SOME HISTOLOGICAL AND HISTOCHEMICAL ASPECTS OF THE CANINE OVARY, OVIDUCT AND UTERUS FOLLOWING THE ADMINISTRATION OF DIETHYLSTILBESTROL FOR PREGNANCY TERMINATION

by

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INTRODUCTION

The veterinary practitioner is occasionally called upon to terminate an unwanted pregnancy in the female of the canine species. While the use of diethylstilbestrol for this purpose has been frequently reported, information on the histological and histochemical changes that occur in the canine genitalia is not, at the present, readily available in the literature.

The purpose of this study was to investigate and report the cytological changes which may be seen in the uterus, fallopian tubes and ovary following the parenteral administration of diethylstilbestrol to induce early abortion.

REVIEW OF THE LITERATURE

The Canine Estral Cycle

The bitch is a monestrus animal, having one estrus per breeding season. Most bitches have two breeding seasons per year, usually in the spring and autumn (Venzke and Donovan, 1966). They can, however, occur at any time of the year (Hancock and Rowlands, 1949). The age at which sexual maturity is reached varies among breeds and individuals within a breed (Hancock and Rowlands, 1949). Smaller breeds tend to have shorter intervals between breeding seasons than larger breeds (Harrop, 1960). Consequently, a small dog may have three or even four breeding seasons a year.

Arenas and Samartino (1939) classified the canine estral cycle according to the follicular activity of the ovary. The stages they listed are: (1) resting period, 2 months, (2) follicular phase, 3-4 weeks, (3) luteal phase, 3 weeks, and, (4) regressive phase, $2\frac{1}{2}$ months.

Evans and Cole (1931) have contributed a classical description of the estral cycle of the dog. They divided the cycle into four stages: proestrus, estrus, metestrus and anestrus. A summary of their histological observations follows.

In <u>proestrus</u>, the endometrium contains simple uterine glands and shows edema of the connective tissue stroma,

hyperemia and extravasations of blood into the subepithelial tissue of the uterus. The ovary contains many follicles of various sizes, the number being markedly reduced at the end of active estrum. In the dog, folding of the follicular wall occurs before ovulation. This folding of the mural granulosa involves the connective tissue and blood vessels of the theca interna. The ovum does not float free in the liquor folliculi until the time of the first acceptance of service. Proestrus is timed from the onset of the sanguinous vaginal discharge to the first acceptance, a period ranging from 7 to 13 days in length with an average duration of 9 days.

Estrus extends from the day of the first acceptance of the male to the day of the last acceptance, a period ranging from 6 to 13 days in length with an average duration of 7 days. Not much change occurs in the endometrium until the sixth day of estrum, at which time the epithelium of the crypts and deep-lying glandular tubules becomes larger and higher. The number of follicles in the ovary has been reduced at the time of first acceptance. Many follicles are undergoing atresia at this time. Ovulation is said to be spontaneous and all ova are expelled within the first 24 hours of the first acceptance of coitus. Griffiths and Amoroso (1939) reported ovulation as occuring in the first 1 to 3 days of estrus. The newly formed corpora lutea cells, as described by Evans and Cole, are not compact but by the eighth day

after the onset of estrus, they are compactly arranged, although large cavities may be seen in the luteal tissue.

Metestrus is the period between the last acceptance of the male and the completion of the regenerative process, a period ranging from 88 to 99 days with an average duration of 90 days. Mitoses are seen in the deepest portions of the uterine glands and the glandular complex increases. Further, Evans and Cole described three distinct zones of the endometrium: (1) a superficial one completely occupied by enlarged crypts and showing columnar epithelium, (2) a middle zone consisting mainly of connective tissue in which are scattered the neck portions of the glands, and, (3) an outer zone crowded with branched and coiled uterine glands. This appearance remains up to the 14th day of metestrus, regardless of the presence or absence of pregnancy. On the 20th day, regression of the glandular complex begins in the non-pregnant animal and is very marked by the 30th day. By the 70th day, the surface epithelium has undergone marked degeneration, being completely replaced by new cells by the 88th day. Regeneration of the uterine glands is also reported and the glandular and luminal epithelium are of the low cuboidal type by the 99th day.

Evans and Cole indicate that lutein cells reach their maximum size on the 10th day of metestrus and degeneration begins on the 30th day. In this phase, cords of epithelial

cells from the germinal epithelium extend into the medulla of the ovary between the corpora lutea. This phenomenon has also been reported by Barton (1945).

In anestrus, the endometrium shows no change from the end of metestrus. This phase extends from the end of the regenerative period to the beginning of the next proestrus, a time which may last for as long as 5 months but usually averages about 2 months in length.

Mulligan (1942) described the cyclic changes of the canine female genital tract. He stated that few changes are noted in the fallopian tubes. A high columnar epithelium was noted throughout the lumen on many mucosal folds of the tubes. Maximow and Bloom (1955) and Trautmann and Fiebiger (1952) indicated that the epithelium varied from simple columnar to pseudostratified columnar. Arenas and Samartino (1939) described tall, ciliated epithelial cells interspersed with non-ciliated secretory cells in the follicular phase of the cycle. As the cycle progressed into the luteal phase, the epithelial cells became a low columnar type and lost their cilia. Harrop (1960) stated that the anestral epithelial cells were columnar in type and lacked cilia. Then in proestrus, they increased in height, became ciliated and developed a granular secretory cytoplasm. The cilia were again lost in metestrus.

Mulligan (1942) described a high columnar epithelium of the endometrium and sub-mucosal glandular structures during

proestrus. He also described hemosiderin-laden macrophages throughout all layers and suggested that this represented the recovery of blood lost through focal extravasations. He divided the endometrium into four layers: (1) a basal glandular layer, (2) a layer containing the tubules of the glands, (3) a stromal layer of connective tissue through which the tubules pass, and, (4) a glandular crypt layer.

The descriptions of Evans and Cole and Mulligan are in agreement as to the cyclic changes that occurred. However, Mulligan further stated that regeneration of the luminal epithelium arose from the crypts. He also stated that the corpus luteum of pregnancy retained about the same morphology at the end of gestation as it had 3 weeks after fertilization. The corpus luteum, 90 days post-partum, corresponded, histologically, to that at the end of metestrus of the non-pregnant animal. Regression of the corpus luteum was characterized by fatty degeneration of the lutein cells, phagocytic infiltration and invasion by connective tissue cells to eventually form the avascular remnant, the corpus albicans (Brambell, 1956).

Mulligan (1942) and Amoroso (1952) described the preimplantation and implantation changes that occurred in the endometrium of the dog. Fertilization of the ova occurred in the uterine tubules and the zygotes, either in the morula or blastocyst stage, entered the uterus 6 to 8 days after ovula-

tion (Griffiths and Amoroso, 1939; Harrop, 1960). The uterine glands became dilated and had a flattened epithelium. The tubules were small remnants and the connective tissue of the stromal layer was stretched and thinned. The crypts became widened into large recesses lined by flattened epithelium, the recesses enclosing a mucinous material. The surface epithelium became hyperplastic, was shed and was utilized as food by the embryo. The chorionic villi of the embryo became intimately associated with maternal capillaries forming the endothelial-chorial type of zonary, deciduate placenta. Implantation occurred around the 17th day after ovulation. Regeneration of the endometrium at the interplacental areas proceeded as previously described. This regeneration was, in the beginning, more complex than that seen in regeneration of the non-pregnant uterus.

Evans and Cole (1931) and Mulligan (1942) indicated the duration of metestrus and anestrus to be 90 and 60 days, respectively. Their classifications were based on the histological aspects of the endometrium and ovary.

Harrop (1960) is of the opinion that, clinically, the two periods, metestrus and anestrus, are 60 and 90 days in duration, respectively.

The Vaginal Smear

The original work of Evans and Cole (1931) is recognized

as the classical description of canine vaginal exfoliative cytology. The cellular changes observed on the vaginal smear closely followed the various stages of the estral cycle. The diagnostic value of the vaginal smear has long been recognized in human medicine (Papanicolaou, 1933). It has also been investigated in the cow (Hansel et al., 1949), and the ewe (Sanger et al., 1958). Many reports on the clinical use of this technique in the dog have appeared in the literature (Roberts, 1956; Gier, 1960; Harrop, 1960; Hooper et al., 1961).

The histological changes that occurred in the canine vagina were described by Evans and Cole and Mulligan. In the anestral animal, the epithelium consisted of two layers, a basal cuboidal and an outer columnar. In proestrus, the vaginal epithelium changed to a stratified squamous type with as many as 20 cell layers. The percentage of cornified epithelial cells that appeared in the vaginal smear gradually increased through proestrus. In estrus, the vaginal epithelium gradually sloughed and neutrophils infiltrated it and the lamina propria. By the first day of metestrus, the epithelium was 3 to 6 layers thick. The anestral appearance had returned and cellular infiltration ceased by the 20th day of metestrus.

The vaginal smear as described by Gier (1960) served as the method of choice in this investigation. The proestral smear was characterized by non-cornified epithelial cells, a

higher percentage of which became cornified as the stage progressed. Erythrocytes were prominent but usually decreased or disappeared toward the end of proestrus. Leucocytes were prominent in the early part of proestrus but disappeared about midway through the stage. Cellular detritus, moderate at first, became light but did not disappear completely.

During estrus, the vaginal smear showed only cornified epithelial cells, no leucocytes in the early part, light cellular detritus and a variable occurrence of erythrocytes. The leucocytes reappeared about one-half to three-fourths of the way through estrus. Evans and Cole associated this with the close of estrus but Gier stated this occurred 36 to 43 hours after ovulation. It may, therefore, signal a decline in the conception rate if breeding occurs after the leucocytes reappear in the vaginal smear.

Cellular detritus became quite heavy in metestrus, frequently obscuring the intact epithelial cells. Many leucocytes were present and, again, the occurrence of erythrocytes was variable. Clumps of bacteria were often seen. All epithelial cells were cornified but these disappeared and non-cornified epithelial cells replaced them as the stage progressed.

At anestrus, all epithelial cells were non-cornified and cellular detritus was minimal. Usually there were scattered leucocytes present but erythrocytes were absent.

Estrogens -- Natural and Synthetic

The nucleus of the naturally occurring steroids is cyclopenteno-perhydro-phenanthrene (Bishop, 1962; Jones, 1957; Cantarow and Schepartz, 1954). Bishop (1962) listed the human estrogens as estrone, estradiol and estriol. He also described how these estrogens were esterified for a more prolonged effect and modified for increased potency.

Metzler et al. (1966) listed the following estrogens as being present in dog plasma in their free forms: estrone, estradiol-17a, estradiol-17b, estriol and 16-epistriol. They reported a progressive decline of the amount of free plasma estrogens in proestrus and suggested that they were being bound at a more rapid rate as the cycle progressed. In the bred animal, estrogen peaks occurred at the 3rd to 4th week of gestation, corresponding to the time of placental formation. An estrogen peak was also seen in the first week after parturition.

Pearlman et al. (1948) and Siegel et al. (1962) reported that estriol was not a major metabolite in the dog. Metzler et al. indicated estriol predominates. While the Metzler group measured only free estrogens, Paschkis and Rakoff (1950) stated canine estrogens were primarily in the esterified form. Metzler et al. suggested, therefore, that, in the canine species, the free and esterified estrogen level may be very high, physiologically.

The changes that occur in the canine endometrium during the follicular stage of the estral cycle can be appreciated by the works of Evans and Cole (1931) and Mulligan (1942). The demonstration of uterine and tubal contractions during estrus of the rat and pig was reported by Corner (1923). The visible signs of an approaching canine estrus, which include vulvar swelling, sanguinous vaginal discharge and attraction of the male, have been summarized by Harrop (1960).

Estrogenic compounds of the stilbene series were first reported by Dodds et al. in 1938. The name "stilbestrol" refers to 4,4' stilbenediol (diethylstilbestrol). In addition to diethylstilbestrol (D.E.S.), dienestrol and hexestrol, both of the stilbene group, were reported. Bishop (1962) mentioned another class of synthetic estrogenic compounds, chemically unrelated to the stilbestrol, the naphthalene derivatives. More recently, Sharman (1964) reported the new synthetic estrogen 3-cyclopentyloxy-oestra-1:3:5 (10)-triene-16a:17B-diol[1]. He stated that this compound was selective in its action and exerted its effect on the lower segments of the genital tract of the human being. It was only slightly effective on the endometrium.

Emmens et al. (1959) reported on estrogen inhibitors of the stilbestrol series. Emmens et al. (1962) listed twelve

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[1]Also known as Pentovis and Colpovis.

alkylstilbestrols and classified them according to their pro-estrogenic, estrogenic and anti-estrogenic activity. It was demonstrated that dimethylstilbestrol inhibited the response to natural and synthetic estrogens (estradiol, estrone, D.E.S.), the former more strongly than the latter.

Anti-estrogenic properties of the compounds tested were determined by their ability to inhibit cornification of the vaginal epithelium of the mouse. Using the mouse mitotic count method, the relative potency of estradiol, estriol, dienestrol, hexestrol and D.E.S. was determined (Emmens et al., 1962). According to this report, estriol and D.E.S. were the most potent.

The estrogenic effects of D.E.S. have been reported by many workers (Doods et al., 1938a, 1938b, 1938c; Folley and Watson, 1938, Noble, 1939; Mazer et al., 1941; Reuber et al., 1961). Dow (1959), in his work on the experimental reproduction of the cystic-hyperplasia-pyometra complex in the bitch, gave an excellent description of the endometrium following the long-term administration of varying amounts of D.E.S. to ovariectomized dogs. He reported that dogs receiving 5 mg. and 10 mg. D.E.S. per day died on the 33rd to the 38th day and 21st to 28th day, respectively. These animals showed thrombocytopenic purpura on postmortem examination. Dogs receiving 0.5 mg. and 1.0 mg. D.E.S. per day survived.

Dow (1959) reported the following histological appearance

of the endometrium following the administration of 0.5 mg. and 1.0 mg. D.E.S. per day for 10 days: (1) edema with dilatation of blood vessels in all strata, (2) diffuse extravasation of blood in the crypt layer without evidence of vascular damage, (3) low columnar luminal epithelium with deep staining basal nuclei, (4) slight increase in the number of crypts, which were lined by an epithelium taller than that of the surface, (5) narrow cryptal and tubular lumina which did not contain any secretion, (6) traces of glycogen in the superficial epithelium.

After treatment for 21 to 28 days, the following picture was described: (1) endometrium edematous but stromal hemorrages less marked and numerous hemosiderin-containing macrophages, (2) tall columnar luminal epithelium, (3) tubules more numerous with coiled basal glands, a few containing a secretion, and, (4) glycogen in the superficial epithelium.

After treatment for 33 to 38 days, the following was noted: (1) a reduction of edema, no evidence of stromal hemorrhage and a few hemosiderin-containing macrophages, (2) reduction in the width of the periglandular and perivascular reticulum network and apparent replacement by fine collagen fibers, (3) more prominent vacuolization of cytoplasm in the luminal and cryptal epithelium, (4) tubules more tortuous with an increase in the number of basal glands, and, (5) glycogen present only in a few cells in the cryptal zone.

Following the administration of D.E.S. for 120 days, Dow described the following changes: (1) general intensification of collagen deposition throughout all strata of the endometrium and myometrium, (2) the epithelium of all glandular structures had become lower and showed no evidence of secretory activity, and, (3) no further increase in the number of glands in the basal zone.

After 400 days of D.E.S. administration, fibrosis of all layers of the uterine wall with associated atrophy of the glandular structures and the cuboidal epithelium of the basal glandular area were noted.

Dow concluded that estrogenic substances alone do not cause cystic glandular hyperplasia of the endometrium. However, he did demonstrate that this condition could be produced by the administration of progesterone, either alone or in the "estrogen primed" animal.

Meyer and Nutting (1964), using the rat, reported the importance of progesterone for implantation and fetal survival. The synergistic action of estrogen and progesterone in the preparation of the uterus for the support of implantation and early embryonic life was also indicated by the works of Hisaw and Leonard (1930), who used the rabbit, Teunissen (1952), who used the dog, and Nutting and Meyer (1964), who used the rat. Hypertrophy of the endometrial glands and the release of their secretion under the stimulation of progesterone has

been reported by Hisaw and Leonard (1930), Bradbury et al. (1950), Teunissen (1952) and Dow (1959).

Pregnancy Prevention and Interruption

Prevenceptives

Suppression of the estral cycle in the dog has been brought about through the use of hydroxyprogesterone acetate[1] (Beard, 1961), repositol progesterone (Burch, 1961), and medroxyprogesterone[2] (Anderson et al., 1965). However, a report appeared on the increased incidence of pyometra following the use of medroxyprogesterone (Anderson et al., 1965). The drug has now been withdrawn from the market by the manufacturer. Research reported in a more recent paper revealed the same results using medroxyprogesterone and repositol progesterone (Brodey and Fidler, 1966).

Abortifacients

Various drugs and procedures have been used for the interruption of an unwanted pregnancy in the dog. These include: (1) ether vaginal douches (Sidney, 1965), (2) estradiol injections (Voute, 1950; Kirk, 1951; Crawford, 1952), (3) diethylstilbestrol injections and/or oral administration (Grieve, 1943; Voute, 1950; Bloom et al., 1951; Kirk, 1951; Harrop,

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[1]Prodox, The Upjohn Co., Kalamazoo, Mich.

[2]Promone, The Upjohn Co., Kalamazoo, Mich.

1960), and, (4) malucidin injections (Williams, 1965). Harrop (1960) stressed the importance of the administration of D.E.S. within 5 to 7 days of the mismating as it may not be possible to counteract the progestational effect after that time.

Friedman (1957) reported the use of the anti-metabolite o-diazoacetyl-l-serine (azaserine) as an abortificient in the dog. He stated it was effective after the 19th to the 23rd day. In 1962, Hershberger reported on a non-steroidal estrogen antagonist used to end pregnancy. This drug, ethamoxytriphetol, was administered to bred bitches for 28 consecutive days after copulation and was found to be very effective.

The discovery that D.E.S. would prevent pregnancy is credited to Parkes et al. (1938). Their observations on the actions of ethinyl estradiol and D.E.S., when used in rabbits, indicated that the progestational proliferation of the endometrium was suppressed by both.

Burdick et al. (1940) demonstrated that testosterone proprionate caused tubal retention of the ova in mice. Their reports of 1935, 1937 and 1938 stated that estrogen administration to mice and rabbits resulted in an acceleration of the rate of passage of the ova through the fallopian tubes. They reported that this acceleration of the rate resulted in damage to the fertilized ova and/or expulsion of them into a

uterus not yet ready to receive them for nidation. Burdick et al. (1940) also suggested "tube-locking" of ova as an explanation for their degeneration.

Whitney and Burdick (1936) described the "tube-locking" phenomenon produced by estrogenic substances in the mouse. They stated that tubal contents are "sucked" back and forth by to and fro movements or peristalsis of the upper portion of the tubes. Below this, the tubes are described as being in a state of spastic contraction. Whitney and Burdick (1938) also reported the accelerated rate of passage of the ova through the fallopian tubes of rabbits following the injection of estrogen. They reported that the endometrial response to progesterone was delayed by the administration of estrogen.

Greenwald (1957, 1958) demonstrated that estrogens were responsible for the synthesis and storage of mucin in the tubal epithelium of rabbits and that progesterone was necessary for the discharge of the secretion. The administration of estrogens resulted in a reduction in the amount of mucin deposited around the ova. Greenwald (1959) reported on the effectiveness of estradiol, estrone and D.E.S. in interrupting pregnancy in the rabbit. All were found to increase tubal motility. He believed that the more rapid transit of the ova through the fallopian tubes, which in itself may result in less time for mucin deposition, was of secondary importance to the interference of mucin release.

Parkes et al. (1938), using the rabbit, Velardo et al. (1956), using the rat, and Stone and Emmens (1964), using the mouse, observed that estrogen administration shortly after breeding resulted in the interference with the development of the proper progestational state of the uterus.

Histochemistry

Histochemical analyses have been conducted on the uterine wall of the pig during the estrus cycle (Austad and Garm, 1959), and on the uterus and ovary of the sheep (Hadek, 1958a, 1958b). Deane (1952) reported on the periodic acid-Schiff (P.A.S.) and alkaline phosphatase reactions in the ovary and oviduct of the rat during the estral cycle.

Fitch (1961) reported on the occurrence of uterine glycogen during the estral cycle of the dog. He demonstrated the presence of glycogen in the epithelium of the necks and deeper portions of the endometrial glands but not in the surface epithelium. In proestrus, he found glycogen present in moderate amounts in the uterine glandular epithelium and more abundant at the peripheral ends of the cells. In estrus, the glycogen content in the secretory parts of the glands increased but was still more concentrated at the peripheral ends of the cells. In metestrus, heavy deposits of glycogen appeared in the deeper, coiled portions of the glands but decreased markedly when the lumina became filled with P.A.S.-positive

material. In regressive metestrus, glycogen again became prominent in the uterine glandular epithelium and remained so during anestrus.

Erichsen (1953) described P.A.S.-positive granules in the supranuclear cytoplasm of the epithelium of the glands and crypts during the proestral-estral period in the dog. He indicated marked depletion of these granules and the appearance of P.A.S.-positive plugs in the lumina of the glands during metestrus.

Neither Fitch (1961) nor Erichsen (1953) employed the alkaline phosphatase technique in their works. However, alkaline phosphatase activity was demonstrated to be particularly pronounced during the late estral-metestral phase of the cycle in the surface and glandular epithelium of the pig (Austad and Garm, 1959) and sheep (Hadek, 1958a). Skjerven (1956) described marked cyclic changes of alkaline phosphatase in the luminal epithelial cells of the bovine endometrium. He demonstrated that, in the bovine species, alkaline phosphatase activity was related to the progesterone level. Skjerven further described an inverse relationship of alkaline phosphatase and glycogen in the luminal epithelial cells. However, Vaczy et al. (1955) demonstrated maximal alkaline phosphatase content in the vaginal epithelium of mice in the proestral phase of the cycle.

Alkaline phosphatase activity was also demonstrated in

the ciliated portion of the rat oviduct (Deane, 1952). Neither P.A.S.-positive material nor alkaline phosphatase was demonstrated in the uterus of the castrated sow, indicating the hormonal influence of estrogen and progesterone on that organ (Austad and Garm, 1959).

Hisaw and Greep (1938) reported that estrone resulted in the deposition of glycogen in the uterine glandular epithelium and that progesterone plays a role in the formation and release of glycogen. Cantarow and Schepartz (1954) stated that progesterone resulted in a decrease of alkaline phosphatase activity.

Atkinson and Engle (1947), using the monkey, reported on the occurrence of alkaline phosphatase activity in the luminal epithelium and uterine glands following the administration of estrogen. This activity decreased with the administration of progesterone. They stated that the appearance of alkaline phosphatase activity during the estrogen phase of the cycle indicated that the enzyme was concerned with protein synthesis. They also stated that its diminution later in the cycle suggested it may be concerned with glycogen formation. Dempsey and Wislocki (1946) reported that alkaline phosphatase can play a role in protein synthesis and glycogen formation.

Kugler and Wilkinson (1960) reported on the discrepancy in the amount of tissue glycogen actually present and that which is demonstrable through histochemical procedures.

Bloom et al. (1951) demonstrated that glycogen exists in tissues in two forms; a protein-bound form and a trichloracetic acid-soluble form. Kugler and Wilkinson concluded that trichloracetic acid-soluble glycogen is the only fraction that enters into the histochemical reaction. They suggested that 6 micrograms of acid-soluble glycogen per 100 mg. of tissue must be present before it is demonstrable by histochemical means.

Bullough (1953) reported increased mitoses in the epidermis of the ear of the mouse following the administration of estrogen which, he stated, stimulated the phosphorylation of glucose. He concluded that if estrogens facilitated the glucokinase reaction, they might also be expected to stimulate energy production and glycogen deposition.

Kerly (1940) demonstrated an increase in the rate of anaerobic glycolysis in rat uteri following the administration of estrone.

MATERIALS AND METHODS

Experimental Animals

Five German Shorthair Pointer female, sibling puppies were obtained for this study. The animals were purchased from a local resident when they were 55 days old. There were 10 puppies in the entire litter; 3 males and 7 females. The bitch was a six year old animal and had had one previous litter, that one being when she was two years of age. The estral cycles of the bitch had been regular and distinct with no history of reproductive problems.

The puppies were whelped without difficulty after a gestation period of 60 days. The time required for the delivery of the 10 puppies was nine hours. The tails were amputated at three days of age, the length remaining being that recommended for the breed. The puppies were weaned at 35 days of age and had been wormed at 38 and 52 days of age. The wormings were not predicated on fecal examinations.

The initial and two subsequent fecal examinations, at two week intervals, were negative for eggs of parasites. The sugar flotation method, as described by Benbrook and Sloss (1955), was employed.

The animals were housed in fiber glass cages and exercised three times daily for periods of 30 to 60 minutes. The diet

consisted of dry dog meal[1] and meat mixed in the ratio of 4:1 by weight. The meat was either ground horse meat or beef. The puppies were fed three times daily until they were 122 days old. At that time, the number of feedings was reduced to two daily and continued throughout the duration of the experiment. A vitamin-mineral supplement[2] was added to the above ration.

All puppies were given anti-distemper-hepatitis-leptospirosis serum[3] at 56 days of age. A tri-valent vaccine[4] was administered to each pup at 77 and 98 days of age. Hemograms, consisting of a total leucocyte count, hemoglobin and packed cell volume, were made at approximately two week intervals. The results obtained up to 212 days of age are listed in Table 1. The hemoglobin and packed cell volume values to 301 days of age are listed in Table 2.

At 56 days of age, the smallest puppy weighed 7.25 pounds and the largest 10.25 pounds. Growth-weight curves for these two dogs to 301 days of age are shown in Figure 1. At 271 days of age, the smallest pup weighed 2 pounds less than on the

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[1]Gaines Dog Meal, General Foods Corp., White Plains, New York.

[2]Vionate, E. R. Squibb Co., New York, New York.

[3]Globulon, Pitman-Moore Co., Indianapolis, Ind.

[4]Tissuevax-4, Pitman-Moore Co., Indianapolis, Ind.

Table 1. Hemograms of experimental animals

Days	W.B.C.	P.C.V.	Hb.
Dog #1			
59	17,400	38	9.58
73	13,100	27	10.35
87	15,250	33.5	9.96
101	15,100	30	9.22
115	12,900	34	11.15
129	18,000	31	10.35
143	18,000	38	11.56
157	16,600	37	10.74
171	18,500	39.5	11.56
185	16,100	38.5	11.96
199	15,500	38.5	11.15
212	10,500	37	11.56
Dog #2			
59	12,150	25	8.86
73	14,950	29.5	9.58
87	13,650	32	9.58
101	13,500	33	9.96
115	15,800	33.5	10.74
129	15,150	34.5	11.15
143	16,100	36	11.56
157	16,900	37.5	10.74
171	15,300	40	11.15
185	12,600	39	13,19
199	13,200	41.5	12,37
212	9,200	38.5	12.37
Dog #3			
59	11,900	26	8.51
73	9,700	24	8.16
87	11,560	29.5	9.22
101	15,000	29	9.58
115	11,450	29	9.22
129	18,000	33	11.15
143	19,000	36	11.15
157	13,400	34	11.15
171	15,950	37.5	10.35
185	14,600	33	11.15

Table 1. (continued)

Days	W.B.C.	P.C.V.	Hb.
185	14,600	33	11.15
199	14,000	38.5	11.56
212	11,000	39	11.56
Dog #4			
59	11,050	24	7.61
73	18,000	25	8.51
87	12,350	32	9.96
101	11,700	30	9.96
115	8,800	32	9.96
129	11,021	33	9.96
143	10,900	34.5	11.15
157	12,500	35.5	11.15
171	10,950	36	10.74
185	13,600	30.5	9.58
199	12,100	40	10.35
212	13,000	34	10.74
Dog #5			
59	15,150	24.5	7.49
73	19,400	31	9.58
87	13,700	29	9.22
101	11,650	30.5	9.58
115	11,450	31.5	9.96
129	11,200	32	10.35
143	14,400	35.5	10.35
157	16,100	37.5	11.15
171	17,150	33.5	10.74
185	15,700	39	11.56
199	11,500	32	11.96
212	12,500	37.5	12.37

W.B.C. = white blood cell count, per $mm.^3$

P.C.V. = packed cell volume in %

Hb. = hemoglobin in grams %

Table 2. Hemoglobin and packed cell volume levels of experimental animals

Dog	229 days		243 days		255 days		271 days		287 days		301 days	
	Hb.	P.C.V.	Hb.	P.C.V.	Hb.	P.C.V.	HB.	P.C.V.	Hb.	P.C.V.	Hb.	P.C.V.
1	11.96	34	13.19	42	12.37	38	15.37	46	15.37	46	14.04	46
2	14.05	44	14.50	37	13.62	42	13.62	44	13.62	44	14.04	45
3	12.77	39	14.05	41	13.19	40	14.05	47	13.62	43	13.62	43
4	11.96	40	11.56	38	12.37	39.5	14.05	45	14.05	44	14.37	44
5	12.37	37	11.62	44	14.04	42.5	12.37	41	13.19	42	13.62	44

Hb. = hemoglobin in grams

P.C.V. = packed cell volume in %

Table 3. Day of estrum when breeding was permitted and the intervals following breeding when left and right genital specimens were surgically recovered

Animal Number	Days of Estral Cycle														
	9	10	11	12	13	14	15...	18...	21...	26	27...	31...	33...	38...	40
1				A		A'			C					C'	
2		A		A'			C				C'				
3			A		A'		B					C			C'
4		A		A'		B			C				C'		
5		A		A'		B	C			C'					

A = day of first breeding

A' = day of second breeding

B = day of diethylstilbestrol administration

C = day of surgery for recovery of left ovarian, fallopian and uterine specimens

C' = day of surgery for recovery of right ovarian, fallopian and uterine specimens

previous weighing date. The largest pup gained no weight in the same time period. A diarrhea of suspected dietary etiology afflicted all the puppies at 268 to 271 days of age and probably accounts for the temporary set-backs.

The animals were identified by numbers tatooed on the inner surface of the ear pinna. The numbers were simply 1 through 5.

The onset of the estrus cycle

The first day of sanguinous vaginal discharge was noted as follows: #1, 565 days of age; #2, 566 days of age: #3, 568 days of age; #4, 448 days of age; #5, 438 days of age. The onset of proestrus of the individual dogs by number and in sequential order was #5, #4, #1, #2, and #3. The time for breeding was determined by the vaginal smear method. Although the duration of proestrus varied, the vaginal smear permitted breeding of each female at comparable stages of the cycle. Two matings were allowed each dog; one on the first day of estrus and the second two days later. Two known fertile adult males were used; one for Bitches #4 and #5 and one for Bitches #1, #2 and #3. At each mating, a "tie" was observed, the duration of which was at least 15 minutes. This so-called "tie" is caused by the constriction of vaginal muscles behind the bulbus glandis of the glans penis. Although the "tie" is a natural procedure, it is not necessary for conception (Harrop, 1960).

Experimental Procedures

Three of the animals were given repositol diethylstilbestrol[1] two days after the second breeding. The other two animals were bred and maintained under identical conditions as controls. Specimens of the uterus, fallopian tubes and ovaries for histological and histochemical study were surgically removed at various intervals after breeding. The left side was removed at the first operation and the right side, including the body of the uterus, at the second operation. See Table 3 for time intervals between breeding and the surgical removal of specimens.

The dosage of diethylstilbestrol was 0.50 mg. per pound of body weight, administered intramuscularly. The manufacturer of the product used states its effectiveness is sustained for 10 to 20 days. The total dosages for the three experimental dogs were: Bitch #3, 30 mg.; Bitch #4, 25 mg.; Bitch #5, 30 mg.

The dog has a bicornuate uterus and removal of one ovary, oviduct and cornu is a relatively simple procedure. This can be accomplished without interruption of the pregnancy in the opposite cornu as shown by Friedman (1957). The tissues were carefully handled and no clamps were used until the specimens

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[1]Pitman-Moore Co., Indianapolis, Ind.

for study had been removed. The areas from which tissues were excised are illustrated in Figure 2. After the removal of the cornu, oviduct and ovary in the first surgical procedure, the cornual stump was sutured with an infolding suture using size 00 chromic catgut and an atraumatic needle. All vessels were ligated with size 00 chromic catgut. After the removal of the remaining cornu, oviduct and ovary in the second surgical procedure, the existing portion of the uterine body was also removed by the usual method (Lacroix, 1957). All abdominal closures were accomplished in the usual manner (Lacroix, 1957).

Although a strict aseptic surgical technique was used, an antibiotic[1] was administered after each operation. At the time of the second operation on Bitch #1, adhesions with several very small abscesses were observed around the stump. This followed the first surgical procedure. Abscessed stumps did not occur in any of the other animals.

Sections of the cornu and oviducts were removed from the middle portions of the organs and the ovaries were incised longitudinally. These specimens were kept for histological and histochemical study and the sections were identified by animal number and by surgical procedure. For example,

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[1]Bicillin, Wyeth Laboratories, Philadelphia, Pa.

#51 refers to Bitch #5, first surgical procedure; #52 refers to Bitch #5, second surgical procedure.

Histological and Histochemical Techniques

After surgical excision of the tissues, one piece of each organ to be studied was fixed in cold 80% ethanol and in 10% formalin. The alcohol-fixed tissues were handled in the following manner (Davenport, 1960):

1. fixation of tissues in 80% ethanol for 24 hours at 4° C.
2. transfer of tissues to 2 changes of 4° C. absolute alcohol for 12 hours each,
3. transfer of tissues to two changes of benzene at 4° C. for one hour each,
4. infiltration of tissues in 3 changes of paraffin wax, 58° C. for 30 minutes each,
5. embedding of tissues in paraffin wax,
6. storage of tissue blocks at 4° C.

The formalin-fixed tissues were dehydrated, infiltrated with and embedded in paraffin wax according to usual procedures (Davenport, 1960; A.F.I.P., 1960).

All sections were cut at 5 to 8 microns. The alkaline phosphatase procedures were conducted on the alcohol-fixed tissues. The periodic acid-Schiff procedure and hematoxylin and eosin staining were accomplished on the formalin-fixed tissues.

Periodic acid-Schiff (P.A.S.) procedure: The method of McManus (1946) was used for the demonstration of P.A.S.-positive material. Control sections were diastase treated. The quantity of P.A.S.-positive granules demonstrated was indicated on an arbitrary scale of + to ++++.

Alkaline phosphatase procedure: The method of Gomori (1952) was used for the demonstration of alkaline phosphatase activity. Control sections, demonstrating the presence of native phosphate, were also prepared. The comparative alkaline phosphatase activity was indicated by an arbitrary scale of + to ++++.

The photomicrographs were taken with a MP-3[1] camera.

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[1]Polaroid Corp., Cambridge, Mass.

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS

Some histological and histochemical characteristics were in agreement in the same organs of both the experimental and control animals. These characteristics will be considered first and, because they apply to all animals, will not be repeated under the individual protocols of individual animals discussed later. There will, however, be some aspects that will receive individual consideration even though they may be the same or similar in all like tissues. This is being done for purposes of emphasis.

Special attention was directed toward the endometrium as significant changes were anticipated here. Mulligan (1942) divided the endometrium into four layers as follows: "(a) basal layer, consisting of polyhedral glands; (b) tubular layer, the tubules of which connect the basal glands with the surface; (c) stromal layer, connective tissue relatively poor in epithelial elements, but through which the tubules pass toward the surface; and (d) crypt layer, consisting of crypts between which extends the surface covering epithelium."

The uterine glands are simple branched tubular glands (Trautmann and Fiebiger, 1952). They are especially coiled toward their ends; the development of the glands being pronounced in the luteal phase of the cycle. (Arenas and Samartino, 1939).

Microscopic Characteristics of Tissues From Experimental and Control Animals

Ovary

All sections of the ovaries contained follicles in various stages of maturation. They were particularly concentrated around the periphery of the ovary (Figure 14). Cords of cells from the germinal epithelium, carrying primordial follicles deeper into the ovarian stroma, have been described by Barton (1945). In the sections examined, the cords of epithelial cells and developing follicles occurred deep in the ovarian stroma and between the corpora lutea (Figure 15).

Atretic follicles were also found in all ovarian sections. Atresia can occur in all stages of follicular development and is characterized by: (1) pyknosis of the nucleus and fatty degeneration of the oocyte, (2) collapse of the follicle, (3) invasion of the follicle by cells of the theca interna and connective tissue, and (4) obliteration of the follicular cavity with lipid-containing cells and connective tissue (Trautmann and Fiebiger, 1952; Maximow and Bloom, 1955; Nalbandov, 1964). No attempt was made to count the number of developing or atretic follicles in the ovarian sections. However, both were observed in all sections (Figures 14 and 19).

P.A.S.-positive granules were never seen in lutein cells. They were observed in the granulosa cells of the follicles, in the endothelium of the blood vessels of the ovary, and in the endothelium of blood vessels within the corpora lutea (Figures 37 and 38). P.A.S.-positive material was prominent in the zona pellucida (Figure 36).

The theca interna cells were always positive for alkaline phosphatase (Figures 56, 57 and 58). The walls of the blood vessels in the ovary and the corpora lutea were always alkaline phosphatase positive (Figure 55). This was also true of the blood vessels in the sections of all the other tissues examined.

Oviduct

A polysaccharide protein is secreted by mucous or goblet cells on many mucous membranes (Maximow and Bloom, 1955). Goblet cells were found in the luminal epithelium of the oviduct and uterus. The epithelial cells of the oviduct were larger in the earlier than in the later stages of the cycle. No cyclic variation in the number of goblet cells was observed.

P.A.S.-positive material was present along the free poles of some epithelial cells. The reaction was also observed in the control slides but was not as pronounced.

Longitudinal folding of the oviduct mucosa was a constant feature in all animals. The many folds result in protected

recesses of epithelium (Figure 18). The loss of cilia from the oviduct epithelium will be discussed later but it should be mentioned here that the percentage of ciliated epithelial cells was always greater in the aforementioned protected recesses. Furthermore, the cilia were retained longer in the recessed areas (Figure 27).

Uterus

The structures of the endometrium, based on the classification of Mulligan, include the epithelium of the lumen and the epithelium of the various sections of the uterine glands. The glandular epithelium may be further classified as that which occurs in the cryptal, tubular or basal portions (Figure 13).

A P.A.S.-positive reaction was seen along the peripheral border of the luminal epithelium of the endometrium. This was present in all experimental and control slides, although the reaction was not as pronounced in the controls.

Much of the information obtained has been summarized and tabulated for easier reference. The epithelial elements and thickness of the endometrium were measured and compared (Table 4). The average luminal diameters of the various portions of the endometrial glands have been listed (Table 5).

An increase in the number and size of the endometrial glands was observed, being more pronounced in the tissues of

Table 4. Length and diameter of various epithelial elements of the uterus and thickness of endometrium on day of cycle stage indicated

Dog	Epithelial cells, in microns				Endomet. thickness	A	B	C	Stage and day of cycle
	Lumen epith.	Crypt epith.	Tubule epith.	Basal gl. epith.					
51	14x3	11x4	15x6	15x6	1.50mm.	14	1	3	estrus; 5
41	10x3	12x4	12x6	12x5	1.40mm.	18	5	7	metestrus; 2
52	14x4	11x6	13x6	18x6	1.90mm.	26	13	15	metestrus; 10
31	8x3	9x4	13x4	13x3	1.05mm.	31	16	18	metestrus; 13
42	9x3	6x4	9x4	13x4	0.95mm.	33	20	22	metestrus; 17
32	14x3	11x3	10x4	7x4	1.14mm.	40	25	27	metestrus; 22
Controls:									
21	11x3	9x3	14x4	16x5	0.80mm.	15	-	3	estrus; 5
11	13x3	10x3	13x5	17x4	1.05mm.	21	-	7	metestrus; 2
22	10x3	12x5	12x4	13x4	1.05mm.	27	-	15	metestrus; 10
12	22x8	14x6	12x5	11x5	1.70mm.	38	-	23	metestrus; 19

A = the day of the cycle

B = the number of days after administration of diethylstilbestrol

C = the number of days after the second breeding

Table 5. Average luminal diameters of cryptal, tubular and basal glandular portions of the endometial glands (Measurements given in microns)

Dog	Crypts	Tubules	Basal gl.
51	22	12	23
41	22	14	26
52	25	10	4
31	12	5	2
42	12	5	3
32	9	9	5
Controls:			
21	15	12	11
11	11	10	2
22	11	11	2
12	19	16	20

some animals than in others and at different stages of the cycle. The comparative development of the endometrial glands in the tissues was tabulated. An arbitrary scale of + to +++ was used based on the number and size of the cryptal, tubular and basal portions of the glands (Table 6).

Table 6. Comparative development of endometrial glandular elements

Dog	Cryptal portion	Tubular portion	Basal portion
51	+	+	+
41	++	+	+
52	+++	++	++
31	++	++	+++
42	++	++	+++
32	+	++	++
Controls:			
21	++	+	+
11	++	+	++
22	++	++	+++
12	+	+	++

Another table using the same type of arbitrary scale was prepared for recording the alkaline phosphatase activity

Table 7. The occurrence of alkaline phosphatase and P.A.S.-positive granules in the endometrium

Dog	Day of cycle	Alkaline Phos. activity: Luminal epith.	Cryptal epith.	Tubular epith.	Basal gl. epith.	P.A.S.-positive gran.: Luminal epith.	Cryptal epith.	Tubular epith.	Basal gl. epith	Days after 2nd Breeding
51	14	+	++	++	<+	-	+	++	+++	3
41	18	+	+++	+++	+++	-	++	+++	+	7
52	26	+	+	+	+	-	-	++	++	15
31	31	++	++	+++	+++	-	-	+	+	18
42	33	++	+	++	++	-	-	-	-	22
32	40	++	++	++	+++	<+	<+	+	+	27
Controls:										
21	15	++	+++	+++	++	-	++	++	++	3
11	21	++	++	+++	+++	-	+	+++	+++	7
22	27	++++	++	+++	++++	-	-	+	+	15
12	38	+	++	++	+	-	-	-	+	23

and P.A.S.-positive granules in the endometrial glandular and luminal epithelium (Table 7).

The presence or absence of a secretion in the lumina of the various portions of the endometrial glands is recorded in Table 8.

Table 8. Presence of secretory material in the lumina of the various areas of the endometrial glands

Dog	Crypt lumina	Tubule lumina	Basal lumina
51	-	-	-
41	-	-	-
52	+	+	+
31	+	+	+
42	+	+	+
32	+	+	+
Controls:			
21	-	+	+
11	-	+	+
22	+	+	-
12	-	-	-

Experimental Animals

Bitch #51

This animal was given 30 mg. of repositol D.E.S., intramuscularly, two days after the second breeding. The surgical removal of the left ovary, oviduct and uterine cornu occurred on the 14th day after the onset of the vaginal discharge and the 3rd day after the second breeding.

Ovary

Corpora lutea Three corpora lutea were present on the slides studied, the cells of which were small and loosely arranged. The average diameter of the corpora lutea was 3.50 mm.

P.A.S. reaction P.A.S.-positive granules were absent from the lutein cells but present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles. P.A.S.-positive material was observed in the zona pellucida.

Alkaline phosphatase activity Alkaline phosphatase activity was present in all blood vessels of the ovary and the theca interna cells of the follicles.

Oviduct

Luminal epithelium The cells of the epithelium were thick and contained light staining cytoplasm and large, plump nuclei (Figure 20). They were simple columnar to pseudostratified columnar in appearance with the latter

being the more accurate classification. Cilia were present on approximately 95% of the cells.

P.A.S. reaction No P.A.S.-positive granules were present in the epithelial cells.

Alkaline phosphatase activity Alkaline phosphatase activity was present in the free poles of the ciliated epithelial cells (Figure 59). Non-ciliated epithelial cells did not always contain alkaline phosphatase.

Cornu

Endometrial morphology Tall, simple columnar epithelium lined the lumen and glands of the endometrium. Mitotic figures were abundant in the epithelium of the basal area of the glands (Figure 33) and were also seen in the glandular crypts and tubules. Hyperemia and edema of the endometrial stroma were present but focal extravasations of blood were not seen. The thickness of the endometrium was 1.50 mm. (Figure 3).

Glandular secretion No uterine gland secretion was observed on any of the slides studied.

P.A.S. reaction P.A.S.-positive granules were present in the cryptal (+), tubular (++) and basal (+++) epithelium of the uterine glands. P.A.S.-positive granules were not observed in the luminal epithelium of the uterus.

Alkaline phosphatase activity Alkaline phosphatase was present in the luminal epithelium (+). It was

more pronounced (++) in the epithelium of the glandular crypts and tubules. A small amount (<+) was present in the basal glandular epithelium (Figures 64 and 70).

Bitch #41

This animal was given 25 mg. of repositol D.E.S., intramuscularly, two days after the second breeding. The surgical removal of the left ovary, oviduct and uterine cornu occurred on the 18th day after the onset of the vaginal discharge and the 7th day after the second breeding.

Ovary

Corpora lutea Two corpora lutea were present in the slides studied with an average diameter of 3.70 mm. The lutein cells were loosely arranged and a large central cavity was present in the corpora.

P.A.S. reaction The walls of the blood vessels supplying the ovary and corpora lutea, and the granulosa cells of the follicles contained P.A.S.-positive granules but the lutein cells did not. P.A.S.-positive material was present in the zona pellucida.

Alkaline phosphatase activity The theca interna cells of the follicles and the blood vessel walls contained alkaline phosphatase. The lutein cells did not contain alkaline phosphatase.

Oviduct

Luminal epithelium The mucosal epithelium was simple and pseudostratified columnar with large, plump nuclei. The cytoplasm was lightly stained. Cilia were present on approximately 95% of the cells.

P.A.S. reaction No P.A.S.-positive granules were present in the epithelial cells.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells (Figure 65). It was not observed in the non-ciliated cells.

Cornu

Endometrial morphology The epithelium of the lumen and glands was simple columnar. The taller cells were seen in the basal portions of the glands. Mitotic figures were abundant in the basal glandular epithelium but were also seen in the glandular crypts and tubules. No focal extravasations of blood were seen but hyperemia and edema were present. The thickness of the endometrium was 1.40 mm. (Figure 7).

Glandular secretion No uterine gland secretion was observed in any of the slides studied.

P.A.S. reaction P.A.S.-positive granules were present in the cryptal (++), tubular (+++) and basal (+) glandular epithelium. They were absent from the luminal epithelium.

Alkaline phosphatase activity Alkaline phosphatase was demonstrated to be present in the luminal epithelium (+). It was also present (+++) in all areas of the glandular epithelium.

Bitch #52

The surgical removal of the right ovary, oviduct and uterine cornu occurred on the 26th day after the onset of the vaginal discharge, 13 days after the administration of repositol D.E.S. and 15 days after the second breeding.

Ovary

Corpora lutea Two corpora lutea were present on the slides examined. The average diameter of the corpora lutea was 4.90 mm. The lutein cells were large and compactly arranged.

P.A.S. reaction P.A.S.-positive granules were absent from the lutein cells but present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles. P.A.S.-positive material was present in the zona pellucida.

Alkaline phosphatase activity Alkaline phosphatase was present in the blood vessels of the ovary and in the theca interna cells of the follicles.

Oviduct

Luminal epithelium The epithelial cells of the

oviduct were simple to pseudostratified columnar with plump nuclei and light staining cytoplasm. Approximately 65% of the cells were ciliated (Figure 21).

P.A.S. reaction No P.A.S.-positive granules were present in the epithelial cells.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells (Figure 61). Those cells in which alkaline phosphatase was not present were non-ciliated.

Cornu

Endometrial morphology Simple columnar epithelium lined the lumen and uterine glands. Compared to Bitch #51 fewer mitotic figures were seen in the basal, more in the cryptal and approximately the same number in the tubular glandular epithelium. Hyperemia and edema of the endometrial stroma were marked. Focal extravasation of blood was not seen. The thickness of the endometrium was 1.90 mm. (Figure 4).

Glandular secretion A secretion was present in the glandular lumina of the slides studied. The secretion was also present in the P.A.S. and alkaline phosphatase control slides.

P.A.S. reaction P.A.S.-positive granules were present (++) in the epithelium of the tubular and basal portions of the glands. They were absent in the epithelium

of the lumen and glandular crypts.

Alkaline phosphatase activity Alkaline phosphatase activity was present in the luminal epithelial cells (++) and in the glandular epithelium (+++).

Bitch #31

The surgical removal of the left ovary, oviduct and uterine cornu occurred on the 31st day after the onset of the vaginal discharge, 16 days after the administration of 30 mg. repositol D.E.S. and 18 days after the second breeding.

Ovary

Corpora lutea Three corpora lutea were present on the slides studied. The average diameter of the corpora lutea was 4.00 mm. The lutein cells were large and compactly arranged and no large cavities were present in the lutein tissue (Figure 17).

P.A.S. reaction P.A.S.-positive granules were absent from the lutein cells but present in the walls of blood vessels supplying the ovary and granulosa cells of the follicles. The zona pellucida contained P.A.S.-positive material.

Alkaline phosphatase activity Alkaline phosphatase was present in the theca interna cells of the follicles and the walls of the blood vessels.

Oviduct

Luminal epithelium The epithelial cells of the oviduct were compact with dark staining nuclei (Figure 26) and were pseudostratified columnar. Cilia were present on approximately 20% of the cells.

P.A.S. reaction No P.A.S.-positive granules were present in the epithelium.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells (Figure 62).

Cornu

Endometrial morphology The epithelium of the uterine lumen and glands was simple columnar but lower than that seen in the previously discussed cornua. Mitotic figures were seen as in Bitch #52. Hyperemia and edema were present but focal extravasations of blood were absent. The thickness of the endometrium was 1.05 mm. (Figure 11).

Glandular secretion A secretion was present in the lumina of many uterine glands in the slides examined. This secretion was also present in the P.A.S. and alkaline phosphatase control sections.

P.A.S. reaction P.A.S.-positive granules were absent in the luminal and cryptal epithelium. Small amounts (+) were present in the tubular and basal glandular epithelium (Figures 51 and 52).

Alkaline phosphatase activity Alkaline phosphatase was present (++) in the luminal and cryptal epithelium. It was more pronounced (+++) in the lower epithelial portions of the uterine glands (Figure 68).

Bitch #42

The surgical removal of the right ovary, oviduct and uterine cornu occurred on the 33rd day after the onset of the vaginal discharge, 20 days after the administration of repositol D.E.S. and 22 days after the second breeding.

Ovary

Corpora lutea Six corpora lutea were present in the slides examined, having an average diameter of 4.50 mm. The lutein cells were large and compactly arranged. No large cavities were present in the lutein tissue.

P.A.S. reaction P.A.S.-positive granules were absent from lutein cells but present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles. P.A.S.-positive material was seen in the zona pellucida.

Alkaline phosphatase activity Alkaline phosphatase activity was present in the theca interna cells of the follicles and the walls of the blood vessels.

Oviduct

Luminal epithelium The epithelium of the ovi-

duct was compact pseudostratified columnar, of which about 50% of the cells were ciliated (Figure 27).

P.A.S. reaction P.A.S. granules were not present in the epithelium of the oviduct.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells. Most non-ciliated cells did not contain alkaline phosphatase.

Cornu

Endometrial morphology The epithelial cells of the lumen and glandular crypts and tubules were a lower columnar than in Bitch #41. The basal glandular epithelial cells were slightly larger. Some mitotic figures were seen in the epithelium of the crypts and basal glandular areas. Hyperemia and edema of the endometrium were less pronounced than in the tissues of the animals previously studied. Focal extravasations of blood in the sub-epithelial tissue were not present. The thickness of the endometrium was 0.95 mm., 0.45 mm. less than that in Bitch #41 (Figure 8).

Glandular secretion A secretion was present in the lumina of the uterine glands in the slides studied. The secretion was also present in the P.A.S. and alkaline phosphatase control slides.

P.A.S. reaction P.A.S.-positive granules were absent from all epithelial elements in the slides studied.

(Figure 50).

Alkaline phosphatase activity Alkaline phosphatase was present in all epithelial elements (++) but less pronounced in the cryptal epithelium (+).

Bitch #32

The surgical removal of the right ovary, oviduct and uterine cornu occurred on the 40th day after the onset of the vaginal discharge, 25 days after the administration of repositol D.E.S. and 27 days after the second breeding.

Ovary

Corpora lutea Four corpora lutea were present, having an average diameter of 3.90 mm. The lutein cells were large and compact. No large cavities were present in the lutein tissue.

P.A.S. reaction No P.A.S.-positive granules were present in the lutein cells but were seen in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles.

Alkaline phosphatase activity Alkaline phosphatase was absent from the lutein cells but present in the theca interna cells of the follicles.

Oviduct

Luminal epithelium The epithelial cells of the oviduct were compact, pseudostratified columnar with dark

staining nuclei. Approximately 20% of the cells were ciliated.

P.A.S. reaction No P.A.S.-positive granules were present in the epithelial cells of the oviduct.

Alkaline phosphatase activity Alkaline phosphatase activity was present in the free poles of the ciliated epithelial cells. Cells without alkaline phosphatase activity were non-ciliated.

Cornu

Endometrial morphology Simple columnar epithelium lined the lumen and the crypts and tubules of the glands. The epithelium of the basal portions of the glands was low and more cuboidal than columnar. Mitotic figures in the glandular epithelium were uncommon. Focal extravasations of blood in the endometrial stroma were prominent, many associated with endothelial damage (Figure 35). The thickness of the endometrium was 1.14 mm. (Figure 12).

Glandular secretion A secretion was present in the lumina of the uterine glands in the slides studied. The secretion was also present in the P.A.S. and alkaline phosphatase control slides.

P.A.S. reaction Small amounts (<+) of P.A.S.-positive granules were present in the epithelial cells of the lumen and glandular crypts. Larger (+), but still relatively small, amounts were seen in the epithelium of the

lower portions of the glands (Figure 54).

Alkaline phosphatase activity Alkaline phosphatase was present in the epithelium of the lumen and glandular crypts and tubules (++). The activity in the basal portions of the glands was more pronounced (+++).

Control Animals

Bitch #21

The surgical removal of the left ovary, oviduct and uterine cornu occurred 15 days after the onset of the vaginal discharge and 3 days after the second breeding. No repositol D.E.S. was administered to this animal.

Ovary

Corpora lutea Three corpora lutea were present on the slides studied. The cells of the corpora lutea were loosely arranged (Figure 15). Large cavities were present in the lutein tissue. The average diameter of the corpora lutea was 4.60 mm.

P.A.S. reaction P.A.S.-positive granules were present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells (Figure 38) of the follicles but absent from the lutein cells. P.A.S.-positive material was present in the zona pellucida.

Alkaline phosphatase activity Alakline phosphatase activity was present in the walls of the blood vessels

and in the theca interna cells of the follicles.

Oviduct

Luminal epithelium The epithelial cells of the oviduct were simple to pseudostratified columnar. They were thick and contained lightly-stained cytoplasm and plump nuclei. Cilia were present on approximately 95% of the cells (Figure 22).

P.A.S. reaction P.A.S.-positive granules were not seen in the oviduct epithelial cells.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells (Figure 60). The cells without alkaline phosphatase were not ciliated.

Cornu

Endometrial morphology The epithelium of the lumen and glands was simple columnar. Mitotic figures were common in the basal glandular epithelium but also seen in the cryptal epithelium. Hyperemia and edema were present but focal extravasations of blood were absent. The thickness of the endometrium was 0.80 mm. (Figure 5).

Glandular secretion A secretion was present in the glandular tubules and basal portions in the slides studied. The secretion was also present in the P.A.S. and alkaline phosphatase control slides.

P.A.S. reaction P.A.S.-positive granules were

present (++) in all areas of the glandular epithelium (Figure 46) but absent from the luminal epithelium.

Alkaline phosphatase activity Alkaline phosphatase was present (++) in the luminal and basal glandular epithelium but more pronounced (+++) in the glandular crypts and tubules (Figures 66, 72, and 74).

Bitch #11

The surgical removal of the left ovary, oviduct and uterine cornu occurred on the 21st day after the onset of the vaginal discharge and 7 days after the second breeding. No repositol D.E.S. was administered to this animal.

Ovary

Corpora lutea Three corpora lutea were present in the slides examined. The average diameter of the corpora lutea was 4.70 mm. and the lutein cells were compactly arranged.

P.A.S. reaction P.A.S.-positive granules were present in the granulosa cells of the follicles and in the walls of the blood vessels supplying the ovary and corpora lutea (Figure 37). They were absent from the lutein cells. P.A.S.-positive material was present in the zona pellucida.

Alkaline phosphatase activity Alkaline phosphatase was present in the walls of blood vessels and the theca interna cells of the follicles (Figures 55 and 56).

Oviduct

Luminal epithelium The epithelial cells of the oviduct were pseudostratified columnar. They were compactly arranged and had darkly stained nuclei. Approximately 70% of the epithelial cells were ciliated (Figure 24).

P.A.S. reaction P.A.S.-positive granules were not seen in the epithelial cells of the oviduct in the slides studied.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells and absent from most non-ciliated cells.

Cornu

Endometrial morphology The epithelium of the lumen and glands was tall, simple columnar. Mitotic figures were abundant in the basal glandular epithelium but also present in the cryptal and tubular epithelium. Endometrial hyperemia and edema were present but focal extravasations of blood were absent. The thickness of the endometrium was 1.05 mm. (Figure 9).

Glandular secretion A secretion was present in the glandular lumina of the slides examined. The secretion was also present in the P.A.S. and alkaline phosphatase control slides.

P.A.S. reaction P.A.S.-positive granules were abundant (+++) in the tubular and basal glandular epithelium

(Figure 48). They were present (+) in the cryptal epithelium and absent from the luminal epithelium.

Alkaline phosphatase activity Alkaline phosphatase was present in significant amounts (++) in the epithelium of the lumen and glandular crypts. It was pronounced (+++) in the glandular tubules and basal portions.

Bitch #22

The surgical removal of the right ovary, oviduct and uterine cornu occurred on the 27th day after the onset of the vaginal discharge and 15 days after the second breeding. No repositol D.E.S. had been given this dog.

Ovary

Corpora lutea Three corpora lutea were present in the slides studied. The average diameter of the corpora lutea was 4.30 mm. The lutein cells were large and compactly arranged.

P.A.S. reaction P.A.S.-positive granules were present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles. They were absent from the lutein cells. The zona pellucida was P.A.S.-positive.

Alkaline phosphatase activity The theca interna cells of the follicles and the walls of the blood vessels contained alkaline phosphatase.

Oviduct

Luminal epithelium The epithelium of the oviduct was a low, pseudostratified columnar. The cells were compact with darkly-stained nuclei. Approximately 25% of the epithelial cells were ciliated (Figure 23).

P.A.S. reaction P.A.S.-positive granules were not seen in the epithelial cells of the oviduct.

Alkaline phosphatase activity Alkaline phosphatase was present in the free poles of the ciliated epithelial cells and absent from most non-ciliated cells.

Cornu

Endometrial morphology The epithelium of the lumen and glands of the endometrium was simple columnar. Mitotic figures were present in the cryptal (Figure 32) and tubular epithelium but more common in the basal glandular epithelium. Hyperemia and edema were present but focal extravasations of blood were not seen. The thickness of the endometrium was 1.05 mm. (Figure 6).

Glandular secretion A secretion was present in the cryptal and tubular lumina of the uterine glands. No secretion was present in the basal gland lumina. The secretion was also present in the P.A.S. and alkaline phosphatase control sections.

P.A.S. reaction P.A.S.-positive granules were absent from the epithelium of the lumen and glandular crypts.

They were present (+) in the epithelium of the tubules and basal gland area (Figure 47).

Alkaline phosphatase activity Alkaline phosphatase was present in significant amounts in the cryptal epithelium (++) and very pronounced (+++) in the epithelium of the lumen and the other glandular portions (Figures 67 and 75).

Bitch #12

The surgical removal of the right ovary, oviduct and uterine cornu occurred on the 38th day after the onset of the vaginal discharge and 23 days after the second breeding. No repositol D.E.S. had been given this animal.

Ovary

Corpora lutea Two corpora lutea were present in the slides studied. The lutein cells were compactly arranged. The average diameter or the corpora lutea was 4.90 mm.

P.A.S. reaction P.A.S.-positive granules were absent from the lutein cells but present in the walls of the blood vessels supplying the ovary and corpora lutea and in the granulosa cells of the follicles. P.A.S.-positive material was present in the zona pellucida.

Alkaline phosphatase activity Alkaline phosphatase was present in the walls of the blood vessels and

in the theca interna cells of the follicles (Figure 58).

Oviduct

Luminal epithelium The epithelium of the oviduct was a low pseudostratified columnar. The nuclei of these cells were darkly stained. Cilia were present on approximately 15% of the cells (Figure 25).

P.A.S. reaction No P.A.S.-positive granules were present in the epithelial cells of the oviduct.

Alkaline phosphatase activity Alkaline phosphatase activity was present in the free poles of the ciliated epithelial cells. Cells without alkaline phosphatase were non-ciliated.

Cornu

Endometrial morphology The luminal epithelium contained large, "fattened" cells with vacuolated cytoplasm (Figure 34). Many areas of sloughing epithelium were present. Mitotic figures were not seen. Hyperemia and edema of the endometrium were marked and extravasations of blood were present. Leukocytic infiltration through all areas of the endometrium was present. The thickness of the endometrium was 1.70 mm. (Figure 10).

Glandular secretion No secretion was present in any area of the uterine glands in the slides studied.

P.A.S. reaction P.A.S.-positive granules were present (+) only in the basal glandular epithelium (Figure

42). The leucocytes were P.A.S.-positive and their infiltration was particularly well demonstrated on the P.A.S. slides (Figure 43).

Alkaline phosphatase activity Alkaline phosphatase activity was present (+) in the epithelium of the lumen and basal gland area but was more pronounced (++) in the epithelium of the crypts and tubules.

DISCUSSION

Estrogenic substances are known to cause hyperemia and edema of the endometrium and proliferation of the uterine glands (Cantarow and Schepartz, 1954; Guyton, 1961). Sharaf and Dabash (1958b) demonstrated a marked increase in the uterine weight of the dog following the administration of estrogens. The thickness of the endometrium was consistently increased in the experimental animals in the presented study (Figures 3 through 12). The thickness of the endometrium of Bitch #51 was 1.50 mm. 1 day after the administration of repositol D.E.S. The endometrial thickness in Bitch #52 was 1.90 mm. 13 days after the administration of D.E.S. The endometrial thickness of Bitch #41 was 1.40 mm. 5 days after the administration of repositol D.E.S. and 0.95 mm. 20 days after repositol D.E.S. administration in Bitch #42. The endometrial thickness in Bitch #31 was 1.05 mm. 16 days after the administration of repositol D.E.S. and 1.14 mm. 25 days after the administration of repositol D.E.S. in Bitch #32.

The endometrial measurements in the control Bitches #21, #11 and #22 were made at the same time of the estral cycle as Bitches #51, #41 and #52, respectively. The endometrial measurements of the control bitches were less than those of the experimental animals (Table 4).

The greater the hyperemia and edema, the greater the endometrial thickness. It appeared that repositol D.E.S.

produced greater endometrial hyperemia and edema (experimental animals) than that seen in the estral cycle physiologically (control animals). Furthermore, this effect decreased markedly after the 13th day following the administration of repositol D.E.S.

The relative development of the uterine glandular elements increased as the cycle progressed in both the experimental and control animals (Table 6). However, the tubular and basal glandular elements were more "spread-out" in the tissues of the experimental animals because of the increased edema of the endometrium.

Mitotic figures in the epithelium of the crypts, tubules and basal glandular area were present in the experimental and control sections. Cells undergoing mitotic division, in anaphase or telophase, were counted in the cryptal, tubular and basal glandular epithelium visible in the field of the light microscope under 450 magnifications. The comparative occurrence of these mitotic figures, on an arbitrary scale of + to +++, is indicated in Table 9. More mitotic figures were seen earlier in the estral cycle in those animals that had received repositol D.E.S. Fewer mitotic figures were seen in the luteal phase of both experimental and control animals. Bullough (1953) observed increased mitoses in the epidermis of the ear of the mouse following estrogen administration. Mitotic figures in the glandular epithelium of the

Table 9. The comparative occurrence of mitotic figures in epithelium of the uterine glands, based on a scale of + to +++

Animal number	Cryptal epithelium	Tubular epithelium	Basal gl. epithelium	Days after 2nd breeding
51	+	+	+++	3
41	+	+	+++	7
52	++	+	++	15
31	++	-	++	18
42	+	-	++	22
32	+	-	+	27
Controls:				
21	+	-	++	3
11	+	+	+++	7
22	+	+	++	15
12	-	-	-	23

uterus of the bitch in the follicular phase of the estral cycle were reported by Evans and Cole (1931) and Mulligan (1942). In the presented study, the administration of repositol D.E.S. appeared to stimulate mitotic division in the uterine glandular epithelium.

The epithelial cells of the lumen and basal glandular areas reached their maximal sizes on the 26th day of the estral cycle in the experimental (Bitch #52) and on the 21st

day of the estral cycle in the control (Bitch #11) animals. The epithelial cells of the crypts and tubules were also larger at these times than later in the estral cycle. The height of the basal glandular epithelial cells in Bitch #32 (Figure 28), 25 days after the administration of repositol D.E.S., was markedly decreased compared to those in Bitch #31 (Figure 29), 16 days after the administration of repositol D.E.S. The difference in the height of the basal glandular epithelial cells in Bitch #52, 13 days after the administration of repositol D.E.S., and Bitch #41, 5 days after the administration of repositol D.E.S., is well illustrated in Figures 30 and 31.

The larger cells contained a granular cytoplasm. The luminal diameters of the basal glandular area of the uterine glands decreased as the cells increased in size. Compared to the control animals, the morphological appearance of the uterine glands of the experimental animals was similar except maximal cell size was attained earlier in the controls. It is suggested that repositol D.E.S. was responsible for the prolongation of and/or the delay in the attainment of maximum cell size. As progesterone produces hypertrophy of the uterine glands (Bradbury, 1950; Dow, 1959; Bishop, 1962; Nalbandov, 1964), a D.E.S.-induced delay in reaching maximal cell size is a possible explanation. As indicated in Table 6, the development of the uterine glands appears to be further

advanced earlier in the cycle of the controls than in the experimental animals.

Focal endometrial extravasations in the proestral-estral period were described by Evans and Cole (1931) and Mulligan (1942). Dow (1959) also observed this phenomenon in castrated bitches receiving either estradiol or D.E.S. These extravasations were observed only in Bitch #32. They were not seen in any tissues obtained before the 31st day of the cycle or in any tissue obtained at the first operation. Significant endothelial damage was observed in the endometrium of Bitch #32 (Figure 35). No plausible explanation is offered for the absence of extravasations of blood in the majority of the sections studied. However, all of the tissues came from animals during or following their first estral cycles. Focal extravasations of blood may be a phenomenon that occurs during subsequent cycles. This is suggested by Mulligan (1942) who did not find hemosiderin or hemosiderin-containing macrophages in the endometrial stroma of puberal bitches. As the presence of hemosiderin is thought to be preceeded by extravasations of blood, the extravasations are probably also absent in "first-heat" bitches. Trauma is suggested as a possible cause of the extravasations observed in Bitch #32.

The morphological appearance of the ovaries was the same in the experimental and the control animals. The loose arrangement of the lutein cells in Bitches #52 and #21 was similar,

as was the more compact arrangement of the lutein cells seen in Bitches #41 and #11. Follicles of various sizes were present at the periphery of the ovary and between the corpora lutea in all sections.

Guyton (1961) stated that estrogens cause an increase in the number of ciliated epithelial cells in the fallopian tubes. In the dog, the cilia are lost in metestrus (Harrop, 1960). With the administration of estrogenic substances, it might be expected that the cilial loss would be delayed. The results in the presented study indicate that this occurs. The percentage of ciliated cells in the experimental animals was higher for longer periods of time than in the controls. For example, Bitch #52 had 65% ciliated epithelial cells in the oviduct as compared to control Bitch #22, which had 25% ciliated epithelial cells. These two animals were at comparable stages of the cycle. This phenomenon supports the suggestion of prolongation of the estral state by the administration of repositol D.E.S.

As the cycle progressed, the cilia seemed to coalesce to form club-like protuberances at the peripheral ends of the cells (Figure 26). These protuberances then disappeared, completing the process of cilia loss.

The control animals were bred and not given repositol D.E.S. The male dogs that were used had each sired litters. No semen studies were conducted on the males other than a

microscopic examination for density and motility. Both were considered to be fertile. However, neither of the controls was apparently pregnant.

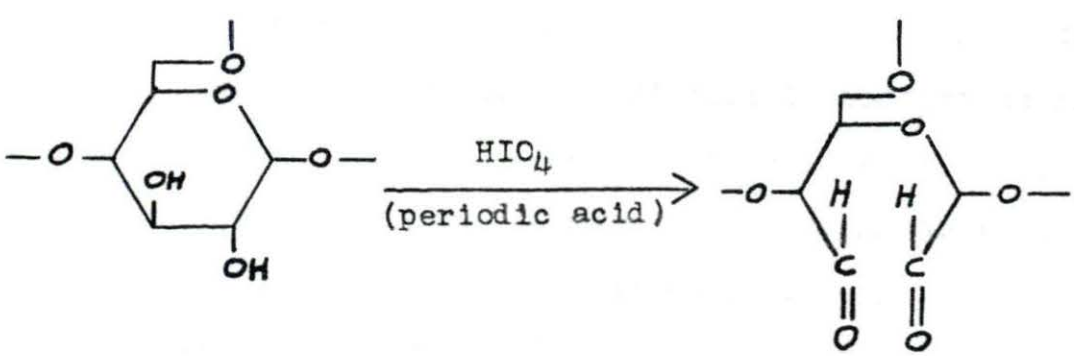
About 6 to 8 days are required for the ova to traverse the fallopian tubes and implantation is said to occur about 14 to 18 days after ovulation (Griffiths and Amoroso, 1939; Harrop, 1960). Uterine enlargements would not be present in the first two control animals at the time the tissues were surgically removed. The first two operations on the control animals were conducted 3 and 7 days after the second breeding.

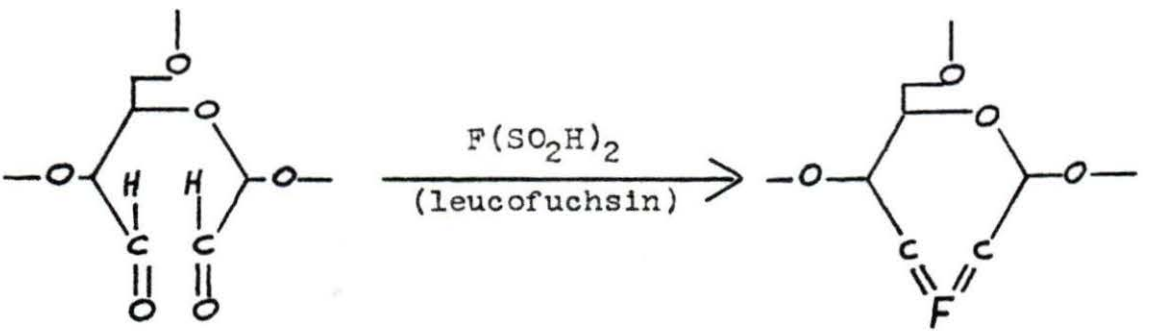
Uterine enlargements would be expected, however, in Bitch #22, which was operated 15 days after the second breeding. Although Friedman (1957) removed a portion of one uterine cornu without disruption of a pregnancy in the remaining cornu, his procedures were carried out on animals more advanced in pregnancy. An explanation for the absence of pregnancy in the presented study is not immediately forthcoming. However, absorption of the conceptus following manipulation of the tissues is a possibility. The only protocol difference in the experimental and control animals was the administration of repositol D.E.S. to the former. Evans and Cole (1931) and Mulligan (1949) have indicated that the endometrial changes in the pregnant and non-pregnant animal are identical in early metestrus. The inclusion of the observations in the control animals therefore is justified.

The studies conducted on Bitch #12 had to be minimized because small abscesses and adhesions were found around the stump probably resulting from the first surgical procedure. This focus of infection was probably the cause of the endometritis present at the time of the second surgical procedure (Figures 34 and 43). The leukocytic infiltration observed in the endometrium was not, however, present in the fallopian tube.

Glycogen is the chief storage form of carbohydrate. It is formed by the linking of carbon atoms of alpha-D-glucose at the 1-6 and 1-4 positions. In glycogenesis, the first step is the phosphorylation of glucose at the C-6 position in the presence of glucokinase. This is changed to glucose-1-phosphate from which glycogen is synthesized (Kaneko, 1963).

In the P.A.S. reaction, periodic acid attacks carbon chains at adjacent hydroxy groups, oxidizing them to aldehydes. Schiff's reagent (leucofuchsin) combines with the aldehyde groups to produce the magenta color (Pearse, 1961). The following illustrates the reaction with glycogen.





Polysaccharides other than glycogen will also be oxidized by periodic acid and therefore their presence will be revealed by the formation of the magenta color. P.A.S.-positive granules of various sizes are stained a deep purple-red. Diastase treated tissue sections that result in the disappearance of these granules prove them to be glycogen (Pearse, 1961).

In the presented study, most of the P.A.S.-positive granules disappeared following incubation of the sections in diastase. The P.A.S.-positive granules were thereby identified as glycogen (Figures 44 and 45).

In the ovary, glycogen was present in the blood vessels and granulosa cells but absent in the lutein cells. This was a constant finding in all sections.

Glycogen was not present in the epithelial cells of the oviduct at any time. Some luminal epithelial cells of the oviduct and endometrium did have a dark P.A.S. reaction at their peripheral ends (Figures 39 and 40). More of this material was seen in the endometrium than in the oviduct.

Although the reaction faded slightly in the control sections, it did not disappear. In the oviduct, the occurrence of this material was not related to the presence of cilia or to the stage of the estral cycle. It is suggested that the P.A.S.-positive material observed could have contained glycogen and a polysaccharide and might have been a secretion of the goblet cells.

Glycogen was not found in the luminal epithelial cells of the endometrium except in Bitch #32. In this section, however, only small traces were evident and these seemed to occur in areas where the glandular crypts opened (Figure 54). The general trend was that glycogen in the epithelium of the uterine glands tended to decrease as the cycle progressed (Table 7). This was true in both the experimental and control animals. However, maximal amounts were seen earlier in the cells of the experimental animals. Glycogen deposition in the canine uterine glands appeared to be influenced by estrogens, which is in agreement with the findings of Erichsen (1953) and Fitch (1961). Because maximal amounts were seen earlier in the experimental animals, it is suggested that the repositol D.E.S. was responsible for this increased glycogen deposition.

As previously mentioned, estrogen administration early in the estral cycle of many laboratory animals resulted in interference with the development of the proper progestational

state of the uterus. The works of Erichsen and Fitch suggested that the disappearance of glycogen from the epithelial cells of the uterine glands and the concomitant appearance of P.A.S.-positive luminal plugs during the luteal phase of the cycle was indicative of the secretion-release function of progesterone. Hisaw and Greep (1938) reported that progesterone plays a role in the formation and release of glycogen.

The appearance of P.A.S.-positive luminal plugs in the uterine glands paralleled a decrease in the glycogen content in the glandular epithelial cells in both experimental and control animals. An estrogen-progesterone formation-release synergism of the secretion is suggested in the works of Erichsen (1953) and Fitch (1961). If progesterone is essential for secretory activity of the endometrial glands and estrogen administration delays progesterone-induced endometrial changes, then a D.E.S.-induced delay of the release of the secretion from the glandular cells might be hypothesized. In the presented study, such a delay was evident. In fact, by the time the P.A.S.-positive luminal plugs appeared in the experimental animals, they had already appeared and disappeared in the control animals.

The phosphatase enzymes are divided into mono-, di- and triphosphatases. The phosphomonoesterases are not specific and will hydrolyze a wide variety of organic phos-

phates. The di- and triphosphatases hydrolyze two and three phosphate esters, respectively. This is followed by monoesterase activity. Di- and triphosphatases cannot be demonstrated unless phosphomonoesterase is absent. Therefore, the phosphomonoesterases are of greater importance in histochemistry (Davenport, 1960; Pearse, 1961).

Alkaline phosphatases are enzymes which will hydrolyze organic phosphates at an optimum alkaline pH. These enzymes are concerned with the transfer of phosphate from one alcohol to another (Pearse, 1961).

The technique of Gomori, for the demonstration of alkaline phosphatase activity, is the cobalt sulfide method. Tissue sections were incubated in a solution containing sodium glycerophosphate (an organic phosphate ester), calcium chloride (the source of the necessary calcium ions), and magnesium chloride (the source of magnesium ions). The solution had a pH of 9.0. The incubation was carried out at a temperature of 37° C.

Calcium phosphate was deposited at sites of enzymatic activity. The magnesium ions served as enzyme activators. Following incubation, the precipitated calcium phosphate was converted to cobalt sulfide, a black precipitate, by passing the tissue sections through a cobalt salt solution and then ammonium sulfate.

Control sections were incubated in a solution not con-

taining sodium glycerophosphate. Some substances present in the tissues, such as native phosphates, will give false positive reactions (Pearse, 1961). These substances are demonstrated in control slides. The differentiation between enzymatic activity and false positive reactions is possible through a comparison of the experimental and control tissue slides.

Pearse (1961) stated that high activity of alkaline phosphatase, as indicated by the presence of a dark reaction, indicates increased phosphate transfer. Dempsey and Wislocki (1946) and Atkinson and Engle (1947) have reported that the enzymes play a part in protein synthesis and glycogen formation. That phosphate activity is related to the progesterone level in the luminal epithelium of the bovine endometrium was particularly well demonstrated by Skjerven (1956).

No differences in alkaline phosphatase activity were observed in the ovaries studied. The blood vessels were always positive, distinctly revealing the vascularity of the corpora lutea (Figure 55). The theca interna cells of the follicles, where estrogen production is thought to occur (Harrop, 1960), were always positive (Figures 56 and 57).

Alkaline phosphatase activity was present in the ciliated epithelial cells of the oviduct. Most non-ciliated cells were negative (Figures 59 through 63). Deane (1952) demonstrated alkaline phosphatase activity in the ciliated cells

of the rat oviduct. It may be that alkaline phosphatase is, therefore, associated with ciliary activity.

Alkaline phosphatase activity appeared to vary inversely with the amount of glycogen in the uterine glands (Table 7). Skjerven (1956) observed the same in the bovine luminal epithelium of the uterus. However, Atkinson and Engle (1947) described the opposite in regard to alkaline phosphatase activity in the uterine glands of the monkey. In the presented study alkaline phosphatase activity was definitely greater in the luminal and glandular epithelial cells of the control animals during the luteal phase of the cycle (Table 7). This increase was delayed, but definite, in the luminal epithelium of the experimental animals but quite variable in the uterine glands (Table 7).

A dark alkaline phosphatase reaction was seen along the peripheral borders of the uterine glandular epithelial cells in the control slides from most experimental and control animal tissues (Figures 68 through 73). The alkaline phosphatase disappeared in the luminal epithelium of all control slides from both experimental and control animal tissues (Figure 69). The occurrence of a positive reaction in the alkaline phosphatase control sections in the uterine glands paralleled the presence of a secretion in the lumina of the basal portions of the uterine glands. The reaction was present in other areas of the glands before the secretion

appeared in the lumina. The presence of native phosphate material in the secretion is evident. However, its occurrence in the uterine glandular cells is variable. These observations have been summarized and tabulated (Table 9).

Table 9. Presence or absence of dark staining reaction in alkaline phosphatase control sections along the free poles of the luminal, crypt, tubular and basal glandular epithelium

Dog	Lumen epith.	Crypt epith.	Tubule epith.	Basal gl. epith.
51	-	+	+	-
41	-	+	+	-
52	-	+	+	-
31	-	+	+	+
42	-	+	+	+
32	-	+	+	+
Controls:				
21	-	+	+	+
11	-	+	+	+
22	-	+	+	+
12	-	-	-	-

Alkaline phosphatase activity was found to be more pronounced in the endometrial luminal epithelium in the post-estral period in the pig (Austad and Garm, 1959), sheep (Hadek, 1958a) and cow (Skjerven, 1956). The same was observed in the dog in the presented study. Not only did alkaline phosphatase become more pronounced in the luminal epithelium as the cycle progressed (Figures 66 and 67), but this increase occurred later in the cycle in the experimental animals than in the control animals (Figures 64, 65, and 68).

If progesterone causes an increase in alkaline phosphatase activity in the luminal epithelium, then a delay in the attainment of the progestational state of the uterus should cause a retardation of alkaline phosphatase activity.

This occurred in the presented study and supports the contention that repositol D.E.S. interferes with the proper progestational development of the uterus.

CONCLUSIONS

The following conclusions are supported by the investigational work:

1. Maintenance of the cilia on the oviduct luminal epithelium is estrogen-influenced.
2. The glycogen content of the uterine glandular epithelial cells decreases in the luteal phase of the estral cycle.
3. The occurrence of glycogen in the epithelial cells of the uterine glands is enhanced by the administration of repositol diethylstilbestrol.
4. Alkaline phosphatase in the luminal epithelium of the endometrium increases in the luteal phase.
5. The increase of alkaline phosphatase in the luminal epithelium of the endometrium is delayed by the administration of repositol diethylstilbestrol.
6. The decrease of alkaline phosphatase in the luminal epithelial cells of the oviduct parallels the loss of cilia in these cells.
7. Mitotic division in the uterine glandular epithelium is stimulated by the administration of repositol diethylstilbestrol.
8. Repositol diethylstilbestrol prolongs the estral

state of the endometrium which delays the onset of the progestational state.

9. An explanation of the value of repositol diethylstilbestrol in terminating pregnancy in the bitch is the lengthening of the estrogen state of the endometrium, thereby delaying the onset of the progestational state in the preparation of the endometrium for implantation.

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Finally, I wish to thank Doctors M. A. Emmerson and J. E. Lovell, whose encouragement, guidance and advice resulted in the completion of this work.

APPENDIX

Figure 1. Days of life (abscissa) versus body weight in pounds (ordinate) for the smallest and the largest of the experimental animals.

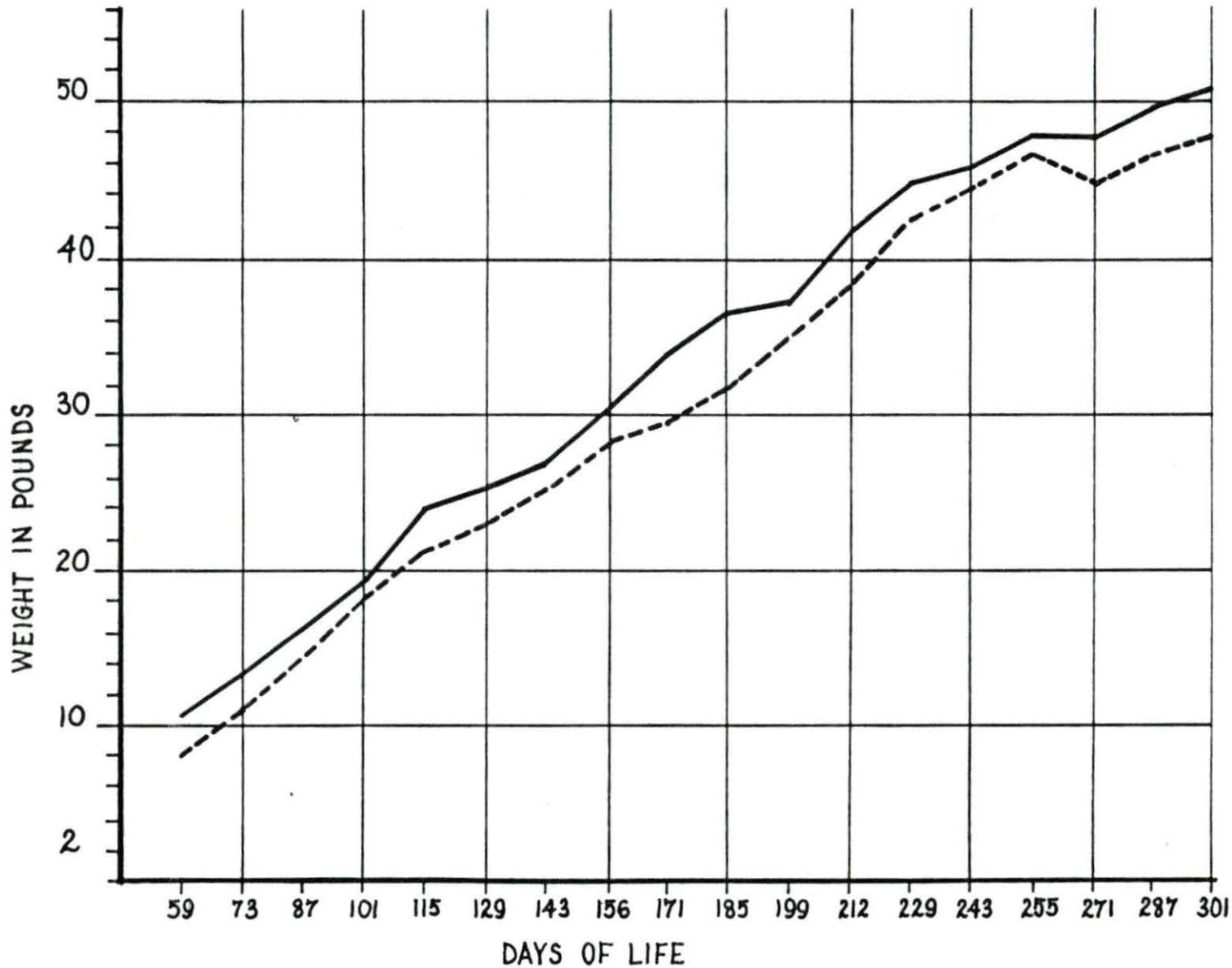

WEIGHT IN POUNDS
50
40
30
20
10
2
59
73
87
101
115
129
143
156
171
185
199
212
229
243
255
271
287
301
DAYS OF LIFE

Figure 2. Schematic of the canine female genitalia showing tissue specimens removed and the amputation sites of the surgical procedures.

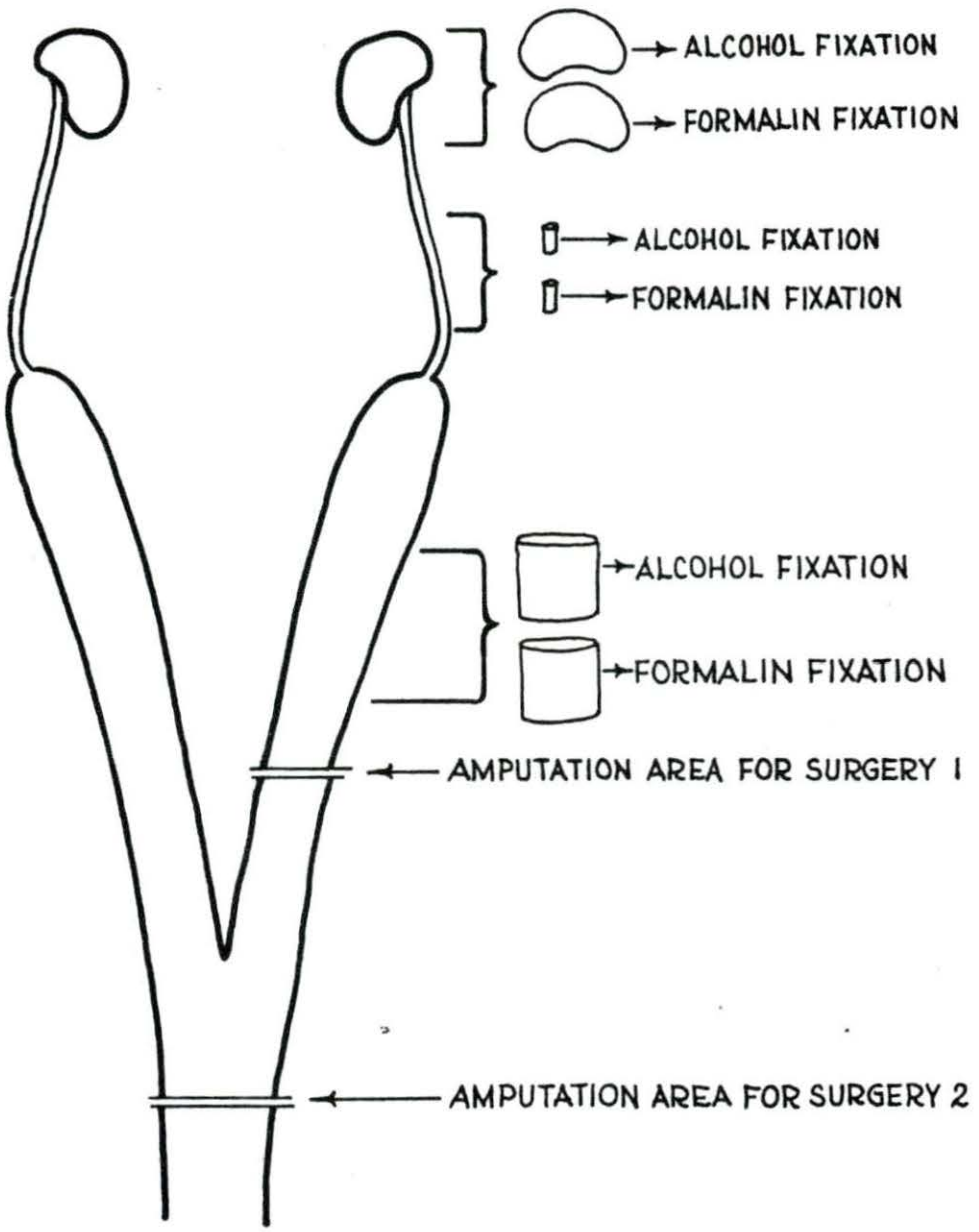
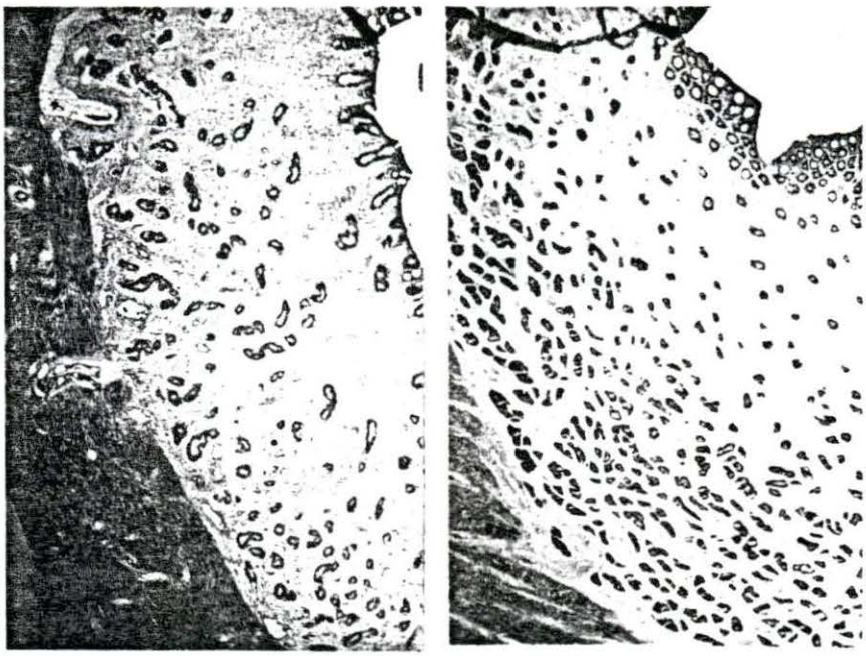

ALCOHOL FIXATION
FORMALIN FIXATION
ALCOHOL FIXATION
FORMALIN FIXATION
ALCOHOL FIXATION
FORMALIN FIXATION
AMPUTATION AREA FOR SURGERY 1
AMPUTATION AREA FOR SURGERY 2

Figure 3 (left). Uterus of Bitch #51, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 4 (right). Uterus of Bitch #52, showing the thickness of the endometrium and development of the uterine glands. H.&E. 35x

Figure 5 (left). Uterus of Bitch #21, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 6 (right). Uterus of Bitch #22, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x



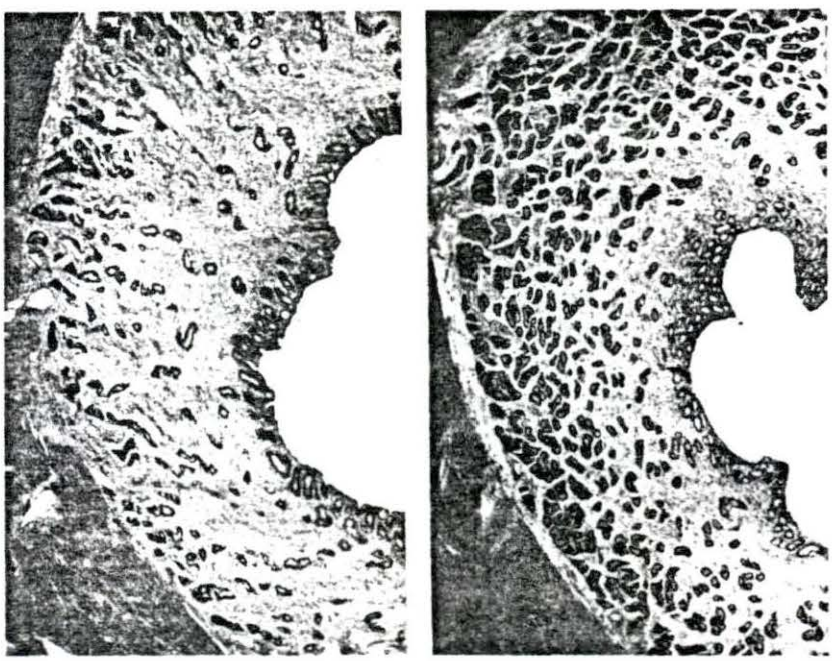
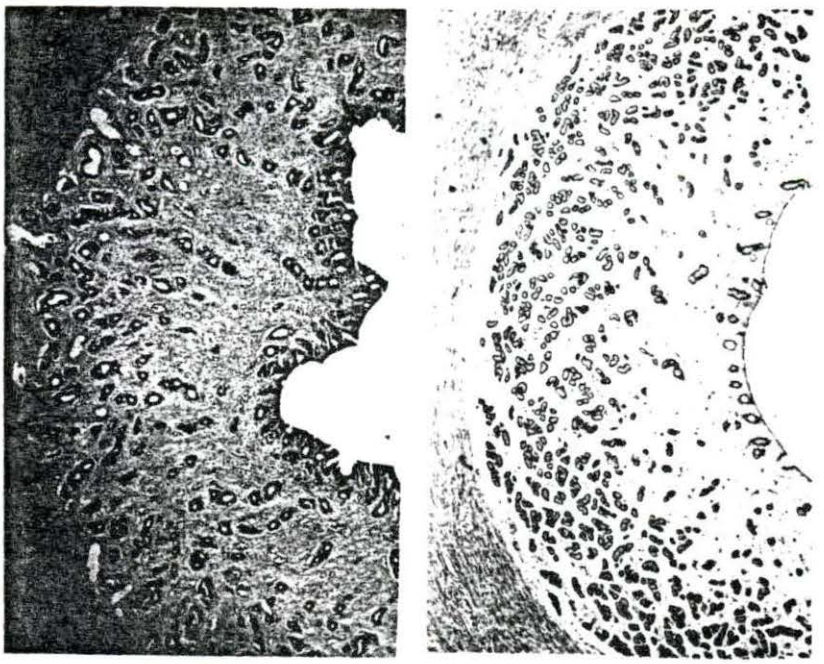


Figure 7 (left). Uterus of Bitch #41, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 8 (right). Uterus of Bitch #42, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 9 (left). Uterus of Bitch #11, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 10 (right). Uterus of Bitch #12, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x



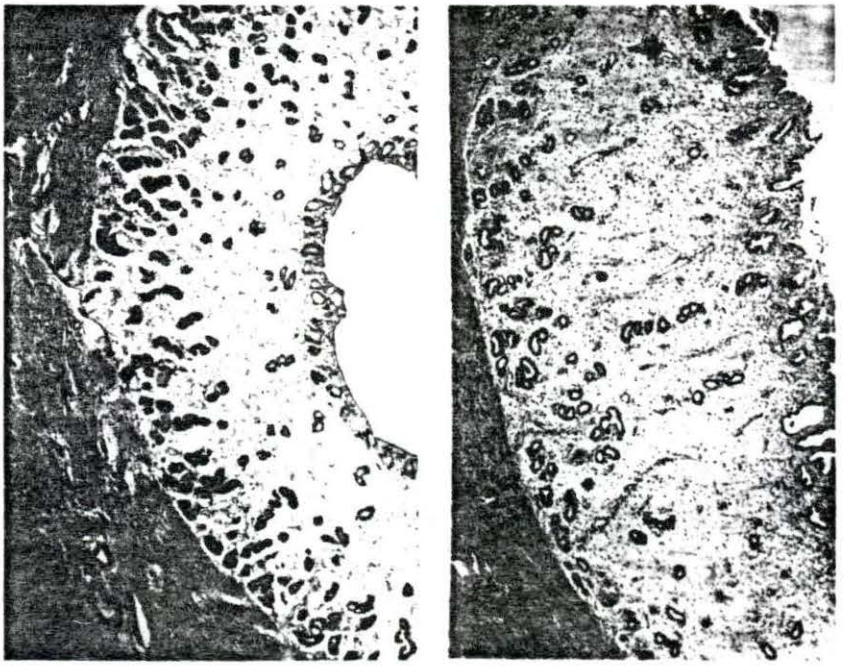
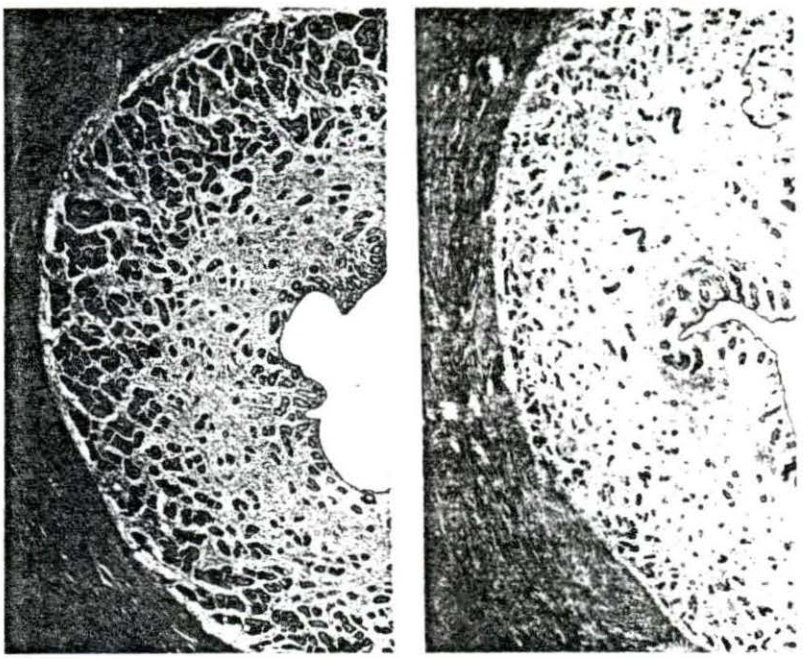


Figure 11 (left). Uterus of Bitch #31, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 12 (right). Uterus of Bitch #32, showing the thickness of the endometrium and the development of the uterine glands. H.&E. 35x

Figure 13. Morphology of the endometrium: a, luminal epithelium; b, crypts of the uterine glands; c, tubules of the uterine glands; d, basal area of the uterine glands; e, connective tissue stroma of the endometrium. From the uterus of Bitch #11. H.&E. 100x



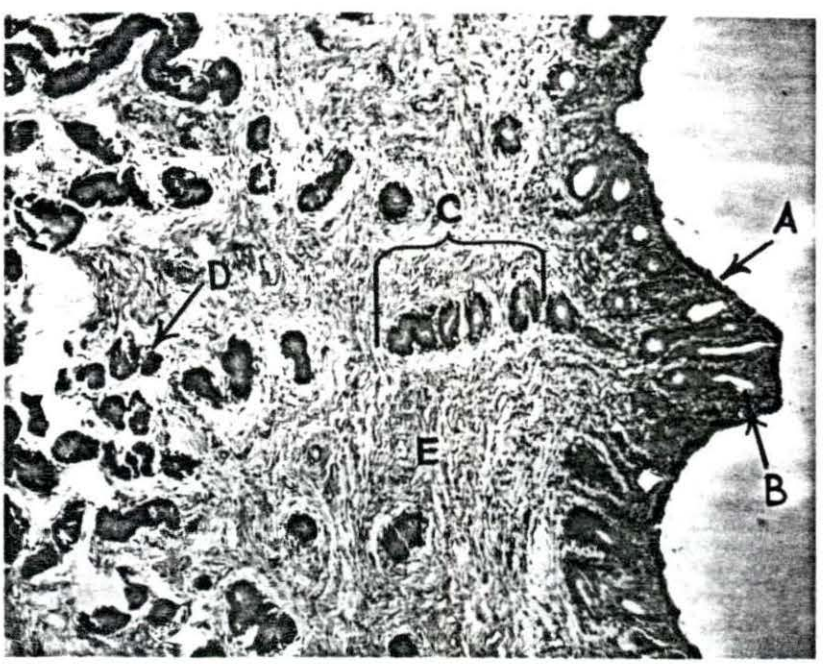
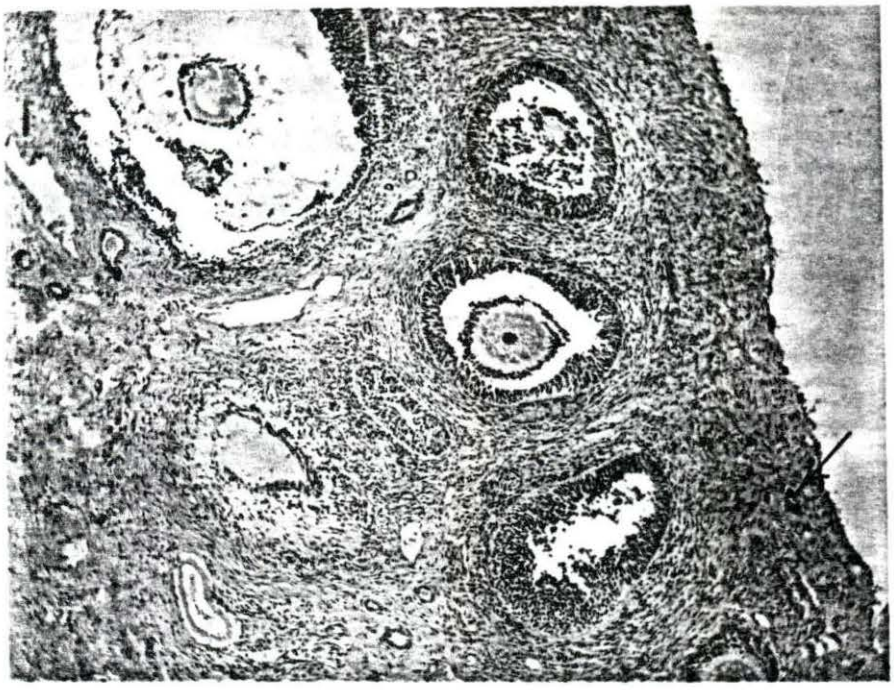

A
B
C
D
E

Figure 14. Follicles of various sizes at the periphery of the ovary. Note the small primordial follicle (arrow). From the ovary of Bitch #11. H.&E. 100x

Figure 15. Cords of epithelial cells (f) from the germinal epithelium of the ovary. The cords are extending into the deep portion of the ovary. Note the primordial follicle (arrow) and corpus luteum (g). From the ovary of Bitch #11. H.&E. 100x



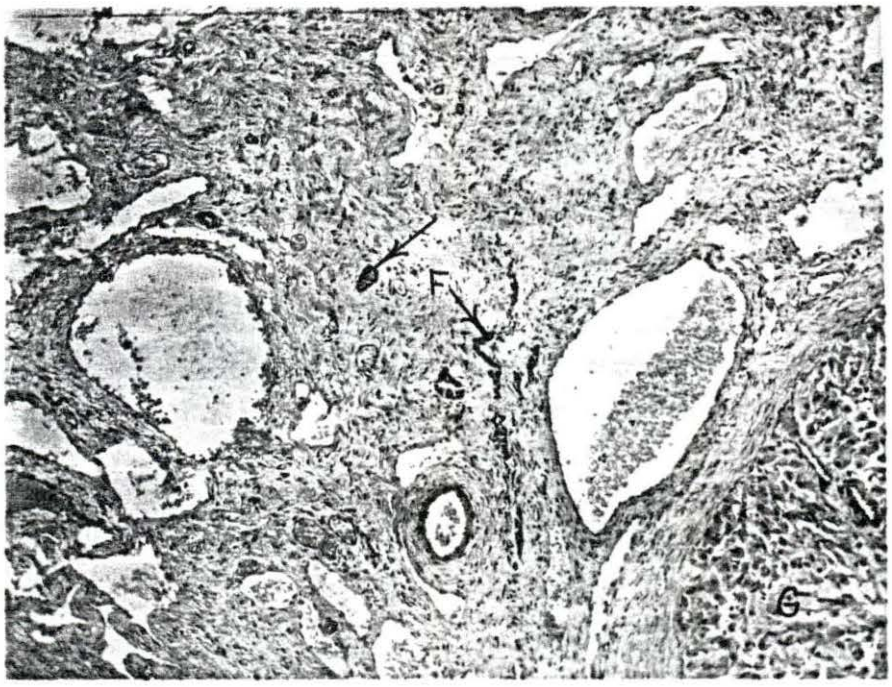

F
G

Figure 16. Corpus luteum of Bitch #21 showing the loose arrangement of lutein cells and centrally located cavity (h). H.&E. 100x

Figure 17. Corpus luteum of Bitch #31 showing the more compact arrangement of large lutein cells. H.&E. 100x


H

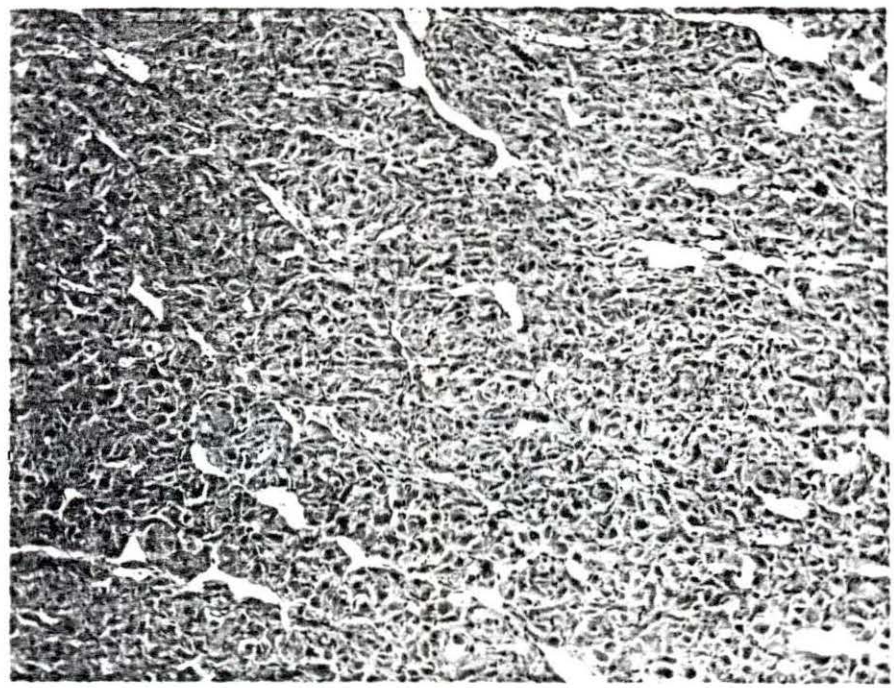
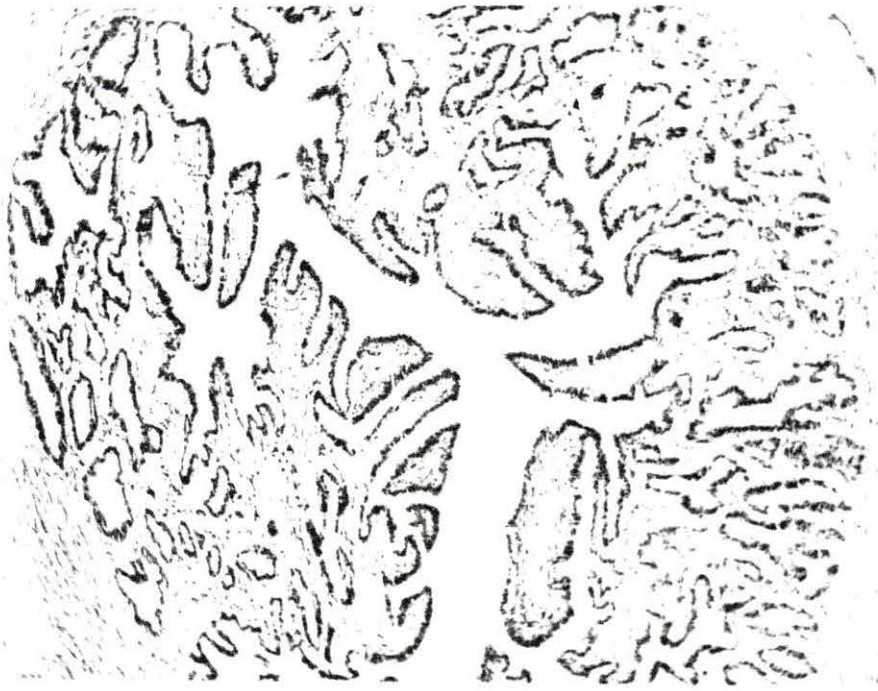


Figure 18. Folding of the mucosa of the fallopian tube and formation of small recesses. From the tissue of Bitch #21. H.&E. 100x

Figure 19. Folding of the follicular wall (arrow) as part of the process of atresia. From the tissue of Bitch #21. H.&E. 100x



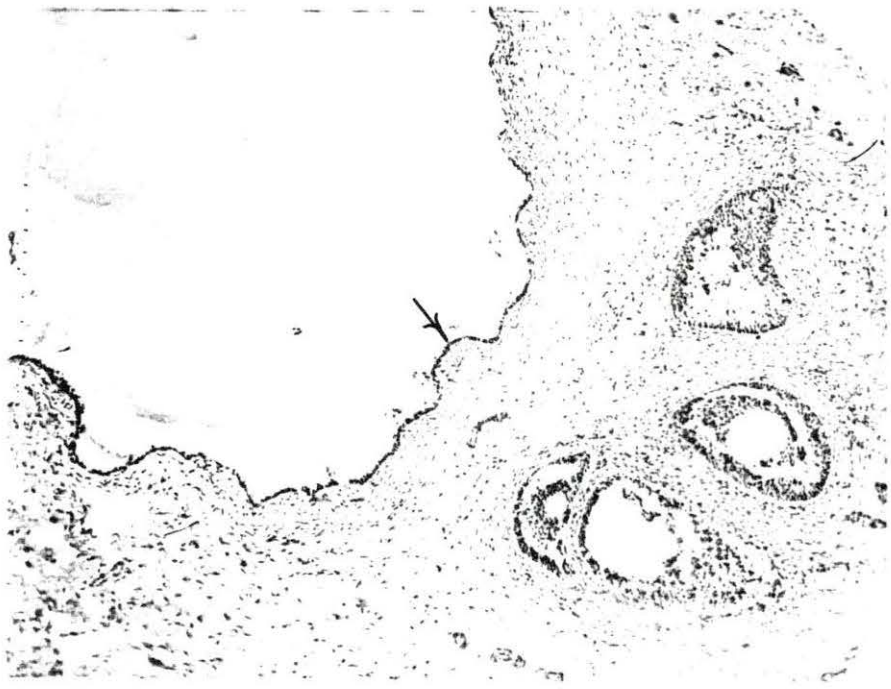
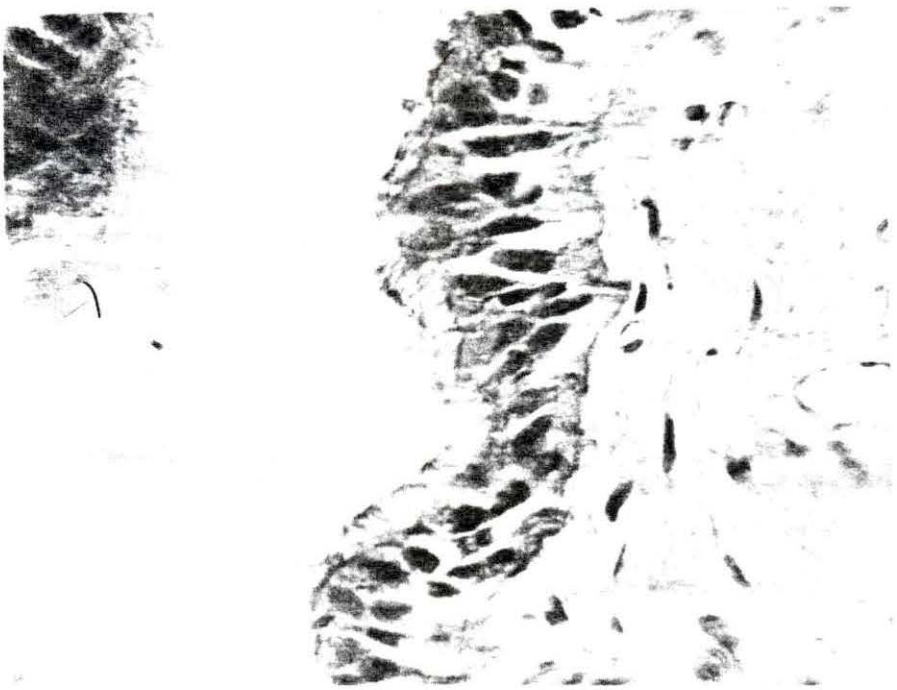


Figure 20. Epithelium of the oviduct of Bitch #51. Note that most (95%) of the large epithelial cells are ciliated. H.&E. 900x

Figure 21. Epithelium of the oviduct of Bitch #52. Note the large cells with plump nuclei. Approximately 65% of the epithelial cells were ciliated in this bitch. H.&E. 900x



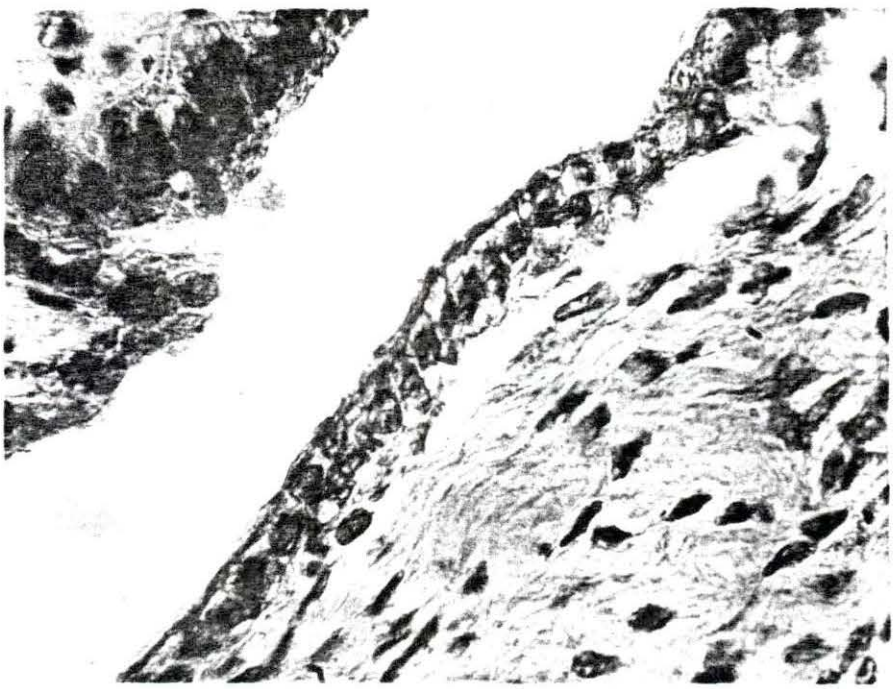
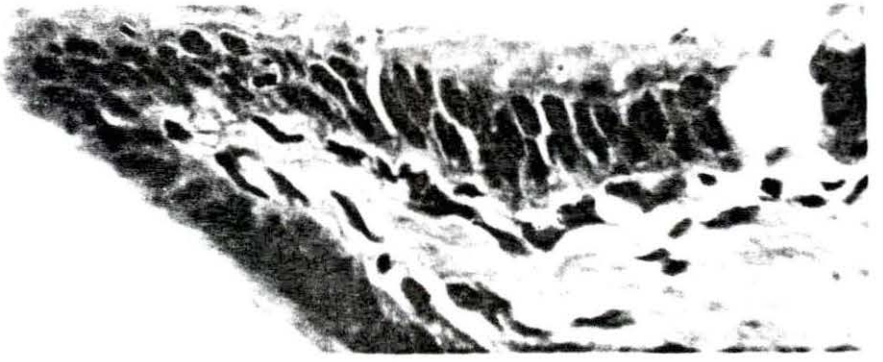


Figure 22. Epithelium of the oviduct of Bitch #21. Note the large, pseudostratified columnar epithelium and many ciliated cells. H.&E. 900x

Figure 23. Epithelium of oviduct of Bitch #22. Note smaller epithelial cells than above. Fewer ciliated cells were present at this time. H.&E. 900x



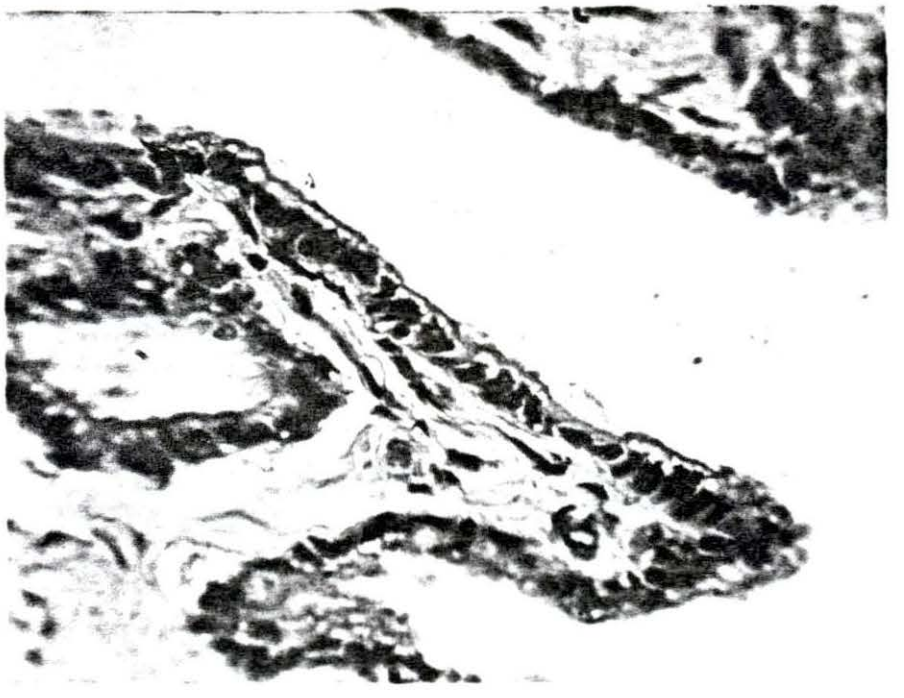


Figure 24. Epithelium of the oviduct of Bitch #11. Approximately 70% of the cells were ciliated. H.&E. 900x

Figure 25. Epithelium of the voiduct of Bitch #12. Approximately 15% of the cells were ciliated. H.&E. 900x

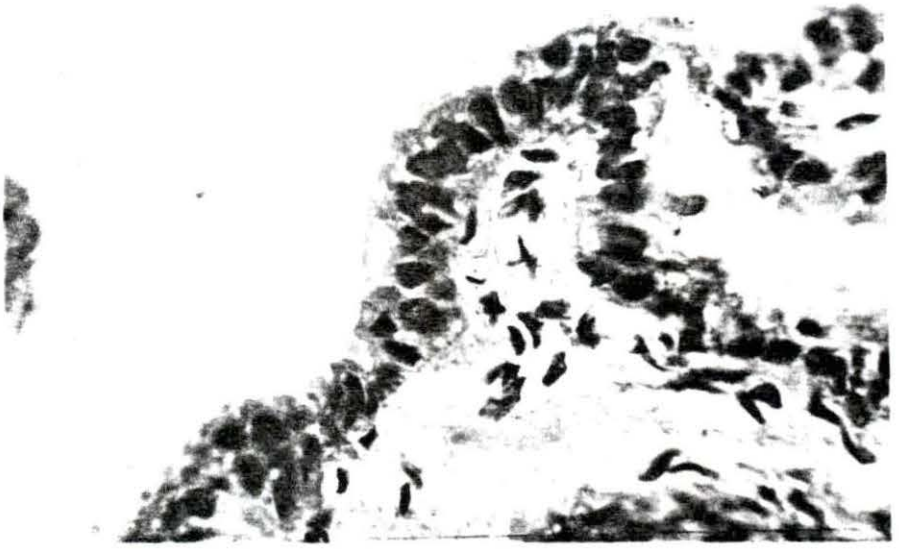


Figure 26. Epithelium of oviduct of Bitch #31. Note coalesence of cilia to form club-like protuberances. H.&E. 900x

Figure 27. Oviduct epithelium of Bitch #42. Note (arrow) that cilia are retained longer on the epithelial cells in the protected recesses. H.&E. 450x

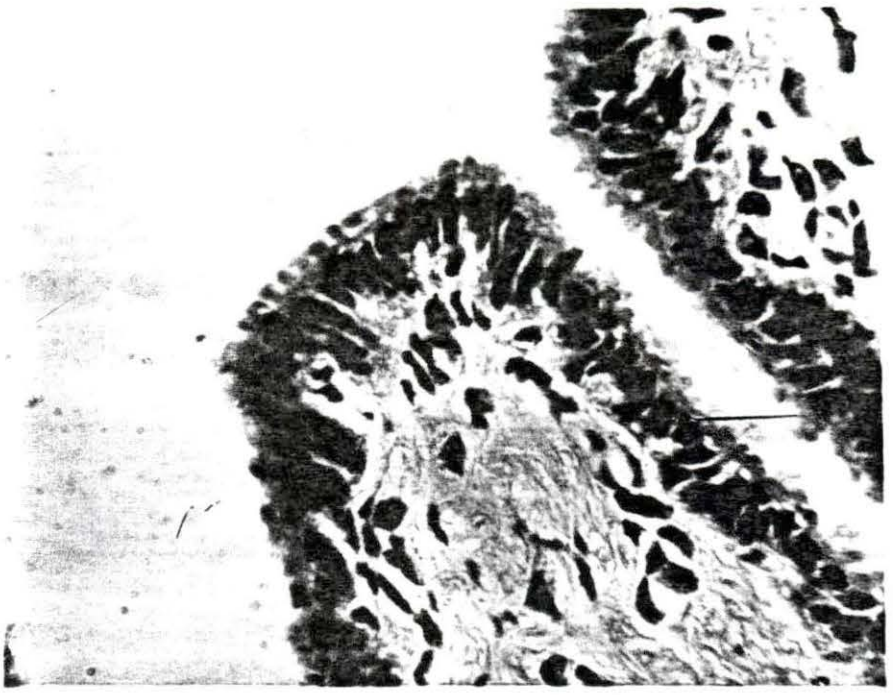
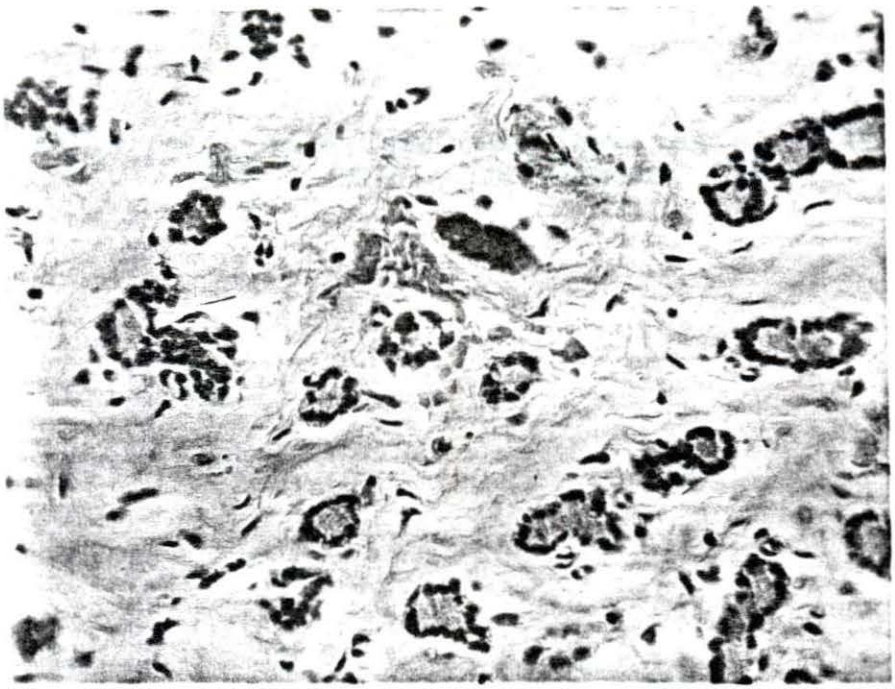


Figure 28. Small basal glandular epithelial cells in Bitch #32. H.&E. 450x

Figure 29. Large basal glandular epithelial cells in Bitch #31. H.&E. 450x



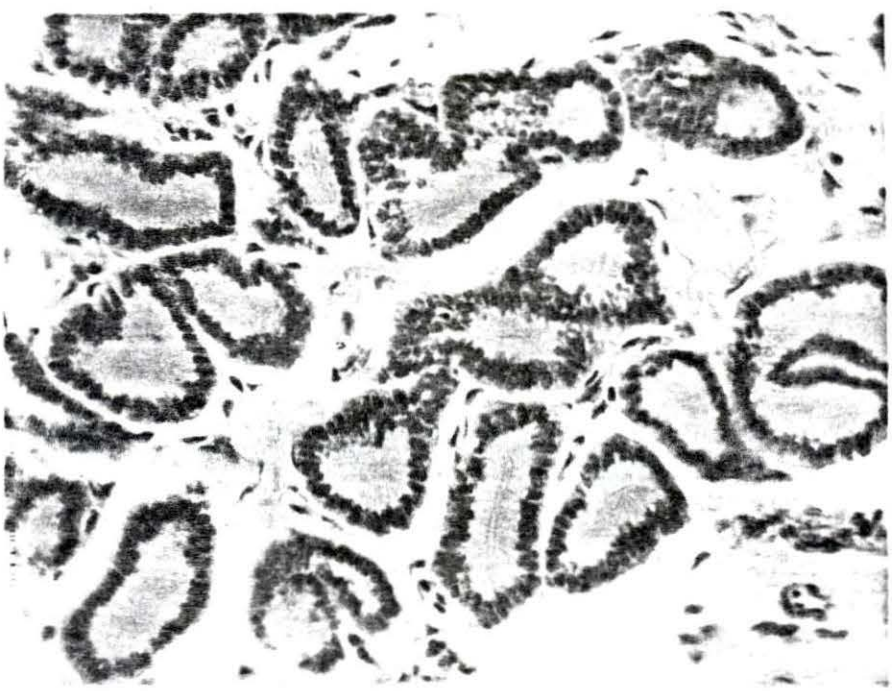
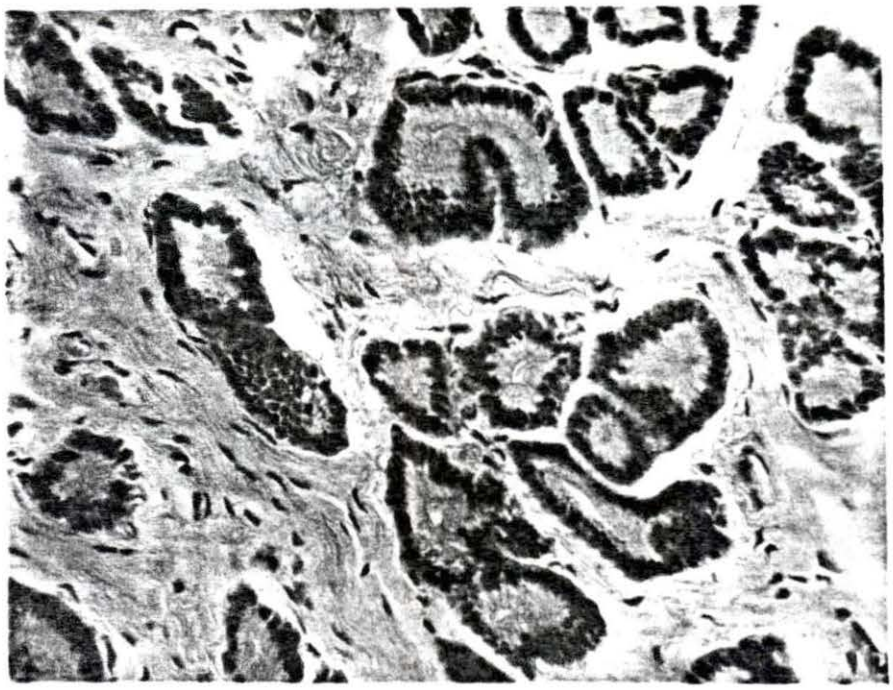


Figure 30. Large basal glandular epithelial cells of Bitch #52 endometrium. H.&E. 450x

Figure 31. Basal glandular epithelial cells of Bitch #41 endometrium. H.&E. 450x



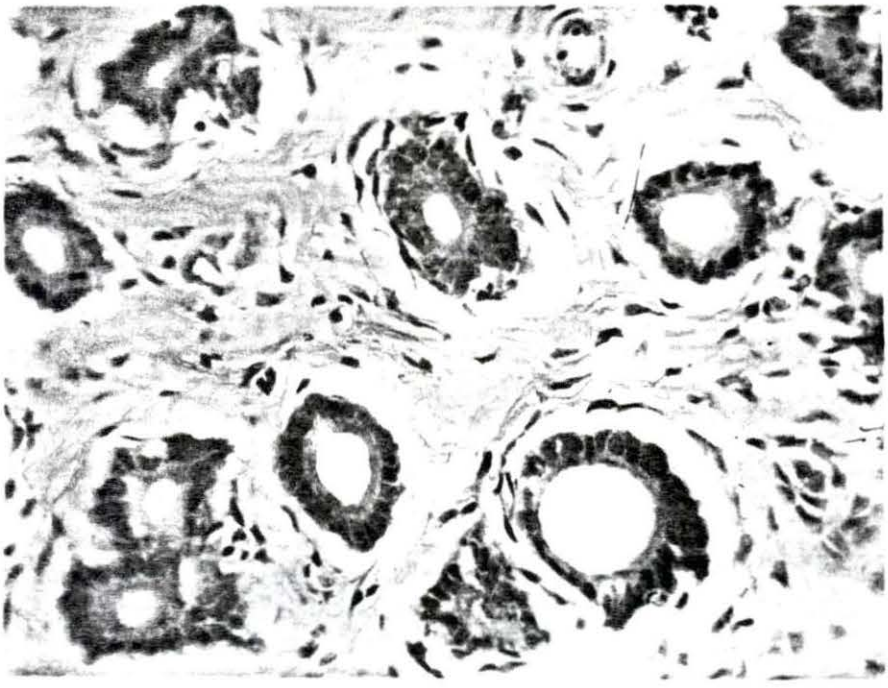


Figure 32. Mitotic figures in telophase (arrow) in the cryptal epithelium of the uterine glands. From the uterus of Bitch #22. H.&E. 900x

Figure 33. Mitotic figures in telophase (arrow) in the basal glandular epithelium. From the uterus of Bitch #51. H.&E. 900x

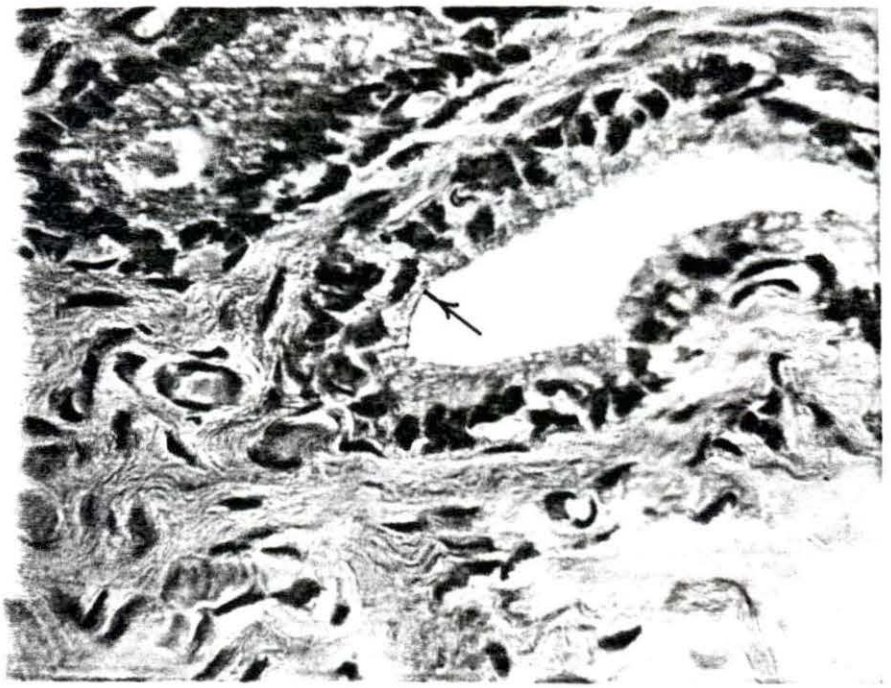
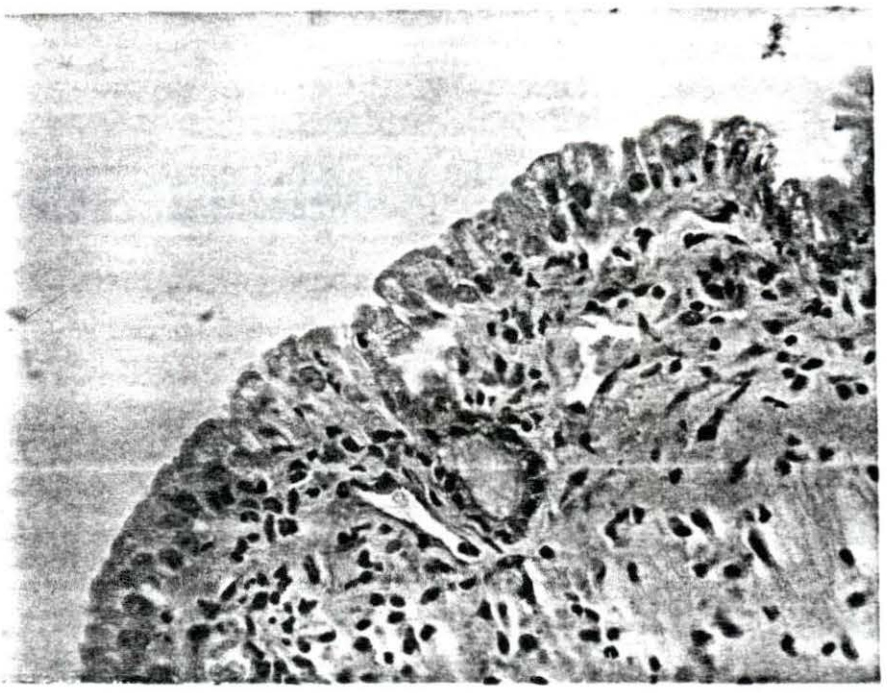


Figure 34. Endometrial luminal epithelium of Bitch #12. Note "fattened" cells with leukocytic infiltration in endometrium. H.&E. 900x

Figure 35. Extravasated blood in endometrium of Bitch #32. H.&E. 450x



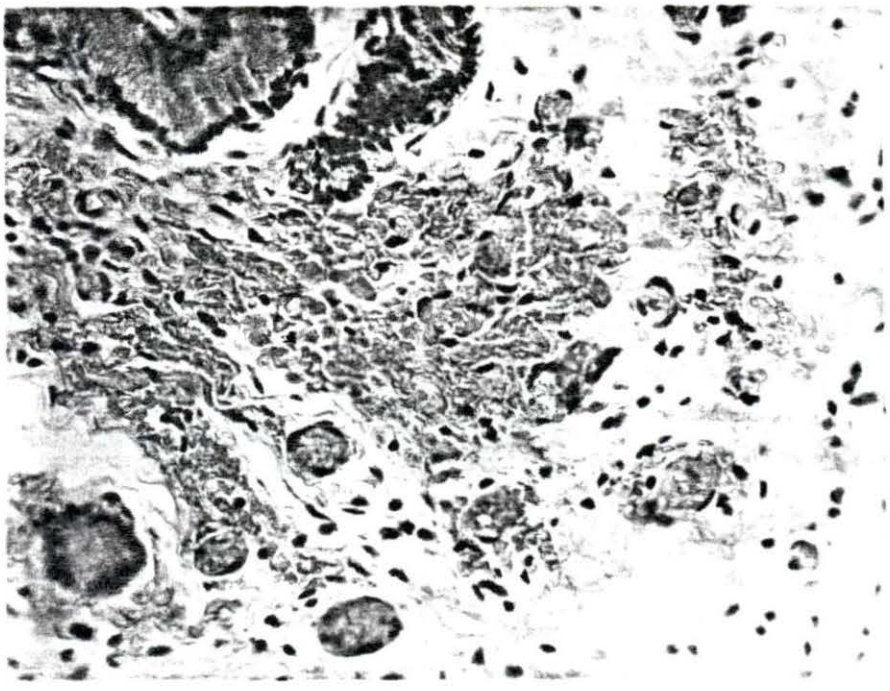
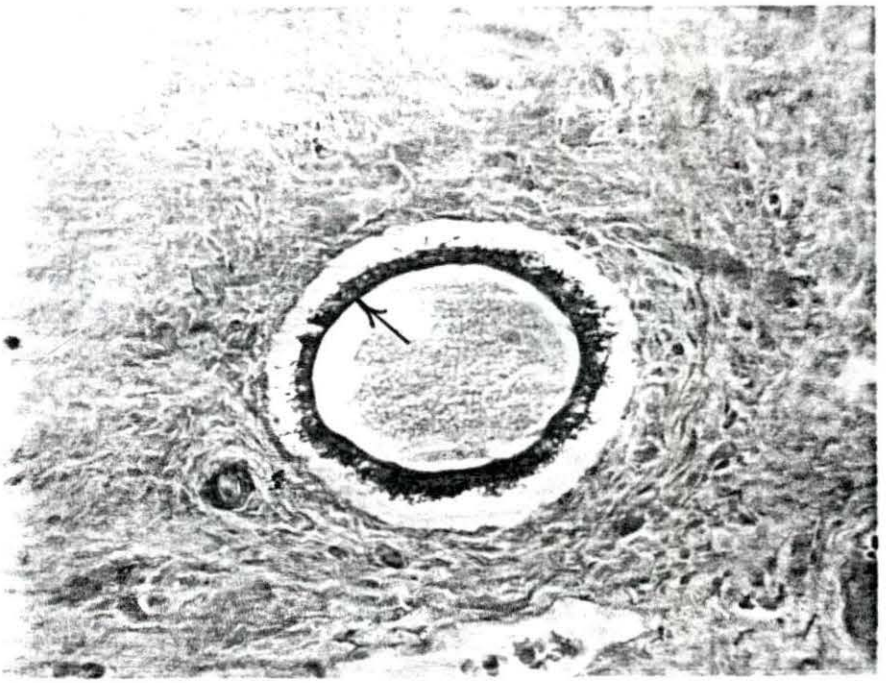


Figure 36. Zona pellucida of ovum (arrow) from Bitch #32. showing dark P.A.S. reaction. 450x

Figure 37. P.A.S.-positive granules in the wall of a blood vessel in the corpus luteum. From the ovary of Bitch #11. 450x



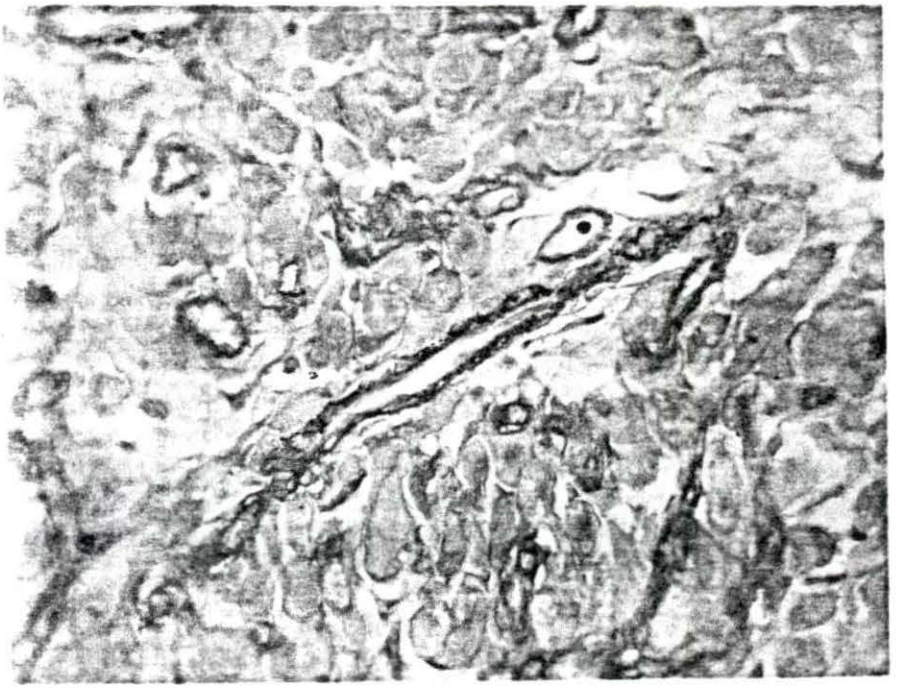
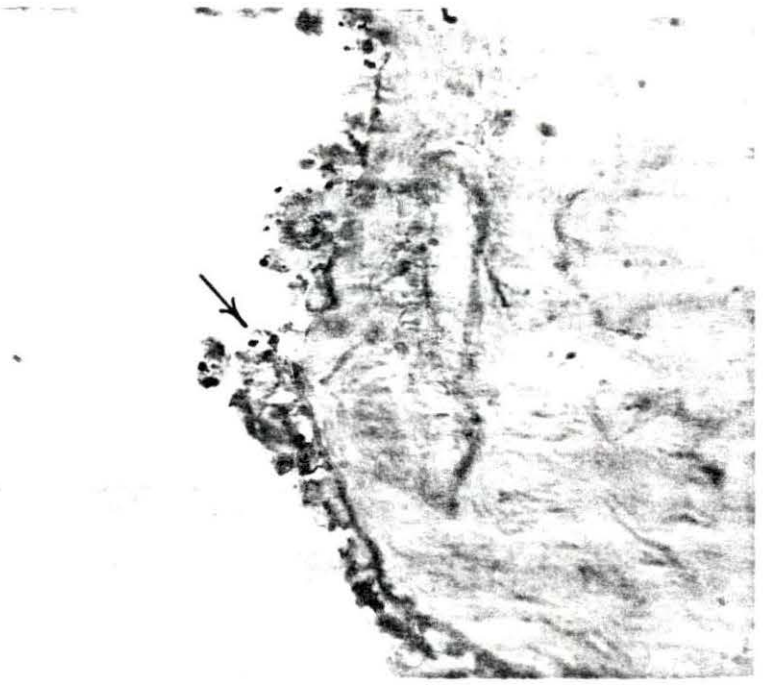


Figure 38. P.A.S.-postive granules (arrow) in the granulosa cells of follicles. From the tissue of Bitch #21. 900x

Figure 39. P.A.S. reaction on oviduct epithelium of Bitch #21. Note absence of P.A.S.-positive granules and dark reaction along free poles of a few epithelial cells. 900x



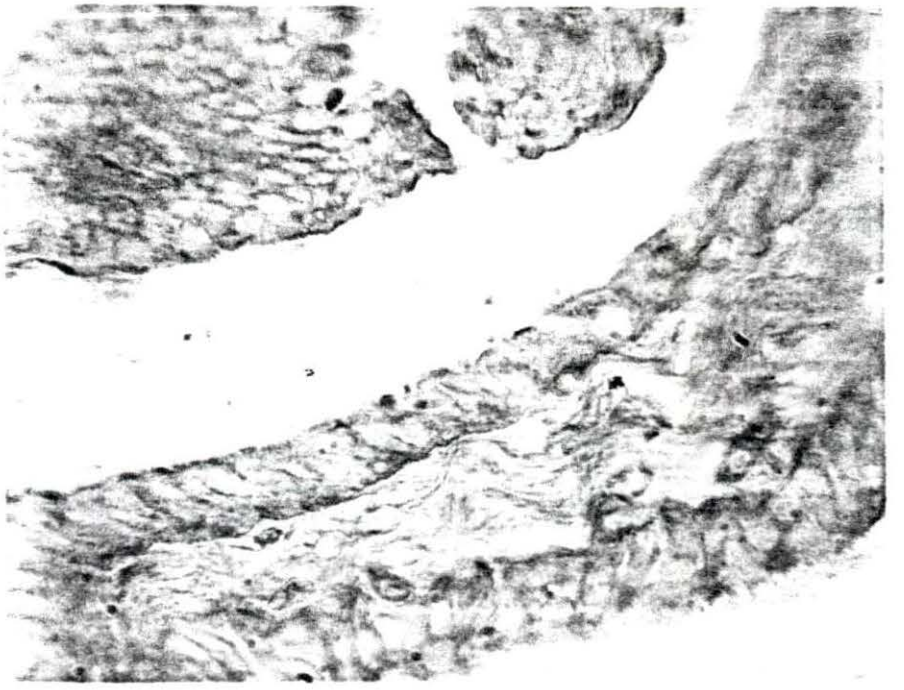
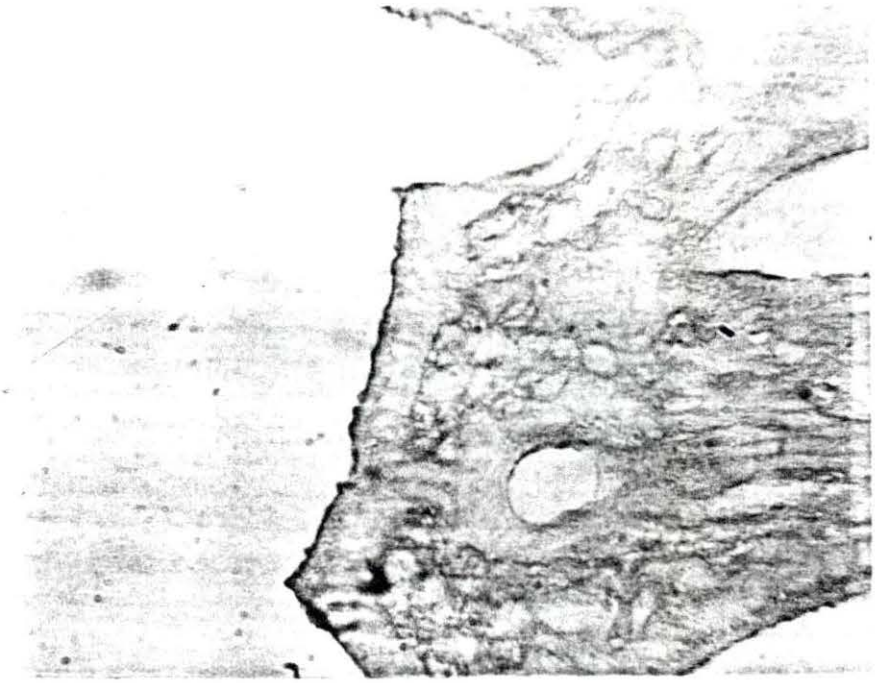


Figure 40. P.A.S. reaction of luminal epithelium of endometrium of Bitch #41. Note absence of P.A.S.-positive granules in epithelial cells and dark staining line along free poles of the cells. 450x

Figure 41. P.A.S. control slide of above. Note that the P.A.S.-positive dark line has lightened somewhat but has not disappeared. 450x



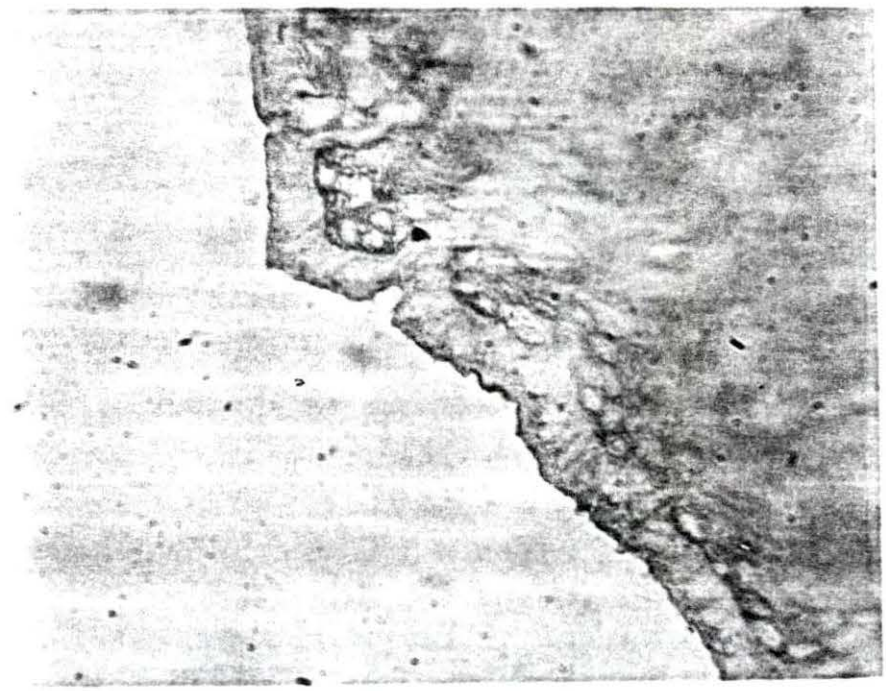
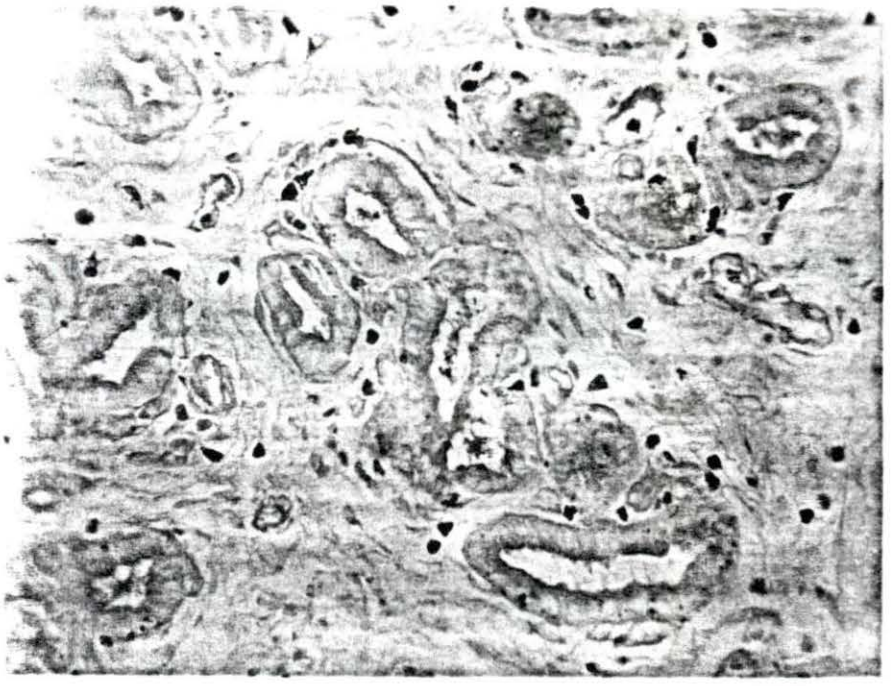


Figure 42. Basal glandular area of Bitch #12. This P.A.S. reaction demonstrates the number of white blood cells, which are P.A.S.-positive, infiltrating the area. 450x

Figure 43. Luminal epithelium of the endometrium of Bitch #12. Note the P.A.S.-positive white blood cells infiltrating the area. 450x



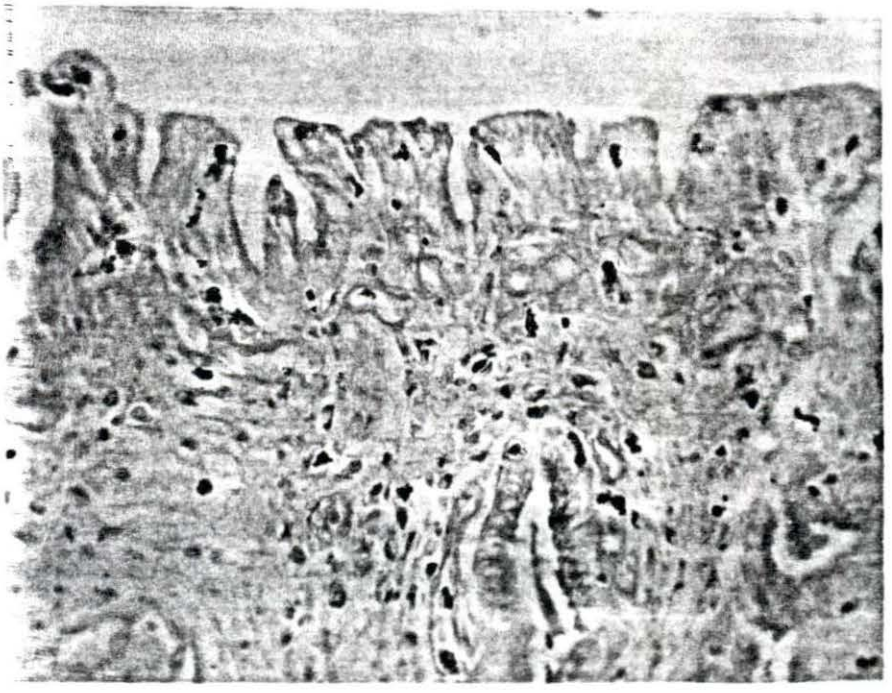
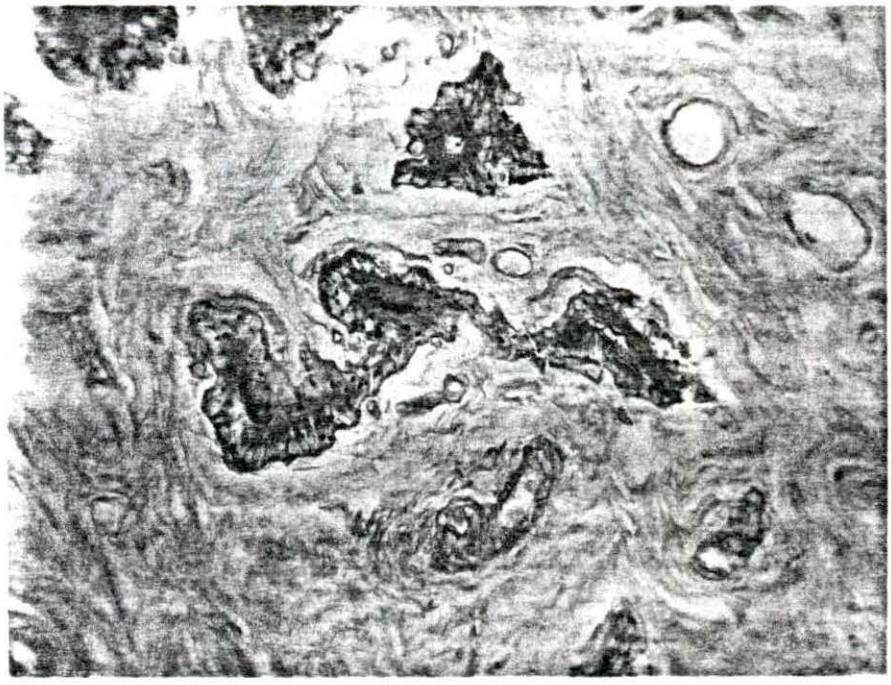


Figure 44. P.A.S. reaction of the basal glandular area of the endometrium of Bitch #51. This represents a +++ reaction for P.A.S.-positive granules. 450x

Figure 45. P.A.S. control section of the above. Note that most all of the granules have disappeared following incubation in diastase. 450x

Figure 46. P.A.S. reaction of the basal glandular area of the endometrium of Bitch #21. This was a ++ reaction for P.A.S.-positive granules. 450x

Figure 47. P.A.S. reaction of the basal glandular area of the endometrium of Bitch #22. This was a + reaction for P.A.S.-positive granules. 450x



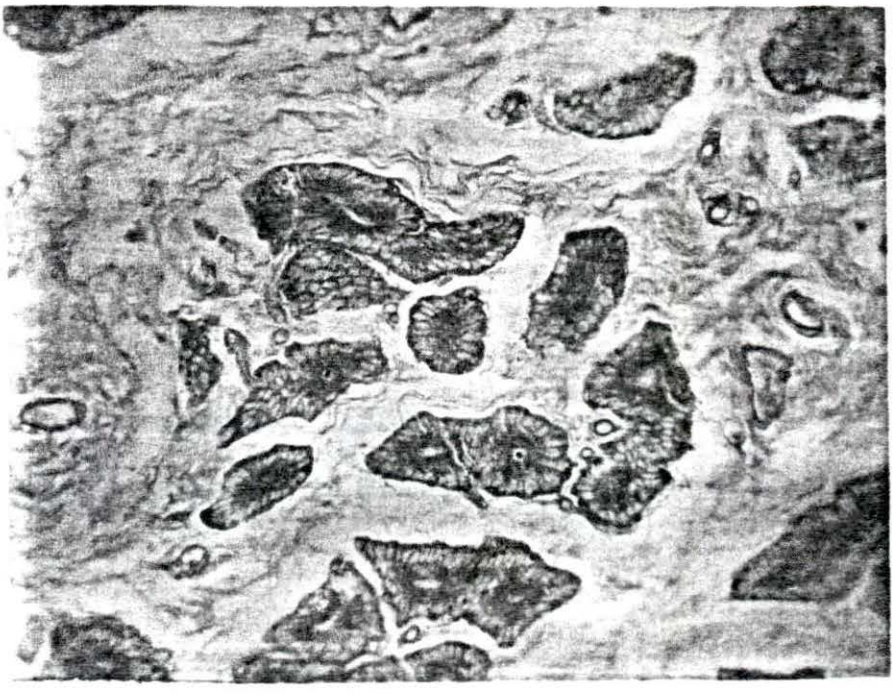
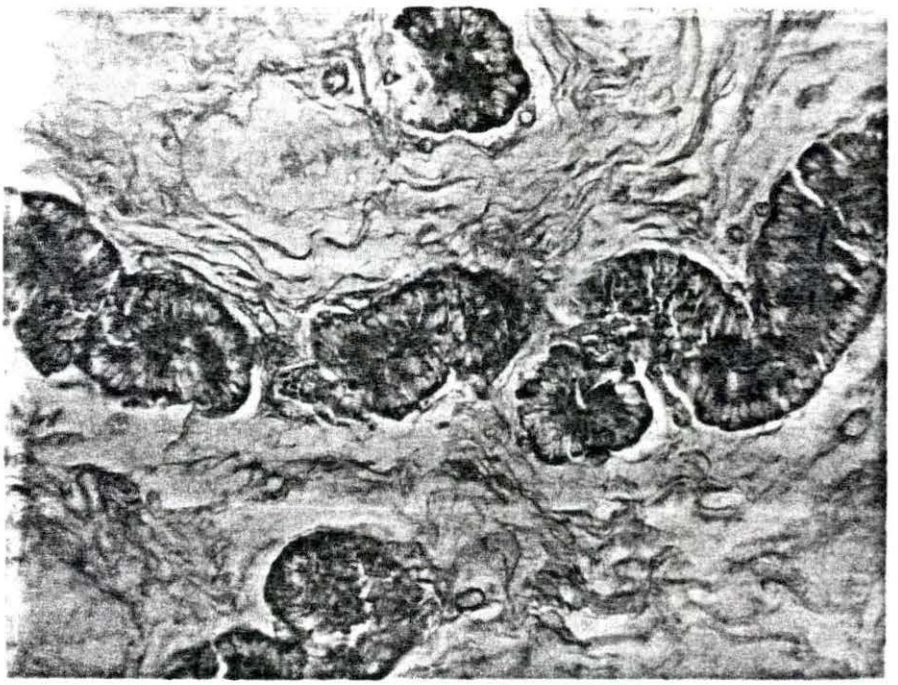


Figure 48. P.A.S. reaction of the basal glandular area of the endometrium of Bitch #11. This was a +++ reaction for P.A.S.-positive granules. 450x

Figure 49. A portion of the basal glandular area of the endometrium of Bitch #51 with a very large number (+++) of P.A.S.-positive granules. 900x



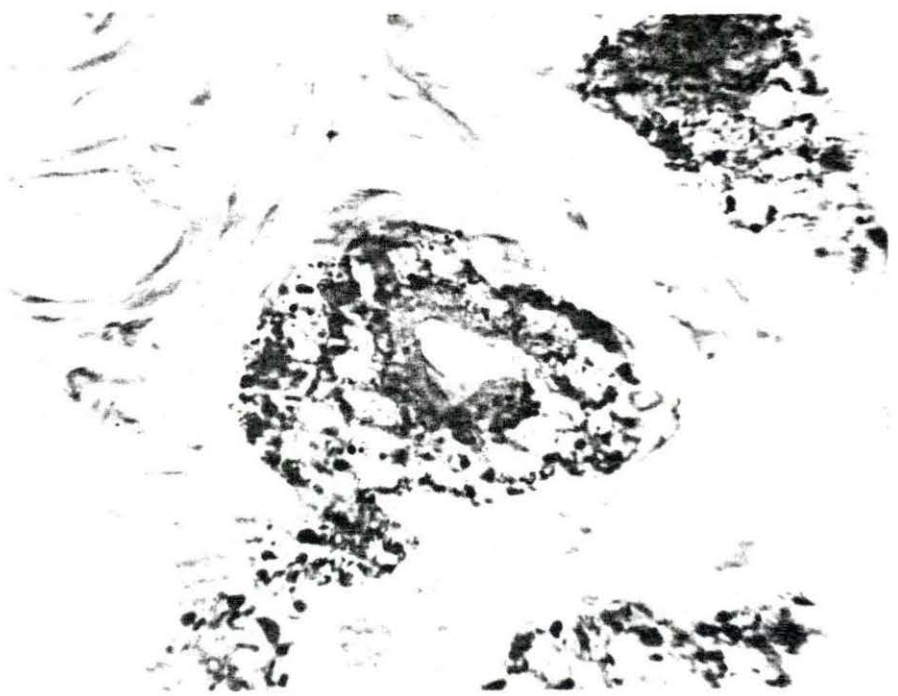
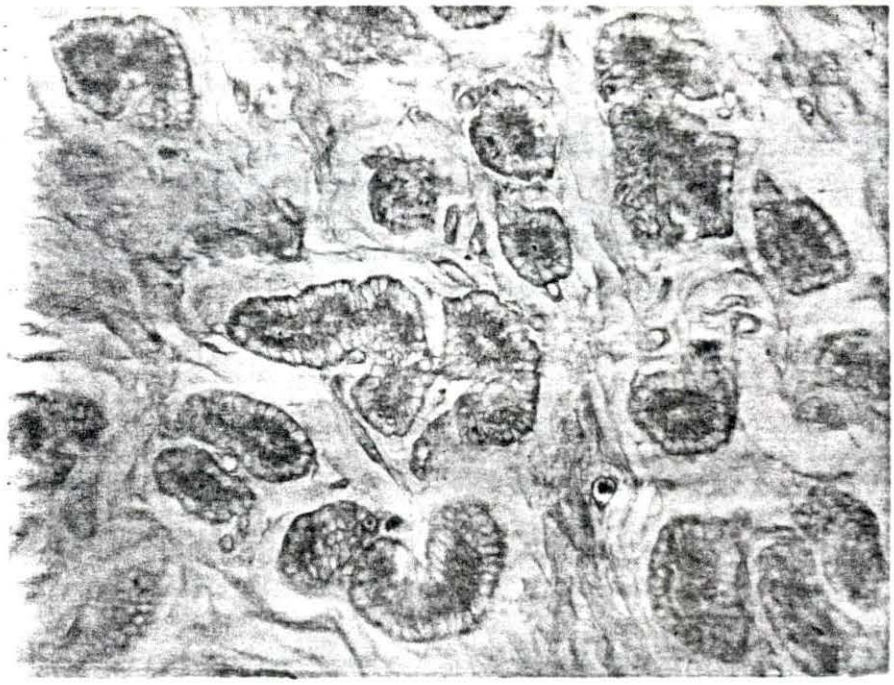


Figure 50. P.A.S. reaction of basal glandular area of the endometrium of Bitch #42. This was a negative reaction for P.A.S.-positive granules. 450x

Figure 51. P.A.S. reaction of basal glandular area of the endometrium of Bitch #31. This represents a + classification for the presence of P.A.S.-positive granules. Note P.A.S.-positive material in the lumina of many of the glands. 450x



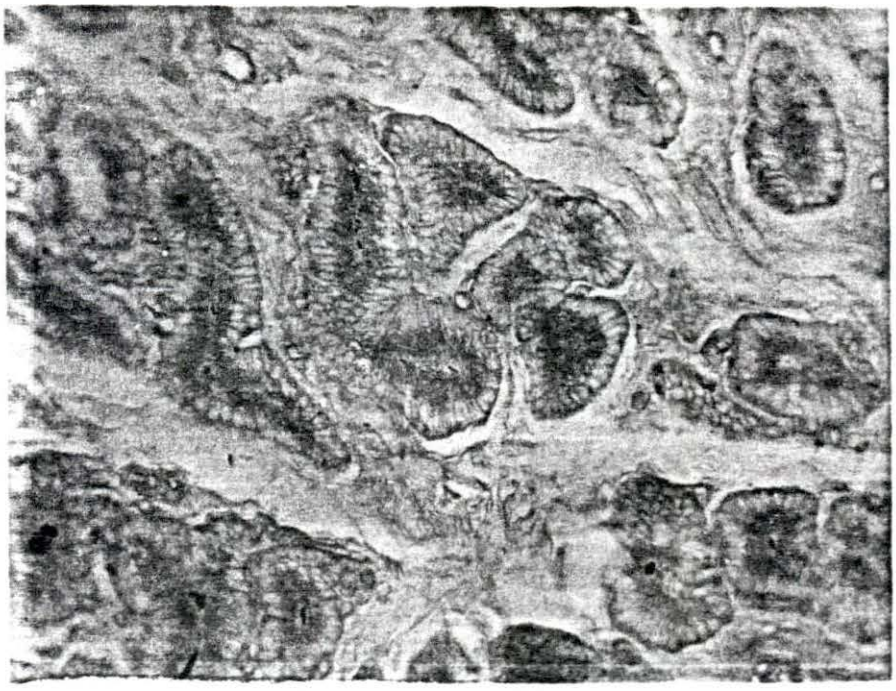


Figure 52. A + reaction for the presence of P.A.S.-positive granules in the epithelial cells of glandular tubules of the endometrium in Bitch #31. Note the presence of secretion in the lumina of the tubules. 450x

Figure 53. P.A.S. control slide for the above. Note that the P.A.S.-positive granules have disappeared following diastate incubation but the secretion in the lumina remains. 450x

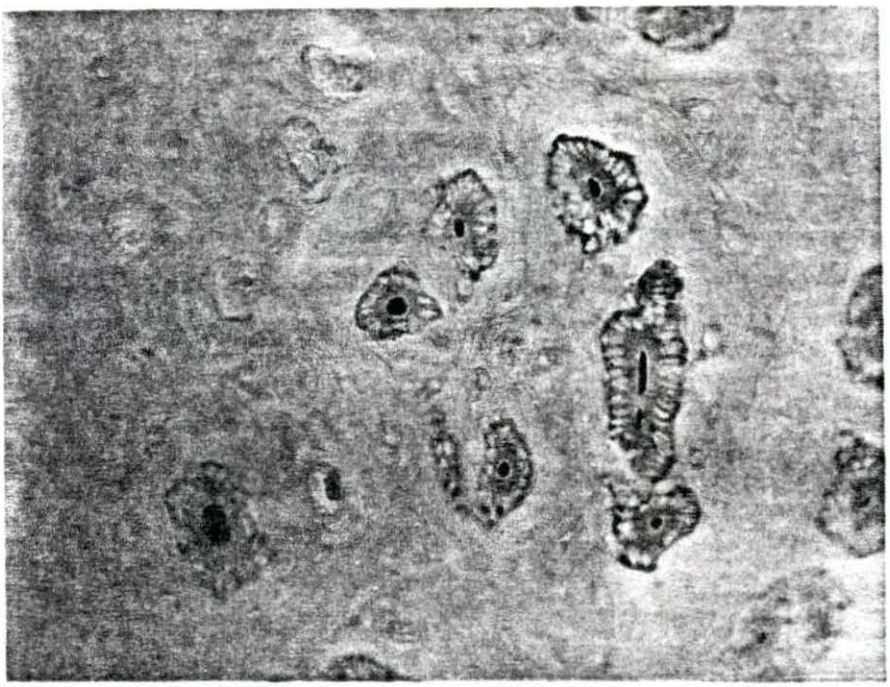
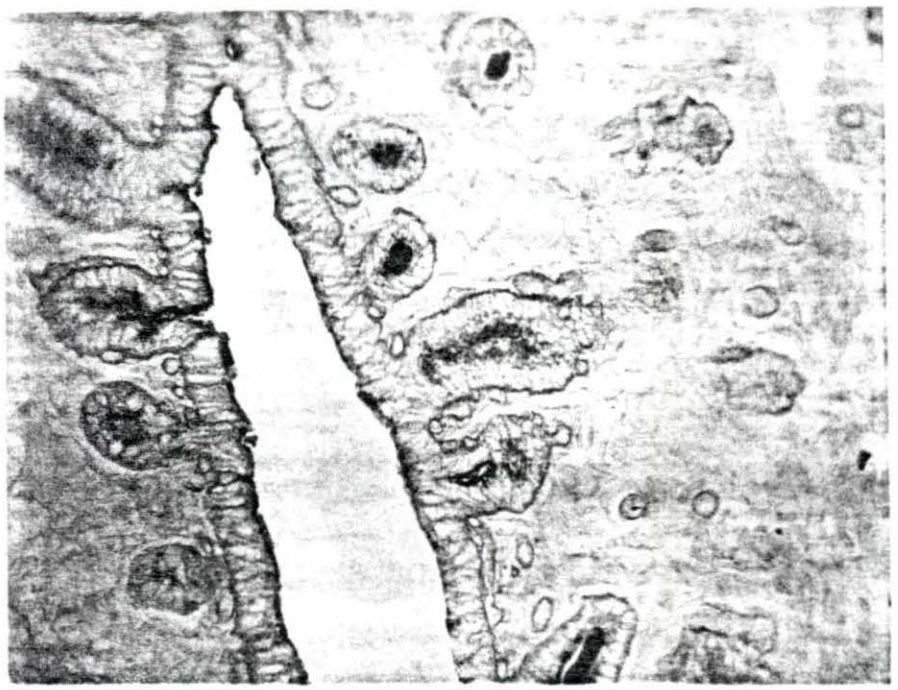


Figure 54. P.A.S. reaction of the luminal epithelium of the endometrium of Bitch #32. Note the dark P.A.S. reaction along the free poles of the epithelial cells and the small amounts of P.A.S.-positive granules in the luminal epithelium in the areas where the glandular crypts open. 450x

Figure 55. Alkaline phpsphatase reaction of the blood vessels in a corpus luteum of Bitch #11. Note the absence of alkaline phosphatase in the lutein cells. 450x



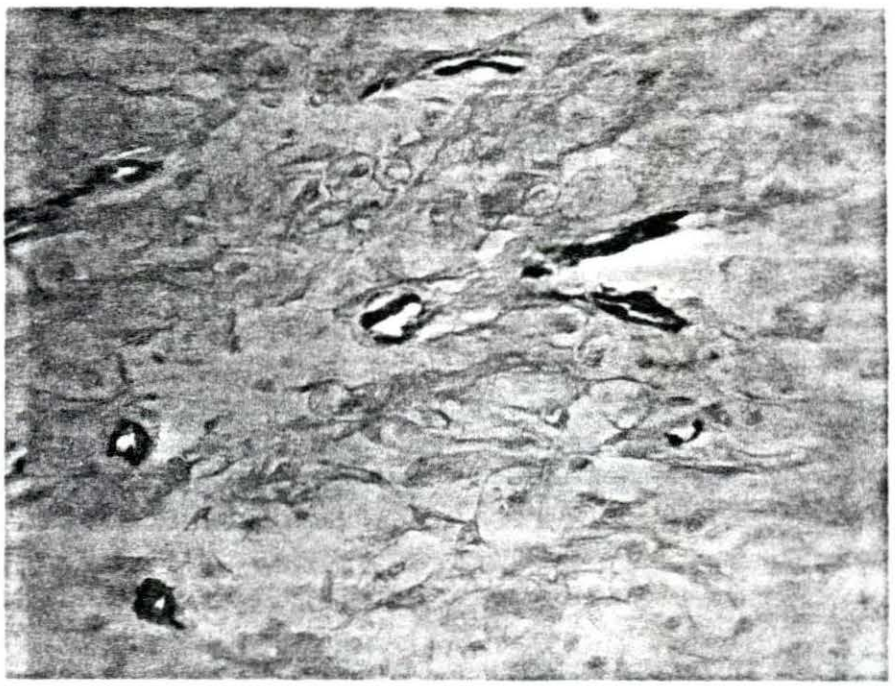
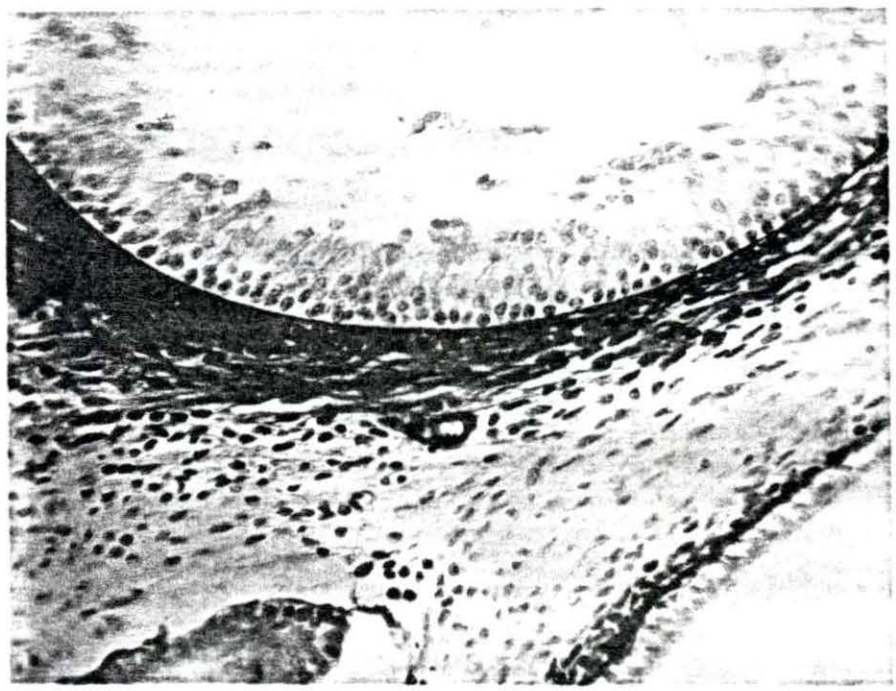


Figure 56. Alkaline phosphatase reaction in the ovary of Bitch #11 demonstrating the positive reaction of the theca interna cells of the follicle. 450x

Figure 57. Alkaline phosphatase control slide of above. This demonstrated that the reaction is due to the presence of enzyme and not native phosphate. 450x



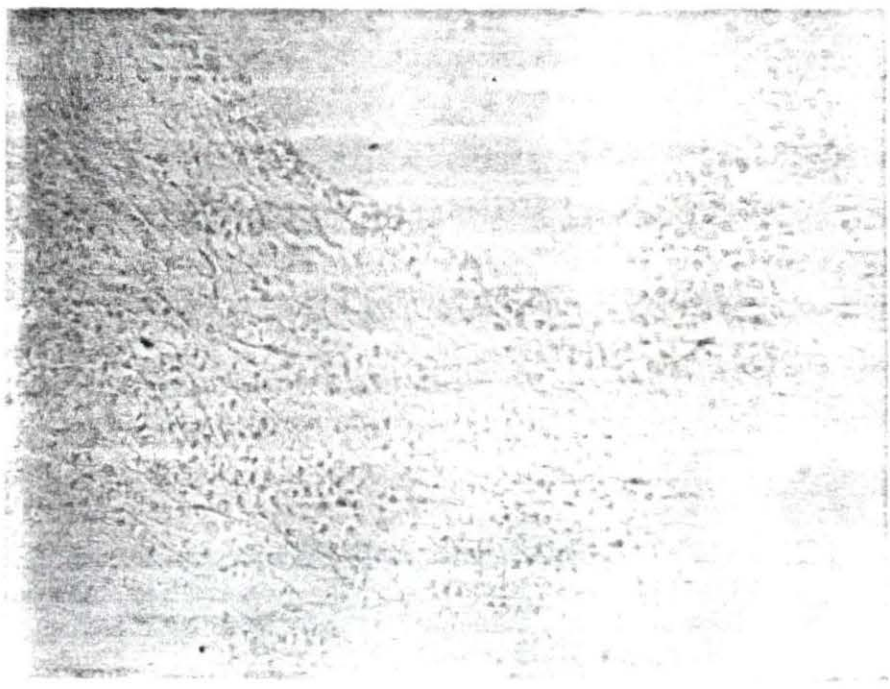
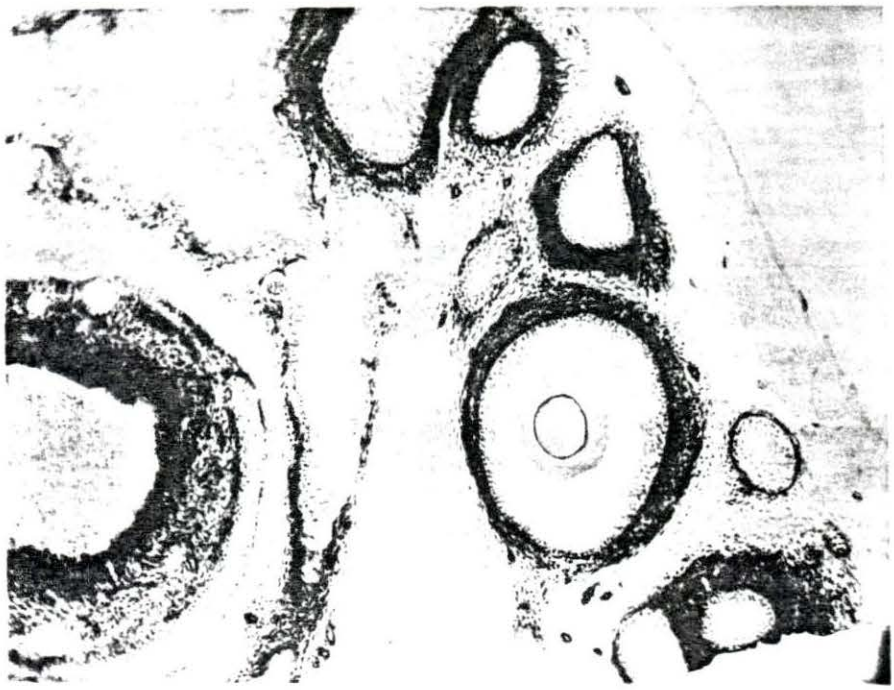


Figure 58. Alkaline phosphatase reaction in the ovary of Bitch #12 showing strong reaction of theca interna cells. 100x

Figure 59. Alkaline phosphatase reaction of oviduct epithelium of Bitch #51. Note the marked reaction along the free poles of the ciliated epithelial cells. 900x



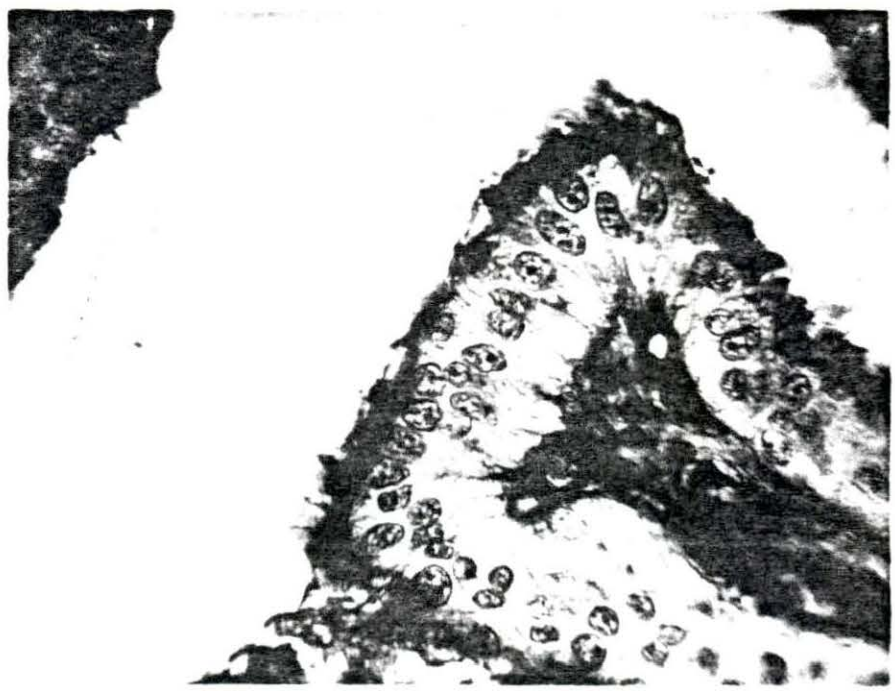
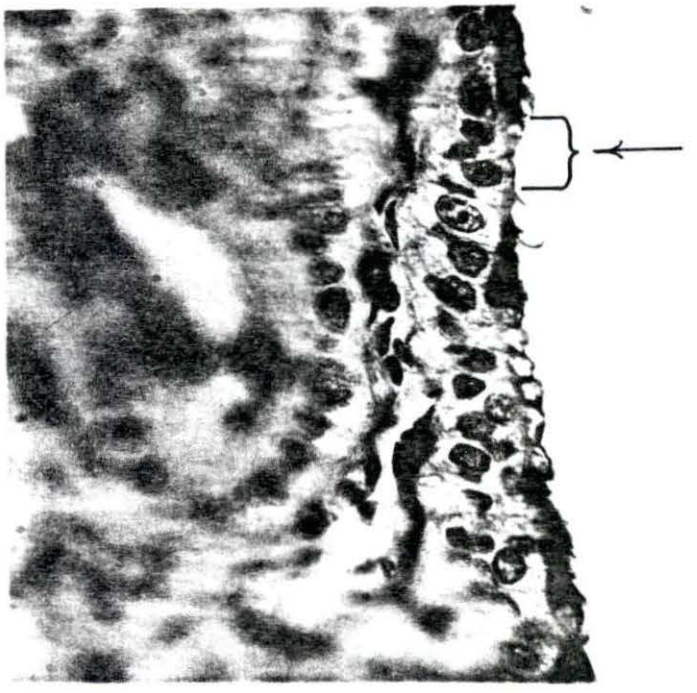


Figure 60. Alkaline phosphatase reaction of the oviduct epithelium of Bitch #21 demonstrating that most of the positive epithelial cells are ciliated. Note group of non-ciliated epithelial cells not containing alkaline phosphatase. 900x

Figure 61. Alkaline phosphatase of the oviduct epithelium of Bitch #52. Most of the ciliated epithelial cells contain alkaline phosphatase. 900x



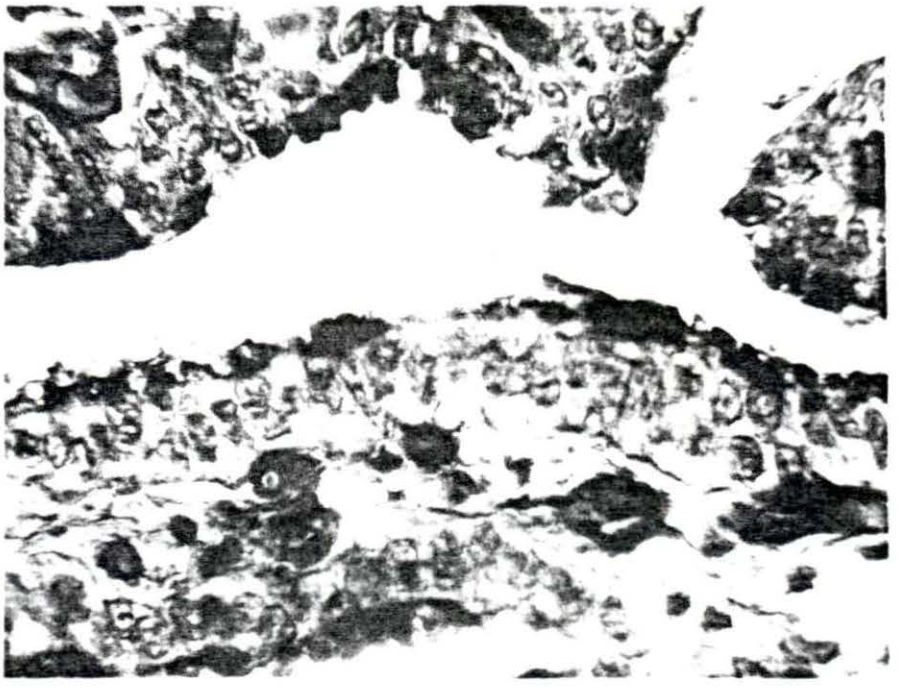
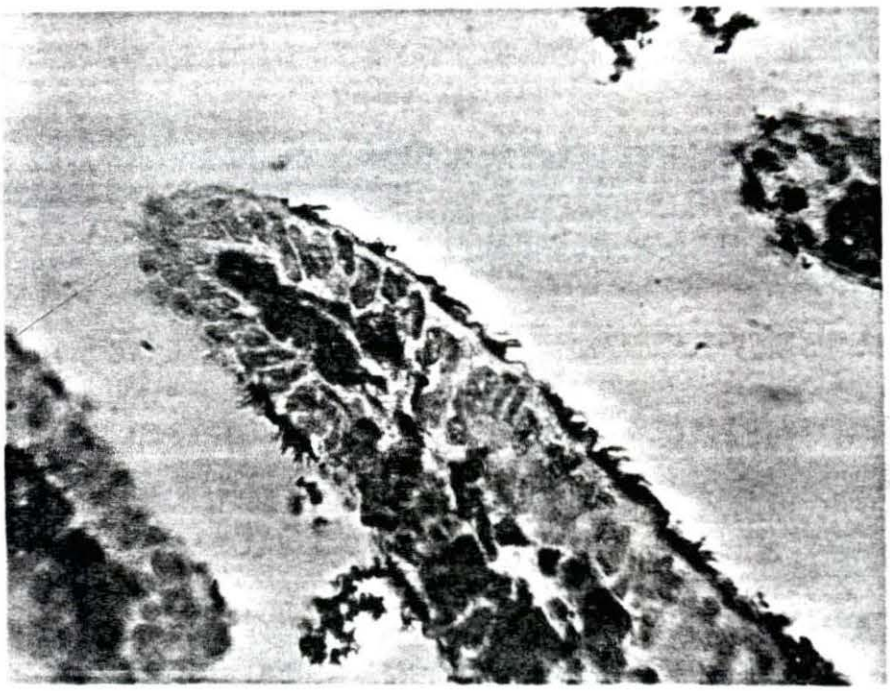


Figure 62. Alkaline phosphatase reaction of the oviduct epithelium of Bitch #31. Note that approximately 20% of the epithelial cells are ciliated. Alkaline phosphatase activity is limited to these ciliated cells. 900x

Figure 63. Alkaline phosphatase control slide of above demonstrating the reaction to be due to enzyme and not native phosphate. 900x



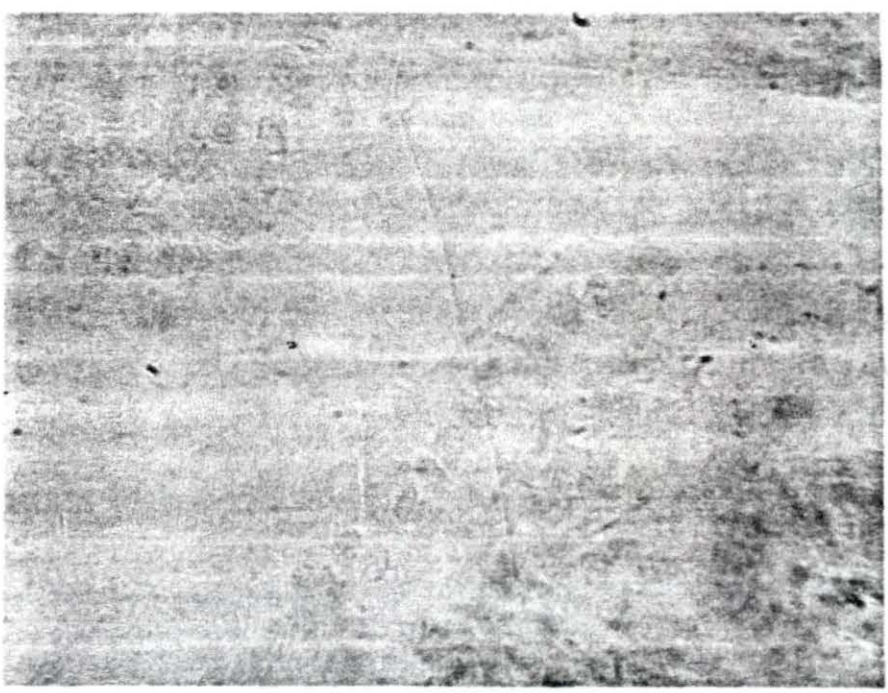


Figure 64. Alkaline phosphatase reaction (+) of the luminal epithelium of the endometrium of Bitch #51. Note that the reaction involves approximately the upper one-fourth of the epithelial cells. 450x

Figure 65. Alkaline phosphatase reaction (+) of the luminal epithelium of the endometrium of Bitch #41. Note that the reaction involves approximately the upper one-fourth of the epithelial cells. 450x

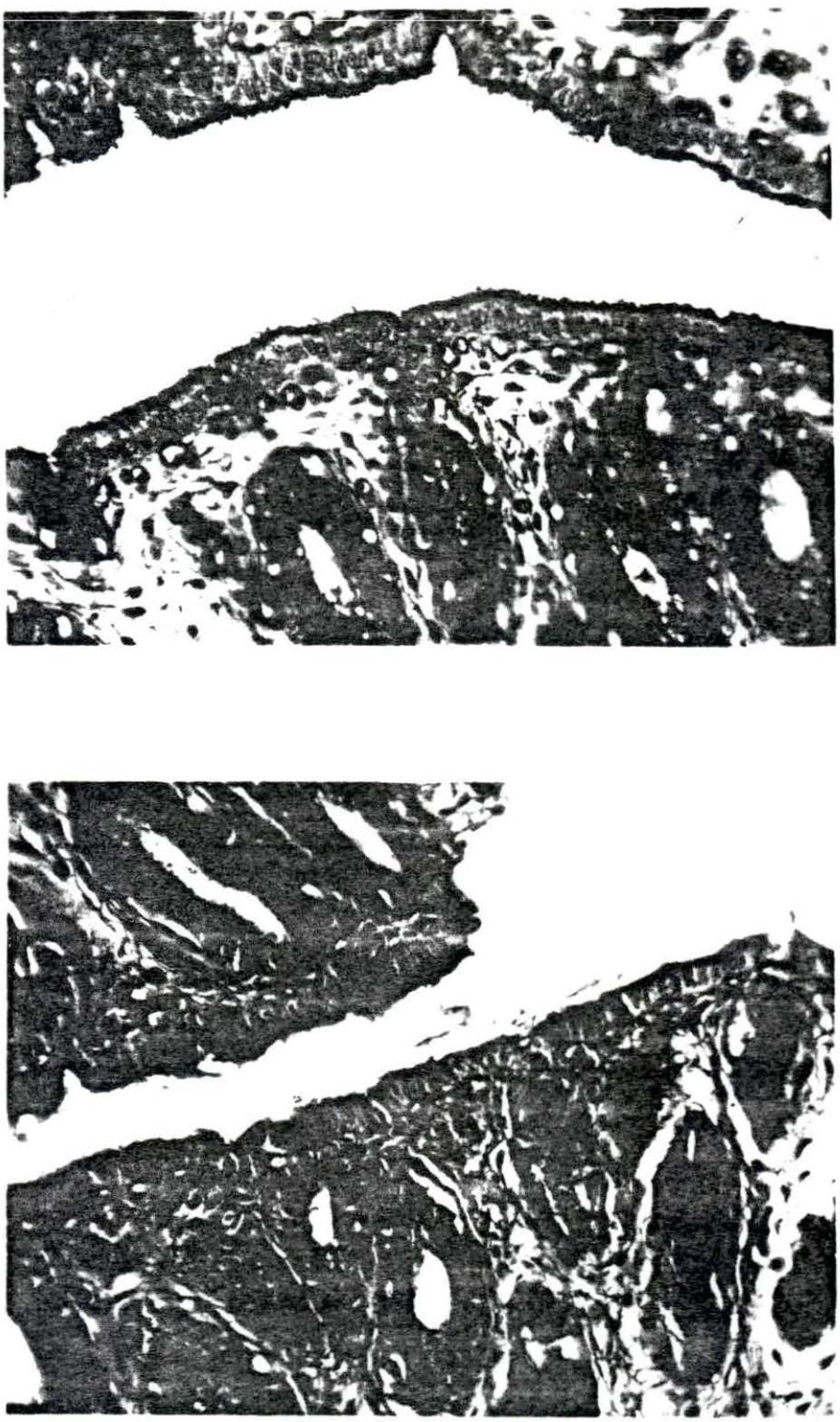
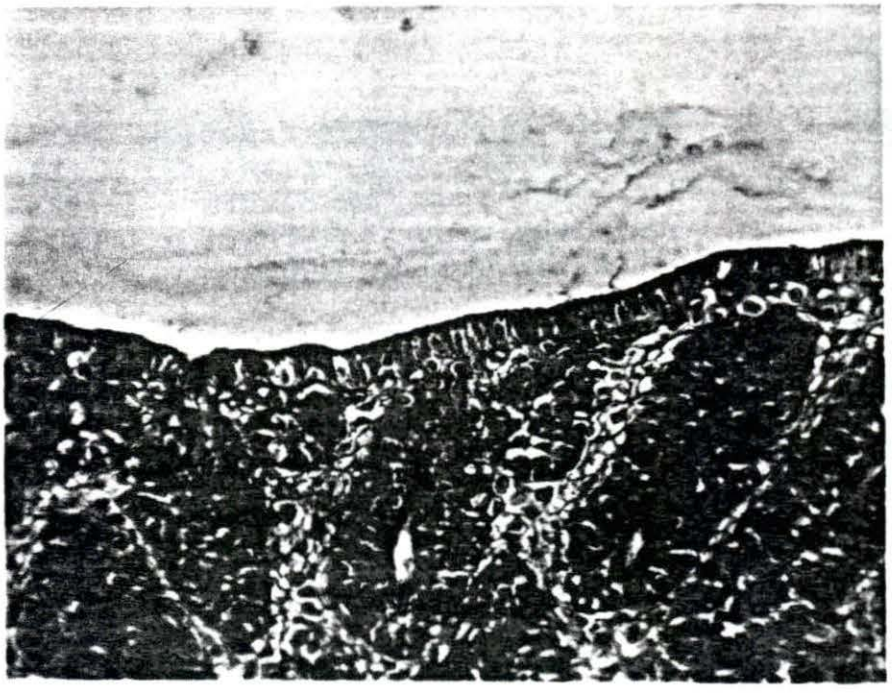


Figure 66. Alkaline phosphatase reaction (++) of the endometrial luminal epithelium of Bitch #21. Note that the reaction involves the upper one-third of the luminal epithelial cells. 450x

Figure 67. Alkaline phosphatase reaction (++++) of the luminal epithelium of the endometrium of Bitch #22. Note that the reaction involved the entire structure of the luminal epithelial cells. 450x



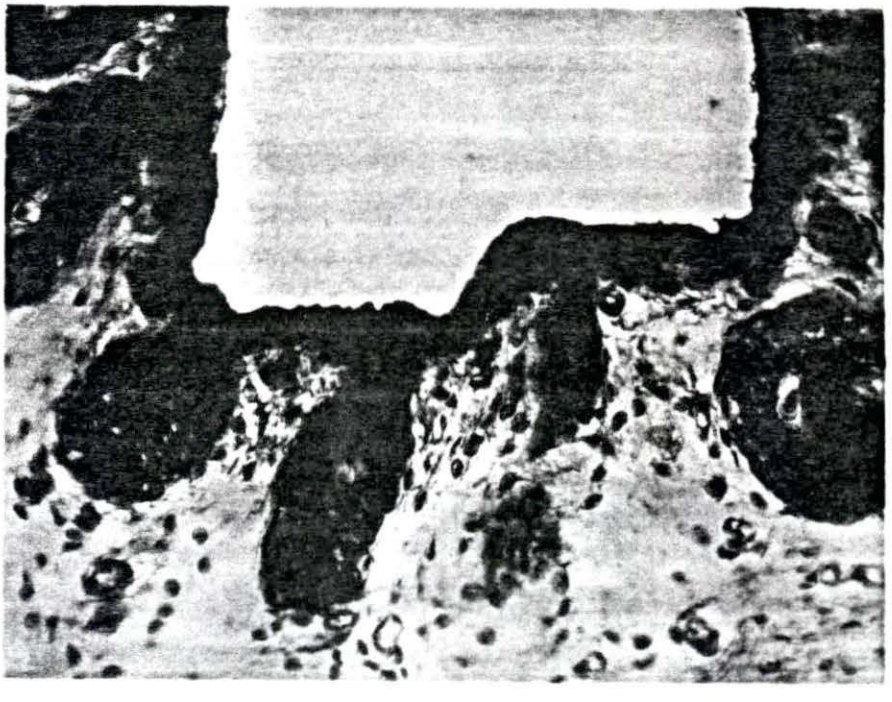
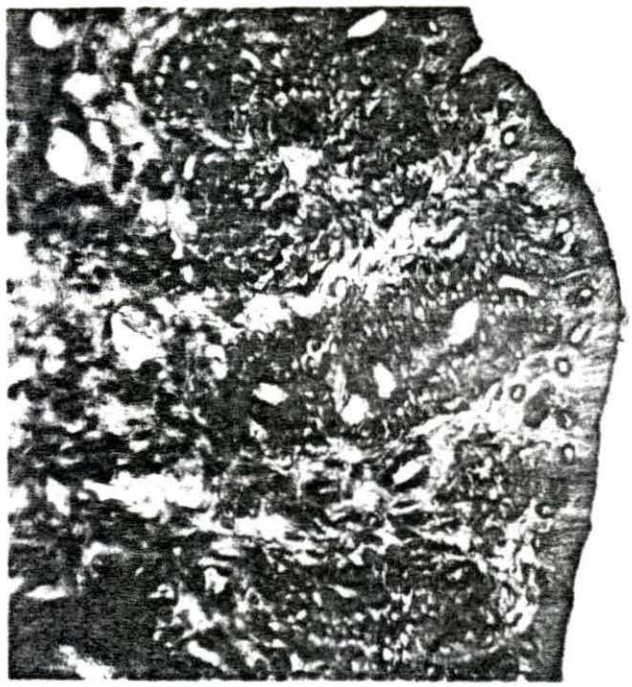


Figure 68. Alkaline phosphatase reaction (++) of the luminal epithelium of the endometrium of Bitch #31. Note that the reaction involves approximately the upper one-third of the luminal epithelial cells. 450x

Figure 69. Alkaline phosphatase control slide of above. Note the disappearance of the reaction from the luminal epithelium but the presence of dark material in the cryptal lumina and the inner poles of the cryptal epithelial cells. 450x



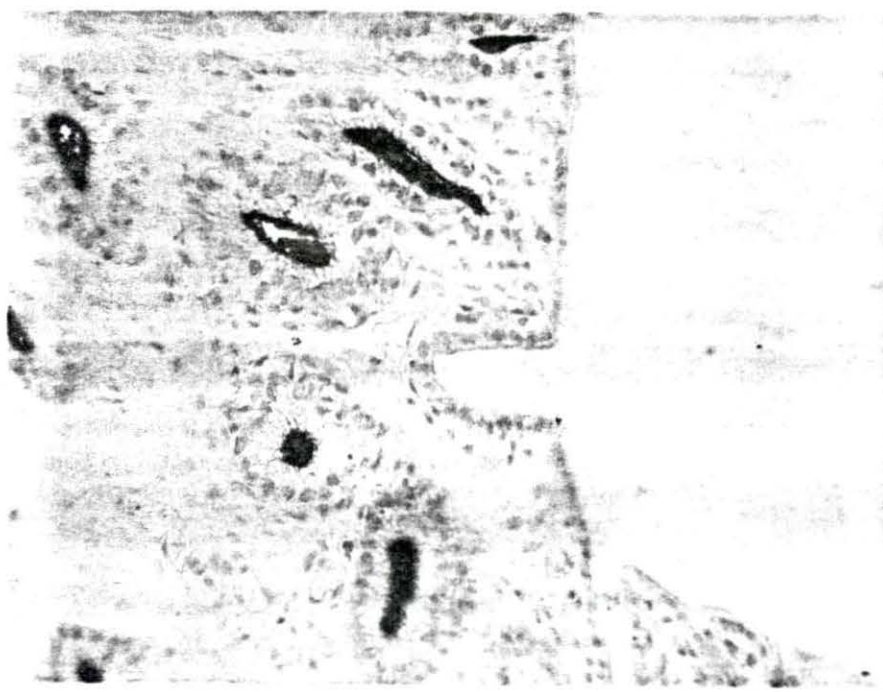
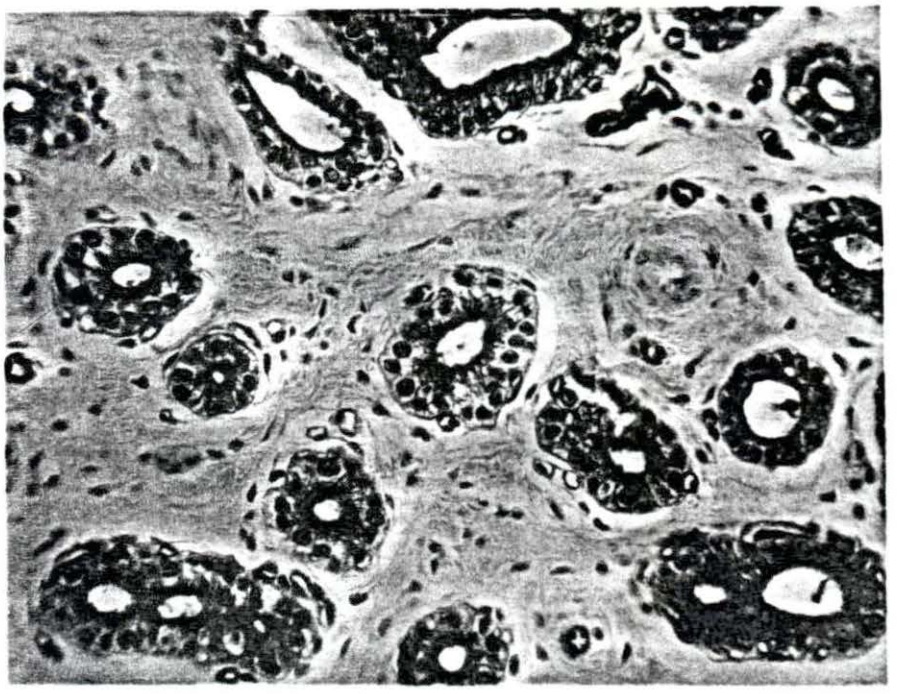


Figure 70. Alkaline phosphatase reaction (+) in the basal glandular epithelium in the endometrium of Bitch #51. 450x

Figure 71. Alkaline phosphatase control slide of above demonstrating the reaction to be due to the presence of enzyme. 450x



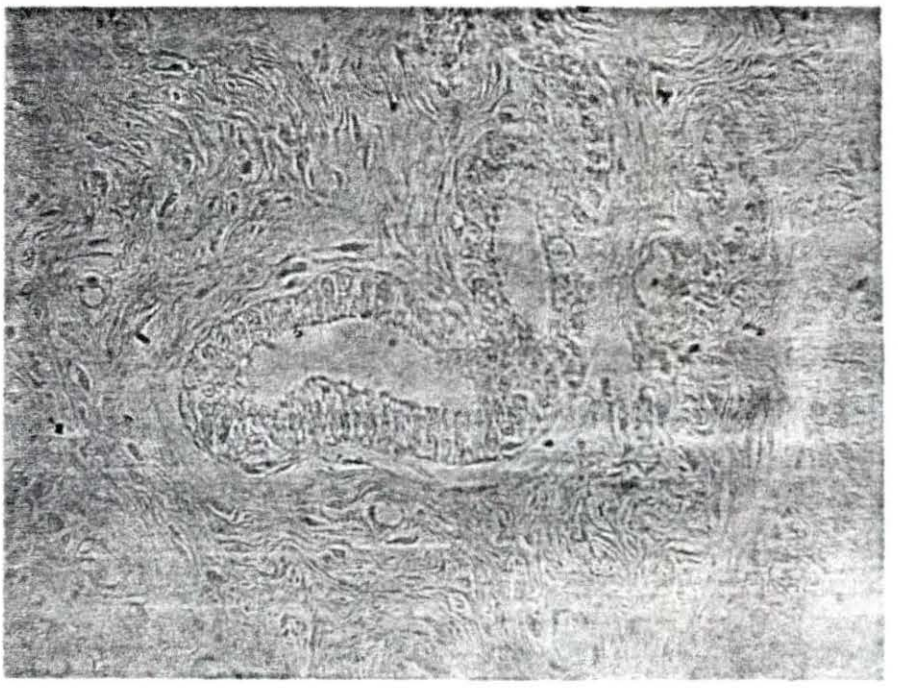


Figure 72. Alkaline phosphatase reaction (+++) in the epithelium of the endometrial glandular tubules of Bitch #21. 450x

Figure 73. Alkaline phosphatase control slide of the above demonstrating the reaction is not entirely from enzyme, but is also due to native phosphate material. 450x

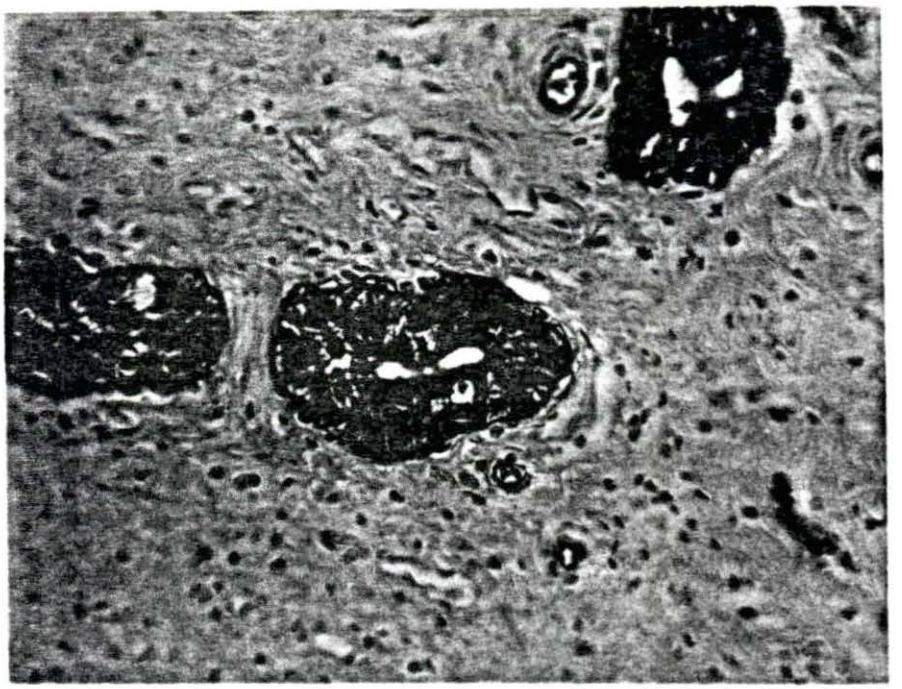


Figure 74. Alkaline phosphatase reaction (++) of the basal glandular epithelium in the uterus of Bitch #21. 450x

Figure 75. Alkaline phosphatase reaction (++++) of the basal glandular epithelium in the uterus of Bitch #22. 450x