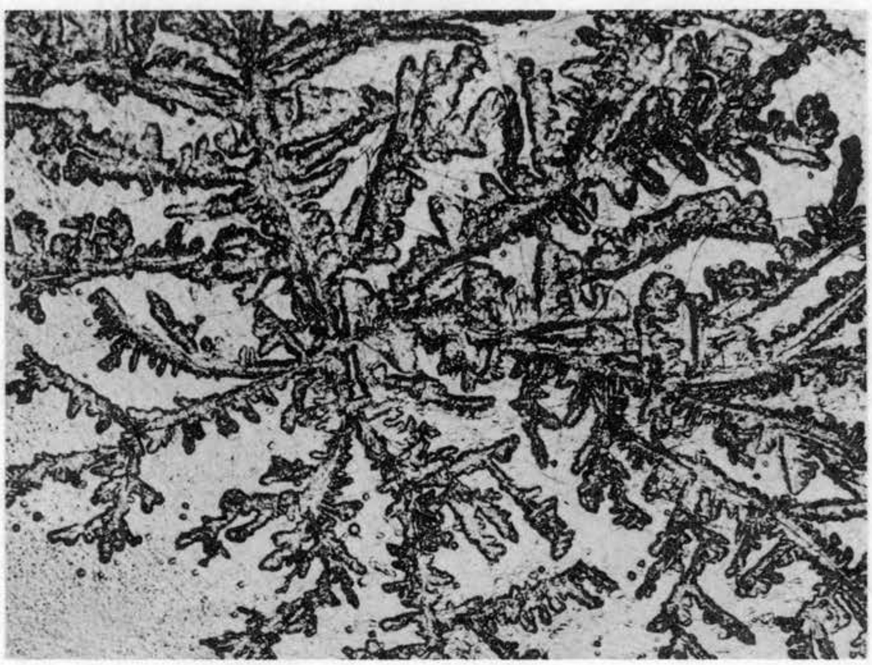

Figure 17. Typical Nasal Pattern Noticed at the Onset of Estrus

components of crystals depend on the mucoidal material present. As shown in Figure 16, the presence of cellular elements in the mucus prevents the formation of fine subvenations on the venations. Similarly, these crystals show great variation in their size and the length of main stems. When the cervical mucus is turbid or translucent and contains more organic material, the crystals appear rough, crude and present a floppy appearance as shown in Figure 15.

In addition to the Types described above, several varieties of intermediate forms were encountered during this study. The variation particularly noted was in branches, sub-branches, the overall size, the fineness of the crystals and the general appearance under a microscope.

Cyclic Changes in the Occurrence of Crystal Pattern Types in Cervical Mucus. The morphological variations in the crystal patterns of cervical mucus, as categorized in 4 main types, were studied in a total of 14 estrous cycles experienced by five Dorset X Rambouillet ewes from August 11, 1966 to October 14, 1966. All the ewes were cycling regularly and were accepting the ram when in heat. The duration of the estrous cycles recorded in this experiment ranged from 17 days to 19.5 days with an overall mean of $18.24 \pm .45$ days.

The crystal patterns described in this study occurred in a cyclic manner beginning near the time of expected estrus. Sample collection at intervals of 4 hours was initiated from the time a recognizable degree of crystallization was noticed in the mucus smears, which generally began one day prior to estrus. The distribution of various patterns observed on the different days of the cycle is presented in Table I and Figure 18 for the pattern Types I, II, III, IV and mixed crystals in relation to first day of esturs.

<u>Cyclical Variation in Type I Patterns</u>. The distribution of Type I pattern in relation to the first day of estrus (0-day) is given in Table I and presented graphically in Figure 18. Beginning from the second day after estrus (+2 day) until the 15th day of the cycle (-4 day), the number of mucus smears exhibiting a pattern of any sort were at a minimum, since less than one-third of the samples exhibited crystallization patterns. However, the crystal pattern that is seen during this period is exclusively Type I. From day -3 until about 13-24 hours prior to the onset of estrus, the frequency of occurrence of this pattern increased in the cervical mucus. However, its disappearance was quite abrupt since none of the mucus specimens examined from 12 hours before until 13-16 hours after estrus showed the Type I patterns. The presence of Type I patterns of the sort illustrated in Figure 3, towards the end of heat was generally associated with the termination of estrus behavious in ewes. Daniel <u>et al</u>. (1960) likewise observed the appearance of amorphous patterns, similar to the Type I described here, within 4 hours after the ewe failed to show signs of heat. Wide variations were observed between days

TABLE I

CRYSTALLIZATION PATTERN TYPES IN CERVICAL MUCUS SMEARS DURING THE ESTROUS CYCLE OF EWE

Days Relative to First Day of Estrus	Hours	No. of Smears Examined	No. Showing Crystal-lization	Percent of the Total Showing Crystal-lization	Percent Showing Crystallization				
					Type I	Type II	Type III	Type IV	Mixed
-9 to -8		22	4	18.18	18.18	-	-	-	-
-7 to -6		37	8	21.62	21.62	-	-	-	-
-5 to -4		36	10	27.78	27.78	-	-	-	-
-3		17	8	47.60	35.30	-	5.87	-	5.87
-2		18	8	44.40	27.77	-	11.11	-	5.56
-1	-24 to -13	23	22	95.65	34.78	-	60.87	-	-
	-12 to -1	23	23	100.00	-	21.70	47.8	-	30.43
Days of Estrus 0	0 to 4	20	20	100.00	-	95.00	-	-	5.00
	5 to 8	20	20	100.00	-	80.00	10.00	5.00	5.00
	9 to 12	19	19	100.00	-	68.43	31.57	-	-
	13 to 16	19	19	100.00	-	47.40	15.80	-	36.80
	17 to 20	18	18	100.00	11.11	22.22	33.33	5.56	27.78
	21 to 24	18	18	100.00	38.90	16.70	27.80	11.11	5.50
+1	+1 to +12	24	18	75.00	41.58	4.16	12.50	-	16.67
	+13 to +24	9	5	55.50	11.11	22.22	22.22	-	-
+2		16	5	31.25	31.25	-	-	-	-
+3		16	4	25.00	25.00	-	-	-	-
+4 to +5		31	4	12.90	12.90	-	-	-	-
+6 to +7		31	6	19.35	19.35	-	-	-	-
+8 to +9		24	3	12.50	12.50	-	-	-	-

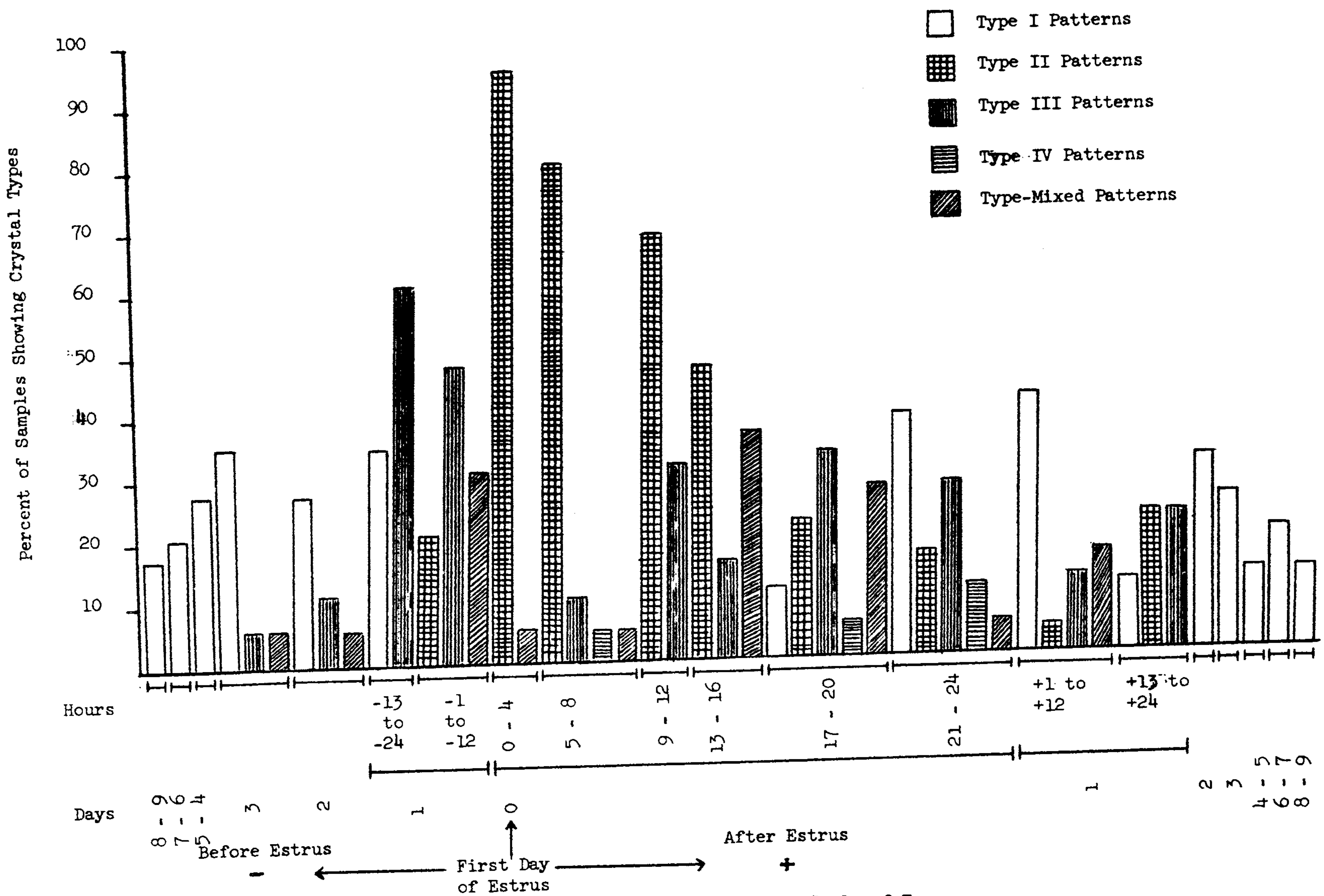

Figure 18. Distribution of Crystal Types at Different Times in the Estrous Cycle of Ewe

of the cycle and between animals. Of the five ewes examined, one did not exhibit the Type I pattern in the period from two days after estrus till one day before the onset of estrus.

The presence of atypical crystal patterns (Type I), or the complete absence of crystallization in the cervical mucus, has been noted during the luteal phase of the cycle in: women (Roland, 1952., Urdan and Kurzon, 1955); cows (Bane and Rajakoski, 1961, Abusineina, 1962); female buffaloes (Quayam and Venkatasami, 1964); and ewes (Daniel et al. 1960). The inhibitory effect of progesterone on crystal formation has been well established (Bone, 1954; Zondek, 1954; 1957; Moberg, 1959). It appears, therefore, that in the period extending from 2 days after estrus until about the 15th day (-4 day) of the cycle, the corpora lutea secretes sufficient progesterone to prevent the occurrence of any other type of crystallization in the cervical mucus. Indirect evidence supporting this fact can be cited from the work of Edgar (1954) and Edgar and Ronaldson (1957) who found an increase in progesterone concentration from the 3rd to the 16th day with a sudden fall on the 17th day in an estrous cycle of 17 days duration in ewes. Neher and Zarrow (1954) found a low level of circulating progesterone at the time of ovulation, with a rapid rise occurring during the post-ovulatory, or luteal phase, of the estrous cycle in ewes. The appearance of Type I pattern at the termination of heat and its subsequent persistence to the onset of the next estrus seems to be associated with the inhibitory influence of progesterone on the formation of organized forms of crystallization.

Cyclical Variation in Type II Patterns. The occurrence of Type II patterns during the various phases of the estrous cycle is shown in Figure 18 and Table I. The first indication of Type II pattern was observed 12 hours before the beginning of estrus when it was observed in 21.7 percent of the samples. However, in most samples this pattern was not seen until about 4 hours prior to heat. At the time the color mark was observed on the ewes (0-4 hours), the Type II pattern was present in all of the cases with the exception of one in which a combination of pattern II and III was seen in the same smear.

Corresponding with the appearance of Type II crystal patterns, the cervical smears exhibited marked crystallizing activity since all samples showed patterns of some type. This increased crystallizing ability of the cervical mucus was sustained for 24 hours after the beginning of estrus in ewes and then reduced gradually with only 31.25 percent of the mucus samples showing crystal patterns, and then exclusively of Type I, on +2 days of the cycle. A great deal of fluctuation in the continuation of Type II patterns during the estrus phase was observed.

In general, this pattern seemed to predominate only during the first 12 hours of heat; however, occasionally it was seen to reappear again towards the end of heat, and was replaced by Type I at its termination. In only 2 of the 19 estrus periods, did the Type II pattern persist for 24 hours to be replaced by Type I which marked the end of estrual activity.

During the estrus period, this variant of crystallization was interspaced by other Types, such as Type III, Type IV or by mixed patterns. In two cycles, the Type II pattern reappeared 44 to 48 hours after the onset of estrus. The occasional reappearance of fern pattern towards the end of heat has also been recorded in Columbia ewes (Daniel *et al*. 1960).

The different varieties of Type II patterns occurring at or during the time of estrus are shown in Figures 4 through 8; however, the types of crystallization present at the peak of estrus activity are shown in Figures 6 and 7. These patterns coincided more precisely with the voluminous, transparent and acellular mucus in the cervix. Fourty-eight hours after the beginning of estrus, till about the onset of next estrus, none of the samples examined revealed this pattern. The patterns similar to those illustrated here have also been observed at the time of ovulation or mid-cycle in women (Papanicolaou, 1946; Campos de Paz and Costa Lama, 1953) and at the time of heat in cows (Abusineina, 1962) and ewes (Daniel *et al*. 1960).

Cyclical Variation in Type III Patterns. As shown in Table I and Figure 18, the Type III pattern precedes and succeeds Type II in occurrence. The increasing crystallizing activity of the cervical mucus during the proestrual period (Day -3 to -1) was first marked by the appearance of the Type III pattern which subsequently increased until about 13-24 hours prior to the actual onset of estrus. In only one sample was it observed at the time of estrus, and not alone but in combination with Type II pattern. The occurrence of Type III patterns during the course of the heat period was very variable; however, its activity seemed to concentrate at about the middle of estrus period. The presence of Type III pattern during the proestrual period and its recurrence during the heat period suggests the possibility that it should also be present at the actual onset of estrus. However, the data reported here suggests that it is not present at the onset of heat since it was never observed in samples collected in the 0-4 hour period. It must be considered that the ewes were observed at 4 hours intervals. Therefore, the interval between actual time of color marking and the collection of mucus sample would be in the range from 1 to 4 hours after the ewe was served. It is likely that no samples were collected exactly at onset of estrus. Generally, in the period from 13-16 hours after the onset of heat until it terminated this pattern was found in combination with other types such as Type II, IV or I. It is interesting to note that as was noted in the Type II patterns, this pattern also disappeared from the cervical mucus within a maximum of 48 hours after the onset of estrus.

<u>Cyclical Variation in Type IV Patterns</u>. Examination of

the data presented in Table I and the distribution of this pattern in Figure 18 would reveal that the Type IV pattern did not exhibit a set sequence of occurrence. Generally, this pattern was more prevalent during the last half of the estrus period; however, as the estrus continued a great difference in the size and shape of this pattern was observed. In most of the cases this pattern was found admixed with Types II and III.

Distribution of Mixed Patterns. More than one pattern could exist on the same slide; therefore, it was necessary to indicate the occurrence of such mixed patterns during the estrous cycle in ewes. Figure 18 illustrated the frequency of their occurrence at various time intervals. The vertical axis shown on days -3 and -2 principally consist of a mixture of Type I and Type III; whereas, those shown at -1 to -13 hours (-1 day) and again at 13-16 hours (0-day) were composed of patterns II and III. The frequency of occurrence of combinations of the major patterns described in this study seems to predominate at the beginning and again at the second half of the estrus period. The distribution points of the mixed patterns indicate a stage when the particular pattern noticed on the previous sampling period undergoes a change to the succeeding Type. For example, the occurrence of mixed patterns of Type I and III on days -3 and -2 coincides with the period when Type I undergoes transformation to Type III. Similarly, the predominance of Type II and III on -13 to -24 hours (-1 day) and again at 13-16 hours (O-day) illustrates how the crystal patterns III and II undergo a change during the different time intervals. These transitional phases of crystal transformation perhaps indicate a change in the physico-chemical properties of cervical mucus at the respective periods of the estrous cycle.

The cyclical variation described for various patterns did not exhibit a definite order of sequence. In 50 percent of the cases, the initially observed Type II pattern at the time of estrus changed to Type III pattern at different intervals during the estrus period. In 37.7 percent of the cases, smears showing the Type II pattern were succeeded by mucus smears showing both varieties; Type II and Type III, in combination. As recorded earlier, in 2 estrus periods the Type II persisted for 24 hours and was replaced abruptly by Type I pattern. In 5 of the 19 periods, Type II again reappeared after the Type III and/or Type IV or mixed patterns had been observed at the previous sampling period. This to and fro change in the occurrence of crystallization seemed to continue throughout the heat period. However, the appearance of Type I at the termination of heat was the most consistent change noted in the different estrus periods studied.

Since the presence of Type II, III, IV and mixed patterns predominated at, or during, the estrus period, their length of duration during an entire estrous cycle was calculated. The mean duration of these types varied from animal to animal, and ranged from 29.25 hours to 61.33 hours with an overall mean of 46.11 hours. In Columbia ewes the arborization (rosette, arbor and fern) were reported to occupy 3 days of the entire estrous cycle (Daniel et al. 1960). In Romney ewes the typical crystal patterns persisted for as long as about 6 days before to 4.5 days after estrus, with an average of 4.5 days for a complete estrous cycle (Raeside and McDonald, 1959). After the onset of estrus the average duration of crystallization (Types II, III, IV, and Mixed) ranged from 20.75 hours to 31.6 hours with an overall mean of 24.91±1.78 hours. In 8 of the 18 (44.4%) estrus periods, these patterns were observed 34.41±11.53 hours prior to the onset of estrus while in remaining cases it did not appear until about the onset of heat.

This experiment has illustrated the periodicity in the occurrence of crystallization pattern Types during the various phases of estrous cycle in Dorset X Rambouillet ewes. The crystal pattern Types II, III, IV, and Mixed occurred with great regularity and their activity seems to be associated only with the follicular activity of the ovary. Arronet (1957) concluded that the intensity of crystallization and their duration depends on the estrogenic activity of ovary. Roland (1952) proposed that by grading the quantity and type of fern the relative height of estrogen may be determined. It is now agreed that presence of extensive arborization in the cervical mucus is associated with increased production of estrogen by the ovaries. It was observed that the Type II crystal pattern described in this study was associated only with the period of estrus, and at the peak of their occurrence, the ewes showed an intense attraction towards the ram.

Cyclical Changes in the Amount, Appearance and Consistency of Cervical Mucus. On the basis of visual appraisal of the amount, appearance and consistency, the cervical mucus obtained from these ewes was graded in the following manner:

Scanty: Amount of mucus enough to prepare more than one but less than two slides.
Moderate: Quantity of mucus sufficient to prepare two but less than three slides.
Copious: Amount of mucus obtained in large quantity to prepare three or more slides.

The viscosity was judged by the force needed to blow the mucus out of the tube in which samples were collected from the cervix, or by observing the stickiness of the mucus while spreading it on the slide. The appearance of the mucus was assessed as turbid or clear by holding the prepared smear against the light and noting the degree of diffusion of the light passing through it. Generally, after the clear watery phase of mucus secretion, small whitish cellular flecks were seen in a drop of mucus which was otherwise transparent. If such material was present in large quantities, it was designated as turbid, otherwise it was placed in the category of clear mucus.

This broad classification of the amount and character of mucus was admittedly very arbitrary. However, since research has shown a relationship between these attributes of mucus and the phenomenon of arborization, it was of interest to study how the changes in these properties of cervical mucus reflected concomitantly on the pattern type variation of crystallization in the smears.

Table II presents the occurrence of these arbitrary evaluations during the various phases of estrous cycle. Generally, about one day before estrus, cervical mucus showed the tendency to increase in amount and tended to become clear, thin, and watery as estrus approached. During the pre-estrual stage the mucus was invariably turbid in appearance and was admixed with a few cellular elements from the cervical canal. This phase of mucus secretion then gradually culminated in a copious, transparent and thin mucus at estrus and the maximum quantity was accumulated 4 to 6 hours after estrus in the anterior part of the vagina. On several occasions this secretion flowed out of speculum after it was inserted into the vagina. The duration of this phase of mucus secretion was variable but tended to last for 8 - 12 hours after the onset of estrus. A gradual change in the appearance and the amount of mucus aspirated was observed, generally, from 12 to 13 hours after the onset of estrus. This mucus contained whitish colloidal particles which gave the mucus a translucent appearance. Correspondingly, the volume of mucus decreased and it tended to become thick in consistency and required more force to blow out of the plastic tubes that were used for collecting the samples. This change was especially evident after the small amount of whitish flecks appeared in a clear, copious

TABLE II

AMOUNT, APPEARANCE AND CONSISTENCY OF CERVICAL MUCUS SAMPLES OBTAINED ON DIFFERENT DAYS OF THE ESTROUS CYCLE IN RELATION TO DAY OF HEAT (DAY-0) AND AT FOUR HOUR INTERVALS DURING ESTRUS PERIOD IN EWES

Days	Hours	Cervix Closed or Dry	Sparce, Turbid, Non-Viscous	Sparce, Turbid, Viscous	Space, Clear, Viscous	Moderate, Turbid, Non-Viscous	Moderate, Turbid, Viscous	Moderate, Clear, Viscous	Copious, Turbid, Viscous	Copious, Clear, Viscous	Total Samples
-9 to -8		50.00[a] (11)[b]	36.36 (8)	9.10 (2)	4.54 (1)	-	-	-	-	-	22
-7 to -6		27.03 (10)	54.05 (20)	18.92 (7)	-	-	-	-	-	-	37
-5 to -4		11.11 (4)	47.22 (17)	16.67 (6)	5.56 (2)	8.33 (3)	11.11 (4)	-	-	-	36
-3		23.53 (4)	17.65 (3)	5.88 (1)	-	5.88 (1)	47.06 (8)	-	-	-	17
-2		5.56 (1)	22.22 (4)	5.56 (1)	5.56 (1)	16.67 (3)	33.33 (6)	11.11 (2)	-	-	18
-1	-24 to -13	-	8.70 (2)	13.04 (3)	-	-	17.39 (4)	8.70 (2)	52.17 (12)	-	23
	-12 to -1	-	-	-	-	-	8.70 (2)	-	34.78 (8)	56.52 (13)	23
Day of Estrus (0-day)	0 - 4	-	-	-	-	-	-	5.00 (1)	-	95.00 (19)	20
	5 - 8	-	-	-	-	-	-	-	10.00 (2)	90.00 (18)	20
	9 - 12	-	-	-	-	-	-	5.26 (1)	26.32 (5)	68.42 (13)	19
	13 - 16	-	-	-	-	-	-	5.26 (1)	42.11 (8)	52.63 (10)	19
	17 - 20	-	5.50 (1)	5.50 (1)	-	-	11.11 (2)	-	33.33 (6)	44.44 (8)	18
	21 - 24	-	-	16.67 (3)	-	-	11.11 (2)	-	50.00 (9)	22.22 (4)	18
+1	+1 to +12	-	8.33 (2)	8.33 (2)	-	20.83 (5)	37.50 (9)	-	20.83 (5)	4.17 (1)	24
	+13 to +24	-	22.22 (2)	-	-	33.33 (3)	11.11 (1)	-	22.22 (2)	11.11 (1)	9
+2		-	50.00 (8)	12.5 (2)	6.25 (1)	12.50 (2)	12.5 (2)	6.25 (1)	-	-	16
+3		6.25 (1)	62.50 (10)	18.75 (3)	6.25 (1)	6.25 (1)	-	-	-	-	16
+4 to +5		9.68 (3)	74.19 (23)	12.90 (4)	-	3.22 (1)	-	-	-	-	31
+6 to +7		22.58 (7)	61.29 (19)	16.13 (5)	-	-	-	-	-	-	31
+8 to +9		37.5 (9)	54.17 (13)	8.33 (2)	-	-	-	-	-	-	24

[a] Figures not enclosed in brackets indicate percent of all samples.

[b] Figures in brackets indicate number of samples.

and thin mucus. Subsequently, these changes occurred in a progressive manner until about the end of estrus when it became moderate in quantity and looked whitish or turbid in appearance. During metestrus and diestrus, only negligible amounts of a whitish, cheesy and highly cellular mucus could be recovered. From about the 3rd to 4th day after estrus until near the onset of proestrus, the cervical os was either closed or a small amount of white material consisting chiefly of cellular debris, was smeared around the external os of the cervix.

Several studies (Cole and Miller, 1931, 1935; Grant, 1934; Sanger et al. 1958) have described these cyclic changes in the cervicovaginal mucus of ewes. However, the specific changes in the mucus collected directly from the cervical canal do not seem to have been examined. The dependence of the secretion of clear, watery and copious, thin mucus on estrogen (Glover, 1959; Abarbanel, 1946), and the characteristic thickening of mucus resulting from progesterone secreted by the ovary have been described (Robinson and Moore, 1956). It appears that the characteristic changes in the physical character of cervical mucus as well as crystallization patterns, are directly governed by the ovarian hormones.

Since these sequential changes in the physical properties of the cervical mucus were paralleled by the degree and form of crystallization observed in the smears, it was interesting to relate these changes with the types of crystal patterns observed in such mucus smears. The results denoting the distribution of various pattern types in the arbitrarily categorized mucus are presented in Table III. It may be observed that, although crystallization occurred in all types of mucus, sparce mucus always exhibited Type I patterns, while the Type II, III, IV, and mixed patterns occurred in various proportions in moderate and copious mucus. Type II patterns were associated almost exclusively with clear, copious and viscous mucus since 94.74 percent of the total Type II patterns were observed in this category of mucus. Type III patterns were present more frequently (80 percent) in copious, viscous but turbid mucus. The Type IV patterns persisted in moderate or copious quantities of mucus which was turbid in appearance and viscous in consistency. A total of 56.52 percent of the mixed patterns occurred in copious, clear and viscous mucus. These mixed patterns consisted principally of the Type II and III or II and IV.

Maximum crystallization occurred in copious mucus since 94.44 percent of the total copious mucus samples showed the crystal patterns.

These changes, in general, illustrate the association in this study between the different characteristics of mucus and the types of crystallization.

Rydberg (1948) observed massive crystallization in limpid and stringy mucus obtained at the time of ovulation in women. Papanicolaou (1946) believed that the form and

TABLE III

DISTRIBUTION OF THE CRYSTAL PATTERN TYPES IN THE VARIOUS CATEGORIES OF CERVICAL MUCUS IN EWE

Amount	Sparce			Moderate			Copious		Total of all the Samples/ Types of Crystallization
Appearance	Turbid		Clear	Turbid		Clear	Turbid	Clear	
Consistency	Viscous	Non-Viscous	Viscous	Viscous	Non-Viscous	Viscous	Viscous	Viscous	
Total Number of Samples	42	132	6	40	19	8	57	87	391
Percent of Samples containing arborization	85.71	7.57	66.67	95.00	10.53	100.00	85.96	100.00	-
No. of Samples containing arborization	36	10	4	38	2	8	49	87	234
No. of Samples containing Type I	36	10	4	27	2	1	2	-	82
No. of Samples containing Type II	-	-	-	1	-	2	1	72	76
No. of Samples containing Type III	-	-	-	4	-	4	40	2	50
No. of Samples containing Type IV	-	-	-	1	-	-	2	-	3
No. of Samples containing Mixed Patterns	-	-	-	5	-	1	4	13	23

structure of crystals is determined by corresponding changes in the viscosity of mucus; whereas, Campos de Paz and Costa Lama (1953) found a close relationship between the phenomenon of crystallization and the spinnbarkeit and cellular content of mucus. Similar observations in cows indicated that the mucoproteins, sodium and potassium chlorides and the increased water contents which are responsible for fernlike patterns are directly under the influence of estrogen secreted by ovary (De Vuyst et al. 1961). These relative references cited above would lead to the suggestion that the variation in the crystal patterns observed during the various phases of estrous cycle are the reflections of the ovarian hormones---estrogen and progesterone, which initiate changes in the physico-chemical properties of the cervical mucus which in turn produce various forms of crystal patterns at different periods of estrous cycle.

Phenomenon of Crystallization in Nasal Mucus. The nasal mucus of the ewes exhibited crystal patterns in various forms and shapes, but it was not possible to categorize them on the same basis described for cervical mucus. Therefore, the types of crystallization typical of the nasal mucus were arbitrarily classified into two main categories, typical and atypical crystallization.

Typical Crystallization: The general appearance of these crystals is shown in Figure 17. Microscopically, the crystals were composed of a main stem arranged in a radial fashion from which branches emerged in all directions. The venation, if present, formed dented projections, and were not very fine or smooth in appearance. In thin and sticky nasal mucus, other varieties of fine fern-like crystals were also present. These, and similar types of organized crystallization, were placed in this category.

Atypical Crystallization: These crystals consisted of various forms and shapes which did not possess the organized assembly as described for typical nasal patterns. Microscopically, these crystals appeared very crude and rough in appearance and were embedded in, or sometimes superimposed on, the cellular matrix. In some samples, the rows of crystal-like structures were arranged in a zig-zag manner, without any characteristic shape or form. At times, cubic shaped crystal nucleii formed the body of the crystal patterns. These patterns were present in a thick, gelatinous or tenacious nasal mucus.

The distribution of crystal patterns in nasal mucus on the various days relative to first day of estrus (day 0) are presented in Table IV and Figure 19. It may be seen that crystallization phenomenon in nasal mucus has a wide range in its occurrence, and typical or atypical patterns seem to persist throughout the estrous cycle in ewes. A tendency was, however, noticed for the typical crystallization to increase three days before estrus. At the time of heat (0-4 hours), 75 percent of the nasal mucus samples exhibited typical patterns compared to 95 percent shown by the cervical mucus at the corresponding stage of estrus. A

TABLE IV

CRYSTALLIZATION PATTERNS IN NASAL MUCUS DURING THE ESTROUS CYCLE OF EWE

Days Relative to Esturs	-9 to -8	-7 to -6	-5 to -4	-3	-2	-1		Day of Estrus (Day-0)						+1		+2	+3	+4 to +5	+6 to +7	+8 to +9
Hours						-24 to -13	-12 to -1	0-4	5-8	9-12	13-16	17-20	21-24	+1 to +12	+13 to +24					
Total No. Samples Examined	22	37	36	17	18	23	23	20	20	19	19	18	18	24	9	16	16	31	31	24
No. Samples Showing Crystallization	19	26	29	15	18	16	20	20	20	19	19	14	9	8	6	12	8	23	27	21
Percent Showing Crystallization	86.36	70.27	80.55	88.23	100.00	69.57	86.95	100.00	100.00	100.00	100.00	77.78	50.00	33.34	66.67	75.00	50.00	74.20	87.09	87.50
Typical Crystallization	[a]13.64 [b](3)	18.92 (7)	25.00 (9)	41.17 (7)	61.11 (11)	17.40 (4)	47.82 (11)	75.00 (15)	50.00 (10)	52.63 (10)	52.63 (10)	27.78 (5)	11.11 (2)	4.17 (1)	11.11 (1)	50.00 (8)	-	12.90 (4)	9.67 (3)	25.00 (6)
Atypical Crystallization	72.72 (16)	51.35 (19)	55.55 (20)	47.06 (8)	38.89 (7)	52.17 (12)	39.13 (9)	25.00 (5)	50.00 (10)	47.37 (9)	47.37 (9)	50.00 (9)	38.89 (7)	29.17 (7)	55.56 (5)	25.00 (4)	50.00 (8)	61.30 (19)	77.42 (24)	62.50 (15)

[a]Figures not enclosed in brackets indicate percent of total samples.
[b]Figures given in brackets are the number of samples.

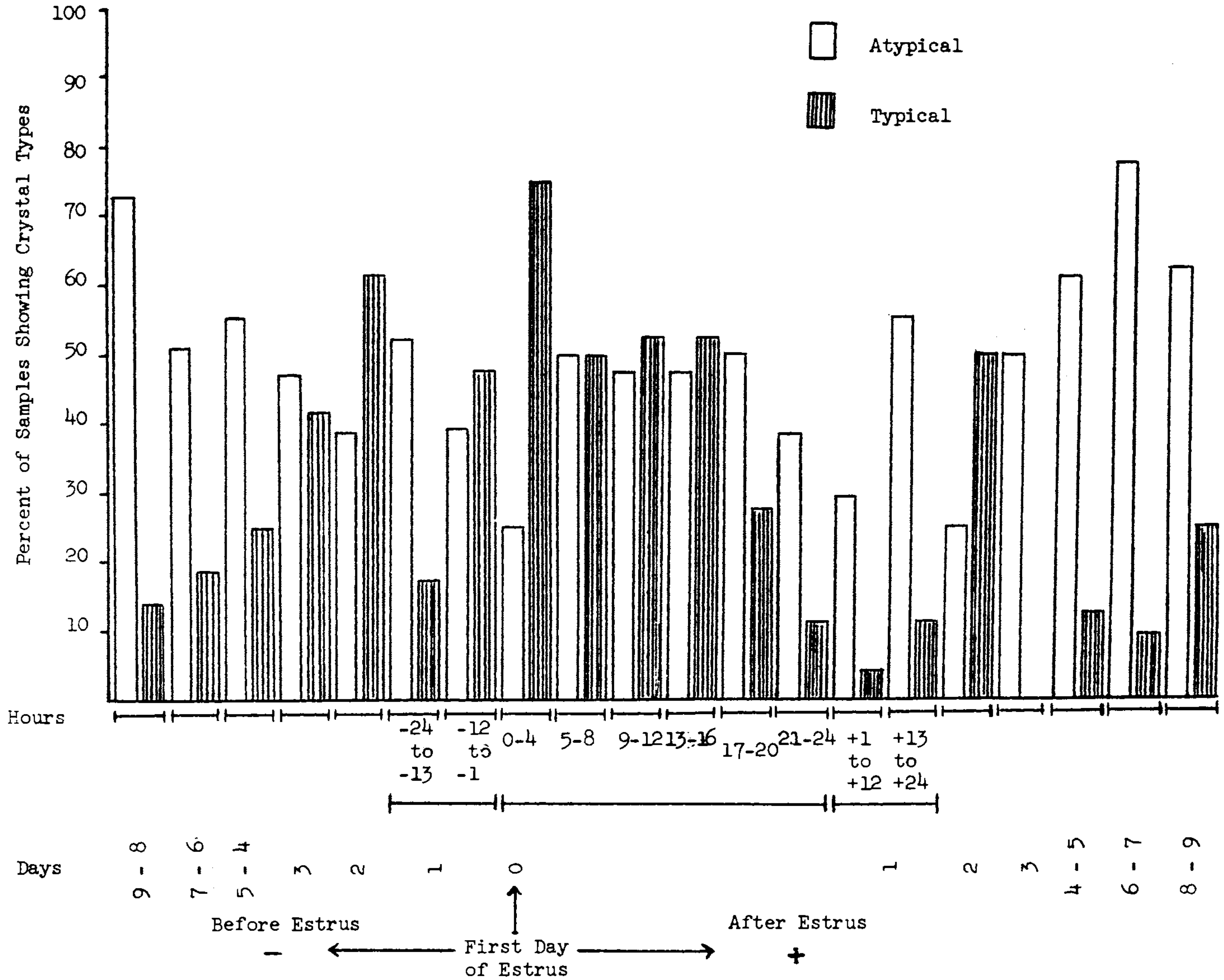

Figure 19. Distribution of Nasal Crystal Patterns at Different Times in the Estrous Cycle of Ewe

marked decrease in number of samples exhibiting typical pattern was, however, observed in the period from about 17 hours until about 48 hours after estrus. Considerable irregularity in the occurrence of typical and atypical patterns during the remaining part of the cycle was observed. The number of samples showing typical patterns, however, remained at low level during the metestrus and diestrus phase of estrous cycle.

During the course of this trial, it was found difficult to obtain nasal mucus free of contamination with dust particles or fragments of grass leaves. Such contamination is probably due to the grazing habits of this species of animals. Contamination of cervical mucus with cellular matter, blood, seminal plasma, lubricant cream, etc., is known to influence the pattern of arborization (Raeside and McDonald, 1959). Similar evidence for nasal mucus is lacking, but it is likely that similar conditions may influence the phenomenon of crystallization in nasal mucus.

In humans crystal patterns in nasal mucus similar to those seen in cervical musuc have been shown to occur at the different phases of menstrual cycle (Davis and Abou-Shabanah, 1958). If such changes in nasal mucus are due to the hormonal influence of gonads, such as appears to be the case in cervical mucus, the typical arborization in the nasal mucus should not be expected to occur during the luteal phase of estrous cycle in ewes. Whether the crystallization phenomenon in nasal mucus is governed by hormones of the ovaries is still left to further confirmation, since in this study clear-cut changes in the arborization pattern similar to those in cervical mucus were not observed. It is doubtful that, at least in ewes, this phenomenon is really cyclic and depends solely on the cyclic hormonal activity of the ovaries. It was, however, interesting to note that whenever the nasal mucus was thin, sticky, transparent and clear, typical arborization occurred irrespective of the stage of estrous cycle. Zondek (1956, 1954) reported the occurrence of nasal crystallization at all the stages of menstrual cycle in women. In the nasal mucus of patients suffering from colds, he was able to obtain typical crystal patterns similar to those observed in cervical mucus at the mid-menstrual cycle. These results indicate that the phenomenon of crystallization in nasal mucus can be influenced by factors other than the ovarian hormones.

In adhesive, thick or tenacious, yellowish nasal mucus, the atypical patterns in various shapes and forms appeared in the smear. These configurations covered the major portion of the mucus smear, but their configurations were composed of discrete cubic shaped nucleii which gave a beaded appearance to the crystal structures. This may be due to low electrolyte concentration in the nasal mucus. Ullery et al. (1959) emphasized the role of electrolyte concentration in the mucus to be the factor responsible for variation in the crystal patterns. McSweeney and Sbarra (1964) noticed cyclic variations in the chloride ion concentration of nasal mucus of women, but the concentration was less than that found in cervical mucus. These findings may explain the presence of atypical beaded crystal structures in the nasal mucus of ewes.

A review of research given for crystallization in nasal mucus in humans indicates a great deal of controversy over the cyclic activity of nasal mucus. Zondek (1953) does not consider that the phenomenon of arborization in any body fluids other than cervical mucus has any significance in studying ovarian activity. Results obtained on the phenomenon of arborization in nasal mucus of ewes does not seem to indicate the clear-cut cyclic activity of crystallization that is evident in the cervical mucus at the different stages of estrous cycle.

<u>Pattern Type Variation</u>. In the classification of the arborization patterns, considerable variation in their form and shape and their appearance under the microscope was noticed. Variation in the pattern types could be attributed to a number of factors, some of which may result as a consequence of physical influence, or may be due to environmental effects. The changes initiated in the physico-chemical properties of the cervical mucus, which seem to be under the sole influence of ovarian hormones, may also be responsible for pattern type variations. These factors may either act individually, or in combination, thereby influencing the morphological appearance of the crystal types.

Among the physical factors, the manner in which the smears are prepared affects the configuration of crystals. Zondek (1954) observed that a smear of moderate thickness would produce a variety of flower patterns; whereas, a thick smear would produce an arborization resembling the pattern of palm leaves. In the present work this appeared to be the case, since on the part of the slide where the mucus was evenly spread, the arboreal patterns shown in Figures 6 and 7 appeared, but at the end of the slide, or at the edge of the smear, the configuration similar to those shown in Figure 8 was present. The presence of dense and compact configuration, as shown in Figure 7, may not be attributable to the thickness or the thinness of the film, because, while observing the process of drying and the crystal formations under the microscope, these configurations were formed later than those seen in Figure 6 on the same slide. These compact crystals (Figure 7) apparently did not have enough space to throw out branching and sub-branching extensively, and, as a result, they assumed the compact and dense appearance shown in Figure 7. During the course of the trial, the thin, slimy and transparent mucus obtained at the time of estrus was placed on the slide. Only half of the drop of thin mucus was evenly spread into a thin film while the remaining half was allowed to dry as it was. Arborization typical of estrus stage was present only in the thin film of mucus, but not in the portion of the mucus, which was allowed to dry in a clump.

Another factor that can influence the arborization pattern is the time interval at which the slides are examined after the smears were dried. Campos De Paz and Costa Lama (1953), De Vuyst et al. (1961) have emphasized the importance of examining the mucus immediately after drying the film. This was investigated in this work. The full fern pattern typical of estrus which was formed in a freshly dried film was left at room temperature for 24 hours. On examination after 24 hours, it was found to have disintegrated and produced the atypical configuration as shown in Figure 9.

Sometimes, the cellular elements in the cervical mucus were seen to influence the crystal patterns as shown in Figure 12. It was found that these cells interfere with the formations of venations and subvenations. Their deposition on crystal structures pose difficulties in categorizing such crystal patterns since the morphological features of the crystals are hidden when viewed through the microscope.

It has been hypothesized that the cervical mucus is not homogeneous in composition (Bane and Rajakoski, 1961). The presence of organic material in the medium in which crystallization takes place has been shown to affect the crystal habits such as length, density and thickness (Abou-Shabanah and Plotz, 1957). The examination of Figures 14 and 15 would reveal the role of organic material in influencing the appearance of crystals under the microscope. The cervical mucus from which both these crystals were obtained, had whitish fine particles in the center of the blob of mucus placed on the slide for preparing the smear. While smearing the mucus, the portion of the film which contained whitish fine particles produced the configuration as shown in Figure 15; whereas, the pattern of the same smear where clear and transparent mucus was present gave the pattern with distinct venations and subvenations shown in Figure 14. Morphologically, both of the crystals are similar in configuration except that the distinct and clear appearance of venations and subvenations was greatly influenced by the organic material which gave a thick and rough appearance to crystal structure (Figure 15). From these observations on the variations in the pattern types of the cervical mucus, it appears that the different patterns exhibited by the cervical mucus obtained during various phases of estrous cycle, generally reflect the alterations in the physico-chemical properties which are under the influence of ovarian hormones. However, certain variations observed in the morphological appearance of the crystals may also result from other factors such as the manner in which the smears are prepared and the time interval at which the slides are examined after they were dried.

SUMMARY

Cyclical variations in the morphological appearance of the crystal patterns in the nasal as well as cervical mucus were followed through 14 estrous cycles in five Dorset X Rambouillet ewes from August 11, 1966 to October 14, 1966. Samples of mucus were collected from the cervix and nose daily during most of the estrous cycle. Sampling at four hour intervals was started generally one day before estrus, extended through estrus and terminated during post-estrual stage. The variations in the amount, appearance and the consistency of the cervical mucus was studied and, depending upon the quantity of the mucus obtained, the samples were categorized as sparce, moderate and copious. The mucus was further divided as turbid or clear, and viscous or non-viscous. The crystal patterns exhibited by the cervical mucus were categorized into 4 main Types: Type I, Type II, Type III and Type IV. The crystal patterns in the nasal mucus were grouped into two main categories: Typical patterns and Atypical patterns.

The results obtained in this experiment indicated that the crystal patterns of Types II, III, IV, and combination of any of these types were associated with proestrus and estrus stages of the cycle and tended to appear in an 18-day cyclic manner, interspaced by Type I patterns. The

onset of estrus was characterized by Type II patterns in the cervical smears; however, occasionally these patterns were observed again towards the end of estrus period. The beginning of proestrus was generally marked by the Type III patterns. These patterns were not seen in the mucus samples collected at the time of estrus but instead, reappeared during the middle of estrus period. Both Type II and Type III crystal patterns disappeared simultaneously from the cervical mucus within a maximum of 48 hours after the onset of estrus. The occurrence of Type IV patterns was variable; however, their activity seemed to predominate during the last half of the estrus period. The mixed patterns consisting of Type I and Type III occurred at the initial stage of proestrus, while the combinations of Types II and III were frequently observed about 12 hours before, and again 13-16 hours after the onset of estrus. The Type I pattern appeared abruptly at the cessation of estrus behaviour and continued irregularly through metestrus and diestrus periods of estrous cycle. The presence of Type II pattern at the onset of estrus, and the abrupt appearance of Type I pattern at the termination of heat was the most prominent and consistent change noted in different estrus periods studied.

The crystal patterns of Types II, III, IV and mixed, occupied about 46.11 hours of the entire estrous cycle; however, after the onset of estrus these patterns persisted for 24.91 ± 1.78 hours.

During proestrus cervical mucus showed a tendency to increase in quantity and invariably remained turbid in appearance until about the onset of estrus when a large quantity of clear, acellular and stringy mucus accumulated into the anterior part of the vagina. Within 8-12 hours after the onset of estrus the cervical mucus progressively decreased in amount while its turbidity increased until the end of estrus period. During metestrous and diestrus period sparce, whitish cheesy material was present.

The distribution of various patterns of crystals in the arbitrarily categorized cervical mucus indicated that Type II patterns predominated in the clear, copious and viscous mucus while the Type III patterns were more frequently associated with copious, turbid and viscous mucus. Type IV patterns were seen in either copious or moderate mucus but turbid in appearance. Mixed patterns occurred in large percentages in copious, clear and viscous mucus.

The typical nasal patterns persisted throughout the estrous cycle in ewes; however, their occurrence showed a tendency to increase 3 days before estrus, and in 75 percent of the mucus samples collected at the onset of heat exhibited typical patterns. During metestrus and diestrus, the typical patterns were present in the nasal mucus but their percentage was considerably reduced.

The factors, other than physiological, responsible for crystal pattern type variations are discussed. The manner in which the smears are prepared and the immediate examination of the slides after drying are stressed.

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VITA

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