

A STUDY OF THE FUNCTIONAL ANATOMY OF THE BOVINE CERVIX WITH
SPECIAL REFERENCE TO THE EPITHELIUM, MUCUS SECRETION AND
SPERM TRANSPORT

by

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(ABSTRACT)

Anatomical features of bovine cervical epithelium were investigated with respect to mucus secretion and sperm transport. Techniques included: 1. Surface staining of fixed tissue blocks in Harris' hematoxylin followed by stereomicroscopic examination, 2. Model construction from serial sections using both computer-aided and plexiglas assembly of epithelial tracings, 3. Histochemical investigation using five follicular phase animals (four bred naturally 8 to 12-h before slaughter) and three luteal phase animals. Cross sections of two samples from each quarter cervix were stained with (1) Alcian blue (AB) at pH 1.0 (sulfomucins) and at pH 2.5 (sialomucins and sulfomucins); (2) periodic acid Schiffs (PAS) (neutral mucins) and AB (pH 2.5); (4) high iron diamine (HID) (neutral and sulfomucins) with AB (pH 2.5). Additional samples were processed for ultrastructural examination. The mucosa was characterized by longitudinal primary and secondary folds which maintained continuity throughout the cervix, with numerous tertiary shallow 'grooves' apparent on all epithelial surfaces. No evidence was found suggesting blind ending glands or crypts. Staining results in follicular animals indicated a predominance of neutral and sulfated mucins in apical areas with secreted mucins extending as sheets from these areas toward the central canal. In basal areas (within grooves) sialomucin production was predominant with secreted mucins evident within grooves and between neutral mucin layers. In luteal phase animals, sulfated and neutral mucins were abundant in both basal and apical areas, while sialomucin production was decreased. Using light and transmission electron microscopy, spermatozoa observed within the cervix appeared

unidirectionally opposed to ciliary beat. Suggested privileged paths for the transport of viable spermatozoa are within grooves, where sialomucins were most predominant.

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Completion of degree requirements for the Master of Sciences marks a personal milestone. With this accomplishment I have gained a new sense of humility and increased awareness of the complex organization which exists at many levels within the world around us. The machinery functions properly at each level only when all the contributing factors are present. These might be a herd of cattle plus farm crew and manager, a pathway plus enzymes and substrates, or a cervix plus folds, grooves and mucins. The completion of a thesis is no exception. It requires the input and encouragement of all associated. I would first like to thank my major advisor, Dr. Richard Saacke, for providing the catalyst. Without his wisdom and encouragement the work would never have been initiated, and it is largely through his example of excellence and patient assistance in helping to chart the course that it has reached completion. Other members of my committee were the supporting structure, valuable and highly skilled in their areas of expertise and at the same time most enjoyable to work with.

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INTRODUCTION

The structure of the bovine cervix as it relates to function regarding mucus production and sperm transport has been considered in part by previous authors. However, due to a lack of agreement among anatomical descriptions of this organ, a clearly defined three-dimensional model of cervical structure has not been established. Similarly, histochemical investigations of mucus production by the bovine cervical epithelium have not provided sufficient information for a concensus among authors. Although there is a substantial literature base available on cervical mucus composition and secretion as well as in vitro studies on mucus/sperm interactions, the functional role of the cervix which provides the structure on and in which the mucus and sperm interact during the process of sperm transport, remains obscure.

In order to establish a valid pattern of mucus production as it occurs in response to hormonal stimulation, it is essential that the anatomical features of this organ first be clearly established. Once this structural model has been established as it occurs in three-dimensions and mucus secretion as it occurs within this structure, studies of sperm transport through the cervix can then be approached more rationally.

Understanding sperm transport through the cervix may provide a useful perspective in solving certain infertility problems which occur in mucus secreting species. This could include fertility failure due to inhibited sperm transport resulting from: 1) abnormal or inadequate mucus secretion, or 2) abnormal ciliary beat resulting in failure to establish adequate mucus flow patterns. Even though the cervix is routinely by-passed in the artificial insemination of dairy cattle, a better understanding of the response within this organ to the natural selection and transport of spermatozoa could provide useful information regarding the role of the cervix and potential consequences of circumventing this organ.

Considering the potential interrelationship of cervical anatomy, mucus production and sperm transport, the following objectives were set forth for this investigation: 1. To develop a three-dimensional model of the bovine cervix. 2. To examine mucoïd secretion within the cervix at the time of sperm transport and establish a secretory pattern of mucoïd types as they relate to this anatomical model, 3. To obtain information on the route(s) of sperm traversing the cervix in mated animals with respect to mucoïd epithelial characteristics.

REVIEW OF LITERATURE

1-1 Introduction

The cervix of bovine, as well as other mucus-secreting species in which semen deposition is vaginal, has been recognized as a barrier to sperm transport. This barrier is effected by interactions which occur between the intricacies of the anatomical structure and the pattern of mucoïd secretion which occurs therein. In order to more completely understand the role of the cervix in sperm transport, we must first gain an understanding of the structure of this organ as it occurs in three dimensions, and secondly determine the pattern of mucus production and flow within as it relates to the cervical structure.

1-2 Cervical Structure - an Historical Perspective in the Bovine

Gross anatomical features of the bovine cervix were presented in some detail as early as 1917 by W. L. Williams. He described the cervical mucosa as consisting of a system of complex longitudinal folds accompanied by circular reflections or annular rings projecting "vaginalwards" with a cervical canal that was extremely narrow and crooked, being "elaborately barracaded by these mucosal folds". Later descriptions of the fine structure of the mucosa have offered alternate views regarding the presence or absence of tubular glands. Hammond (1927) elaborated on hormonally induced changes observed in the bovine cervix and its secretory products at different stages of the Estrous cycle. He included a histological examination of the cervix and reported the existence of a single layer of epithelium which formed simple, sacculated glands. During the follicular stage these epithelial cells were distended with an accumulation of mucus in their apical regions, while very little secreted mucus was visible in luminal spaces. In contrast, during the luteal phase, cells appeared cuboidal with little accumulated apical mucus, but with abundant extracellular mucus filling the luminal spaces. Hammond postulated that a liquefaction of these secreted mucins must take place before estrus in order to allow the ascent of spermatozoa. Reference to the presence of tubular glands in the cervix was also made by Cole (1930). He described bovine cervical glands as tubular and branching within the mucosa, functioning as a mucus reservoir during early stages of pregnancy. During this stage, he described them as spherical or oval in form. However, Herrick (1951) expressed doubts as to the existence of tubular glands by stating, "due to the folding of the mucous membrane of the cervix, the epithelium is arranged so that it only appears to contain many branched tubular glands." He further described the mucosa as "thrown up throughout its length into primary, secondary, tertiary and often quaternary folds". More recent references to bovine cervical structure continue to lack a clear definition.

In 1966, Mattner again referred to the presence of tubular glands in the cervix which he found to function as a reservoir in the storage of viable sperm. However, in 1972(a), Hafez and Kanagawa in a scanning electron microscopic investigation of cervical structure described a system of mucosal "crypts" and folds which delve into the stroma. They suggested that these "crypts" had been incorrectly referred to as cervical glands in previous work, but failed to define the term "crypt" structurally. Reference to a system of "crypts" in the cervix is prevalent in other recent literature, with the crypt described only as a pocket of columnar epithelium. (Elstein, 1978). Whether this "pocket" is a continuous fold or a blind ending tunnel remains to be elucidated.

1-3 Cervical Structure in Related Species

Anatomical descriptions of the cervix of other mucus-secreting species are also prevalent in the literature. By far the most thoroughly investigated of these species would be the human (see Krantz, 1962 for a historical review). As in the case of the bovine literature, there has also been controversy regarding published descriptions of human cervical structure. Opposing views suggest either a system of compound branching tubular glands or an epithelial structure composed of clefts or grooves with "sinuous pits" or "open glands" (rather than tubular glands) in between (Fluhmann, 1957). The meaning of "sinuous pits" and "open glands" is not clear to this reviewer. In his description of cervical structure Fluhmann regards the term "crypt" as being synonymous with "groove" or "cleft". He also states that formations in the mucosa which have been accepted as cross sections of tubular glands should be considered as tangential cuts across irregular grooves between the folds. In 1958, using serially sectioned material, Fluhmann further demonstrated that the basic epithelial structure of the cervical mucosa was that of clefts or grooves which ran in either longitudinal, oblique or transverse directions. He

further described this structure as being accompanied by tunnels which originated within the folds and followed a direction parallel to the folds.

More recently, Singer (1976) investigated cervical structure and described a lobular arrangement of the mucosa, which he referred to as cervical villi, composing the basic epithelial subunit. He described these structures as ovoid, measuring about 0.15 x 1.5mm in diameter and separated from each other by "intervallis crypts". However, Singer (1976) did not define the structural nature of these crypts. Reference to the cervical crypt is, however, prevalent in much of the literature. While a clear definition of its structural nature (whether a blind ending tunnel or open groove) has not been clearly defined, a quantitative study of the number and size of cervical crypts in the human cervix has been attempted by two separate workers with quite different results. One report (Odeblad, 1966) suggested the existence of approximately 100 mucus-secreting gland-like units which he referred to as crypts. The other report (Bernstein et al 1977), using information obtained from serial cross sections taken from 30 consecutive regions of the cervix of estrogen-treated vs control (gestational) women estimated the existence of approximately 9,000 to 10,000 crypts per cervix with the greater number occurring in response to estrogen. It would appear from these variable results (Odeblad, 1966 vs. Bernstein et al, 1977) that the crypt, as a structural unit, lacks the definition necessary for precise quantitation.

Anatomical descriptions of the cervical mucosa of other domestic ruminants have also offered opposing views. The presence of simple tubular, or coiled glands has been described in the cervix of the goat (Dellmann 1968). These glands were described as an invagination and continuation of the deep furrows of the cervix into the lamina propria. Alternatively, Grau and Walter (1958), as cited in Dellmann (1968), interpreted the appearance of glands in histological sections of the goat cervix to be a consequence of the numerous folds of the mucosa and the spiral winding of the cervical canal.

Investigation of the gross anatomical structure of the ovine cervix resulted in it being described as having a highly convoluted surface with invaginations, which have been referred to as glands

(Mattner, 1969), or as a system of large longitudinal folds without evidence of coiled or tubular glands (More, 1984).

1-4 Secretory and Ciliated Cells of the Bovine Cervix

Histological and scanning electron microscopic investigations of the bovine cervical epithelium have revealed two types of columnar cells. (Marinov and Lovell, 1967; Wergin, 1979). The most abundant is the secretory cell, characterized by numerous granules which crowd the nuclei toward the basement membrane during the follicular phase of the Estrous cycle and possess microvilli on their luminal surface. Also present are non-secretory ciliated cells which, according to Marinov and Lovell (1967), comprise approximately 10% of the epithelial cells. Wordinger et al (1973) found no apparent regional differences as to the relative frequency of occurrence of ciliated and non-ciliated epithelial cells when they compared tissue samples from the anterior, middle and posterior cervical mucosa. However, Hafez and Kanagawa (1972a) reported a distinct variation in the relative occurrence of these cell types with 50%, 39% and 29% ciliated cells in the external os, midcervix and internal os, respectively.

Regarding the functional role of ciliated cells, Marinov and Lovell (1967) expressed doubts as to whether they occur in sufficient number to have any significant functional role. However, Hafez and Kanagawa (1972b) found ciliary beat to be in unison toward the vagina and suggested that these cells may function to create a directional flow of luminal fluids, or may facilitate the release of secretory granules adhering to the surface of adjacent secretory cells. Wordinger et al (1973) suggested that the cilia may function in assisting movement of mucus secreted by the non-ciliated cells. The function of the ciliated cells has thus been a topic of speculation, but as yet no proven function has been reported for these cells in this organ.

Variations apparent among secretory cell types regarding their secretory products will be discussed later in this review.

1-5 Mucus Structure

The secretory product generated by non-ciliated cells has also been a topic of investigation. Indeed, the structure of cervical mucus is uniquely complex, and its composition as influenced by hormonal regulation is essential to sperm transport. We must gain an understanding of this secretory product and its character before investigation of sperm transport through the cervix can be meaningfully understood.

Cervical mucus is composed of an aqueous phase and a relatively insoluble glycoprotein polymer, which varies in composition with respect to functional groups. Although both are important in the structure and function of the mucus, this review will direct most attention to the insoluble glycoprotein fraction. Nevertheless, some of the soluble components of the aqueous phase (including NaCl, lipids, fatty acids, prostaglandins, trace metals, serum proteins, enzymes and enzyme inhibitors, etc.) may be involved in providing for the structural integrity of the mucus by influencing its degree of hydration (as will be discussed further in a later part of this review). Other components of the aqueous phase may contribute to spermatozoal survival and/or capacitation (Daunter, 1984). The insoluble or glycoprotein phase is responsible for the rheological properties of mucus, often measured by factors such as viscosity, spinnbarkeit, flow elasticity, plasticity and tack (El-Banna and Hafez, 1972).

Glycoproteins from cervical, respiratory and gastric sources are known to share a common structure (Carlstedt and Sheehan, 1984). This consists of a protein backbone or core which is rich in serine, threonine and proline with a large number of oligosaccharide side chains linked

through O-glycosidic bonds (glycosylation). These side chains can be quite extensive, accounting for as much as 75 - 80% of the glycoprotein molecule (Chantler, 1981). The terminal residue in these side chains is often either sialic acid (sialomucins) or fucose (fucomucins). The concentration of these two terminal residues has been found to be present in a variable but inverse proportion, which may be a contributing factor to cyclic changes in physical properties of cervical mucus (Dische, 1963). Forstner et al (1981) suggested from work using fraction analysis of gastrointestinal mucins, that the sialated mucin species have, on the average, more short glycoprotein chains than non-sialated species. In addition, fucose-containing (neutral) mucins are more highly glycosylated with longer glycoprotein chains. Sialic acid-containing mucins add a negative charge to the structure due to the presence of a free terminal carboxyl group. It has been suggested that one way sialic acid may influence the structural integrity of the glycoprotein complex, is by its tendency to bind water (Daunter, 1984). This could help explain why the sialic acid content of bovine cervical mucus has been shown to increase in proportion to total protein content at proestrus, reaching a maximum at estrus when mucus hydration is also at a maximum (Hamana et al, 1969).

Additional negatively charged glycoproteins which carry a sulfate functional group (sulfomucins) are present among cervical as well as gastrointestinal and respiratory mucins. Due to their relative abundance in the gastrointestinal and respiratory tracts, sulfated mucins from these sources have been most thoroughly investigated. However, due to their structural similarity with cervical sulfomucins some consideration of this work will be included here.

In the gastrointestinal tract, sulfomucins accompany peptic inhibitory activity (Mikuni-Takagaki et al, 1979), thus serving in a protective role. In the human trachea, highly sulfated mucins are associated with stress and disease (Gallagher, 1981), and are also protective in nature. Bronchial washings of healthy human mucosa are, however, rich in sialated mucins but deficient in sulfated mucins, suggesting a protective role for the sulfomucins but a normal clearance function for the less viscous sialomucins. Increases in sulfomucins have also been observed in

cystic fibrosis patients. Mucins in these cases are highly viscous, often blocking epithelial lined ducts (Forstner et al., 1981).

In addition to these charged polyanionic or acidic mucins there are the neutral mucins, including fucomucins, which do not carry a net charge but have a vicinal hydroxyl functional group. There has been little speculation as to their role, although they may constitute a longer chain glycoprotein gel structure than exhibited by the acidic mucins (Gallagher, 1981). Acidic groups in the charged glycoproteins are thought to function by keeping these molecules expanded and capable of binding a very large domain of organized water (Bhushana Rao and Masson, 1977). Considering this as a possible basis for the estrogen effect (increased mucus hydration), Tam and Verdugo (1981) proposed a model of mucus hydration whereby a mucus macromolecular network, or gel composed of high molecular weight neutral mucins might behave as a semi-permeable membrane and prevent the polyionic mucins from diffusing to the outside of the gel. In this manner, they suggest a Donnan equilibrium may be established in which entrapped polyanionic mucins could generate the osmotic drive, while transepithelial movement of water and electrolytes regulate hydration of the mucus structure during estrogen stimulation. In experiments which would support this theory of hydration, using bovine cervical mucus, Tam and Verdugo (1981) found that luteal mucus could be hydrated as a polyionic gel using physiologic ranges of pH and NaCl concentration to become an estrous-like gel. On the contrary, estrous mucus exposed to these same pH and NaCl conditions showed no significant amount of additional swelling as a result of hydration.

1-6 Histochemical Investigation

Histochemical visualization and localization of these epithelial mucins can be accomplished by several techniques utilizing contrasting coloration of the physically similar, but chemically

different mucins. The usefulness of these dyes rely upon the availability of carboxyl groups (sialomucins) sulfate groups (sulfomucins) or vicinal hydroxyl groups of these complex carbohydrates. Mowry (1963) described a preferred scheme for the detection of these particular reactive groups in complex carbohydrates. These are as follows: 1.) for vicinal hydroxyl groups, the periodic acid - Schiff (PAS) reaction; 2.) for carboxyl groups, colloidal iron or alcian blue; 3.) for sulfate groups, metachromasia with toluidine blue-O and; 4.) for general survey of all mucins (especially valuable for epithelial mucins), either colloidal iron - PAS or alcian blue - PAS stains. This survey method (4) was also supported by Spicer (1963). He pointed out that a staining sequence which incorporates a basic dye such as alcian blue or colloidal iron followed by acid Schiff (PAS) allows for the differentiation of blue or purple staining acid mucins from red or magenta neutral mucins, which are non-basophilic components.

The alcian blue-PAS technique was investigated more recently by Jones and Reid (1973) with regard to the effect of pH on the alcian blue (AB) staining properties of sialomucins and sulphomucins. These workers found that in tissue sites known to contain sialomucins alone, no AB staining took place below pH 1.5. They further differentiated the sialomucins by distinguishing between those which were sialidase-sensitive, finding that these mucins stained only between pH 1.7 and 2.6, and those mucins which were sialidase-resistant, finding these mucins reactive at a slightly lower pH range between pH 1.5 and 2.6. Also identified were two types of sulphomucins. These included the most common one, [investigated by Jones and Reid (1973) in tracheal tissue] which was AB positive at all pH levels studied. The other, located in the canine submaxillary salivary gland, stained only at pH levels between 0.5 and 1.5. Therefore, at pH levels ranging from 1.5 to 2.6, AB specifically stains both acid glycoproteins (sialomucins and sulphomucins), while PAS reacts with the neutral glycoproteins. However, if AB is used at pH values below 1.5, no reactivity is observed with sialomucins, while sulphomucins stain blue.

Another technique which has been used in separating sulphomucins and sialomucins involves the use of high-iron diamine (HID) and AB (Pearse, 1985, Cook, 1972). When this

technique is employed, sulphated mucins stain black after the diamine reaction while all other acidic mucins (including sialomucins) stain blue after AB (used in this technique at pH 2.5).

1-7 Histochemical Differentiation Within Cervical

Anatomy

The occurrence of both sulfated and sialated mucins within the cervix of mucus-secreting ruminants (ewe, goat doe and cow) has been investigated histochemically (Wordinger et al, 1972; Hafez et al, 1971; Heydon and Adams, 1979). Hafez et al (1971), using histochemical techniques on paraffin-sectioned tissue, defined 7 different secretory cell types in both the rabbit and bovine cervix, with an additional non-secretory, ciliated cell described in the rabbit. However, they erroneously failed to differentiate ciliated cells in the bovine cervix. Secretory cell types included variations of both "granulated" and "vesicular" epithelial cells. All cell types were found to be present at each reproductive stage studied. This included cows at estrus and 16, 72, and 168 hrs post-ovulation. Secretory granules within the granular epithelial cell types reportedly contained acid mucopolysaccharides, but neither glycogen nor hyaluronic acid were detected. These cells were positive to both PAS and AB (pH of AB not indicated). Vesiculated (non granular) cells, however, contained PAS-reactive homogenous material, with a granular core and "outer zone" which was also AB reactive.

In an investigation of the histology and carbohydrate histochemistry of the bovine cervical mucosa, Wordinger et al (1972) considered the effect of treatment with melengestrol acetate (MGA) for 14 days, starting at day 4 of the Estrous cycle. All experimental animals were slaughtered 3 days following breeding which was carried out at the time of the induced estrus. They described only two cell types: ciliated and non-ciliated columnar cells (as previously de-

scribed in this review) and reported the presence of both sulfated and carboxylic acid mucosubstances in both intracellular regions and extracellular luminal spaces. MGA treatment had no effect on the types of mucosubstances present relative to control animals which were not fed MGA to induce estrus. However, treated animals did present an increase in cell height and cells contained more mucus than was present in control animals. Wordinger et al (1972) reported a regional staining difference in their histochemical investigation. Using an Aldehyde-Fuchsin - Alcian Blue technique, they found cells lining the folds of the cervix to contain bluish-purple stored mucins, indicating the presence of both sulfated and carboxylic acid mucosubstances. Cells in the base of the crypts, however, contained mostly alcian blue positive material, indicating a predominance of non-sulfated carboxylic acid mucins, with ciliated cells devoid of stained material. Trapped, secreted mucins were found to stain identically to mucins within the apical portion of epithelial cells and, therefore, included a combination of both sulfated and carboxylic acid mucins. Likewise, using the high iron diamine (HID) staining technique, more concentrated HID staining within cells lining the folds was found as opposed to cells lining the crypts. However, using the PAS-AB(pH 2.5) technique they found no difference in staining pattern between these two regions (Wordinger et al, 1972).

These regional differences in mucus secretion were investigated further in 1979 by Heyden and Adams. They compared the morphology and mucus histochemistry of the ruminant cervix (cow, goat and ewe) at estrus. The effects of estrogen on the cervical epithelium was investigated in this study using ovariectomized ewes, with and without estradiol benzoate replacement. In all species studied, these workers found the secretory cell at the base of mucosal folds, or secondary indentations to be more numerous and contain more mucus than did cells in regions closest to the lumen. However, cells in all parts of the folds contained only small amounts of mucin in ovariectomized ewes. Those mucins which were present stained positive for HID and densely with AB at pH 1.0, indicating a predominance of sulfomucin in the absence of estrogen. When ovariectomized animals were given estradiol replacement, cervical epithelial cells were taller than comparable cells in untreated ewes, and mucus present in the

apical portions of these cells showed an absence of stain with HID and were less densely stained with AB at pH 1.0. However, a strong positive stain was observed, with AB at pH 2.5, indicating a production of sialomucin in response to estrogenic stimulation. In support of the regional staining differences reported by Wordinger et al (1972), Heyden and Adams (1979) also reported histochemical evidence based on HID and AB staining techniques for the production of sulfomucins from those parts of the cervical folds closest to the lumen, while cells in the crypts or secondary indentations which contained more abundant mucus, stained positive for sialomucins, based on metachromatic staining with azure A and toluidine blue or AB at pH 2.5 or at pH 1.0.

1-8 Histochemical Investigation in Related Tissue

Studies of cell surface coats and of mucus-producing tissues of the respiratory tract have involved the use of a cation such as alcian blue or cetylpyridinium chloride (among others). Along with these gluteraldehyde fixation was used to precipitate and stabilize the acid glycoproteins rendering them susceptible to crosslinking by the fixative (Behnke and Zelander, 1970). The preservation of acidic mucus glycoproteins secreted in the isthmus of the rabbit oviduct was addressed by Jansen and Bajpai (1982). They chose a method of perfusion fixation which included alcian blue in gluteraldehyde. They were successful in preserving mucins both within cellular secretory granules and secreted products within the luminal areas. These secretory products often appeared to be intimately associated with the cilia. Micrographs of cells within the isthmus revealed numerous secretory granules which they described as electron-lucent. Histochemical investigation of adjacent sections revealed that these granules were AB positive at pH 1.0, indicating that these electron-lucent granules were acidic sulfomucins. Micrographs from regions of the ampullary-isthmus junction and the ampulla revealed an absence of the electron-lucent granules but contained, in smaller numbers, electron dense, homogeneous granules which did

not stain (in adjacent thick sections) with AB at pH 1.0. Histochemical staining in these areas revealed a near absence of all cytoplasmic staining except for some cells in the ampullary- isthmus junction which stained positive for sialyated mucins. The cilia in both areas, which have a sialyated glycoprotein coat, stained AB positive at pH 2.5 only.

1-9 Mucus Secretion and Sperm Transport

The secretory pattern of mucins within the cervical structure has been investigated in humans by Hoglund and Odeblad (1977) and Odeblad (1978). Odeblad described a "dynamic mosaic" model consisting of three mucin fractions arranged within the cervical structure. This study was based on 326 samples of ovulatory mucus collected from single crypts, and examined using nuclear magnetic resonance (NMR) techniques. These three mucins were : 1. a viscous fraction labeled E1, for estrogenic "loaves" and comprising 70 - 80% of the total mid-cycle mucus; 2. a low viscosity fraction labeled Es for "estrogenic strings", and comprising 15 - 25% of total mucins. This fraction appeared to flow between the loaves of E1 mucus and was found to permit entrance and propagation of sperm much more readily than E1 mucus and 3.) G or gestagenic mucus, the most viscous fraction and comprising only a small percentage of the total in the estrogenic phase of the cycle. Unfortunately, Odeblad did not attempt to define the chemical nature of these mucin fractions regarding their acidic or neutral properties. In physical terms, Hoglund and Odeblad (1977) described the Es fraction to be composed of 50 to 200 micelles in parallel with wide intermicellar spaces for sperm advance. The E1 fraction, however, did not have a pronounced micellar structure and was considerably less receptive to sperm. They further reported that mucus was relatively more abundant in the proximal than in the distal part of the cervical canal and suggested that the Es fraction formed "highways" by which spermatozoa might advance toward the cervical crypts, while being mechanically and thermodynamically supported by the E1 fraction.

A similar concept for the secretion of mucus and sperm transport within the cervix of the domestic ruminants has been proposed by Mattner (1966). He suggested that the alignment of mucus micelles along lines of strain, originating from the mucosa and passing caudally to the anterior vagina, established "privileged paths" by which spermatozoa, gaining entrance into the secreted mucus are compelled to progress toward the cervical epithelium. On examination of cross-sectioned bovine cervical tissue which had been previously flushed with a saline solution, Mattner found large numbers of spermatozoa remaining in areas he referred to as cervical "glands". In this manner, he suggests, a sperm reservoir is formed within the mucosa from which migration of spermatozoa to the uterus can continue for a prolonged period after mating. In additional work on the distribution of spermatozoa and leucocytes in the genital tract in cows and goats, Mattner (1968) found a majority of leucocytes to occur in the central mass of the mucus, with very few apparent between the folds or within the glands. However, spermatozoa tended to be aggregated in the vicinity of the cervical mucosa as previously reported (Mattner, 1966). This orientation of sperm results in a separation of sperm and leucocytes which he suggests may be important to the survival of a sperm population. Mattner's theory of sperm orientation toward the mucosa is supported by Tampion and Gibbons (1962), who found that spermatozoa traveling in a thread of mucus are preferentially oriented in the direction in which the thread has been drawn out.

1-10 Transcervical Sperm Migration

The possibility that cervical mucus as it is secreted and aligned in vivo plays an active rather than passive role in the transport of sperm has been considered by several authors (Odeblad, 1962; Tampion and Gibbons, 1962; Mattner, 1966; Davajan et. al, 1970; Hogland and Odeblad, 1977; Katz and Overstreet, 1981). Odeblad (1962) estimated the swimming rate of human sperm in midcycle mucus to be about $0.005\mu\text{m}/\text{sec}$. (based on a mucus viscosity measurement of about

10 poise). This implies a period of several weeks would be required for transcervical sperm migration based on their own motility rates in this media. However, using nuclear magnetic resonance techniques, he found that in fact a much lower viscosity, about 0.03 poise, was apparent between macromolecular chains, indicating that the internal mobility of water molecules was close to that of free water. This concept of free-water mobility within the glycoprotein structure was supported by Katz and Singer (1978). Considering the flexible nature of the macromolecular network and the free water mobility within its structure, Odeblad (1962) developed a model of sperm transport which suggested that micellar segments within the network may function as a harmonic oscillator. Between these oscillating chains, Odeblad suggests, spindle shaped cavities filled with a low viscosity aqueous fraction may then be created. He presented a mathematical model in support of this concept which would utilize the thermal energy present at body temperature. It is within the spindle shaped aqueous cavities created between oscillating macromolecular chains, Odeblad suggests, that sperm which possess the appropriate morphology and motility may be propagated through cervical mucus structure at minimal energy expense. Non motile particles, however, are removed with the bulk mucus flow. Although this concept of sperm transport and the energy requirements of such a system remain to be clearly defined, it is presented as a model which may help to explain sperm transport within the complex mucus structure.

In a review of spermatozoan transport in cervical mucus, Davajan (1970) addressed Odeblad's model. He suggested that an energy source for maintaining macromolecular oscillations, rather than purely thermal agitation, as suggested by Odeblad, could be supplied by cervical cilia which beat in a propagating manner toward the vagina (Hafez and Kanagawa, 1972b). These cilia could consequently serve to maintain a consistent oscillation frequency throughout the cervical canal. Davajan (1970) pointed out that ciliary action has been shown to propagate mucus in respiratory tissues at average rates of 1-7mm/min, which has been shown to be sufficient to maintain phase separation of high and low viscosity fractions (Quinlan et al, 1969 as reviewed in Davajan et al, 1970) in macromolecular solutions. Cervical cilia, he suggests,

could provide the necessary stress required for micelles within the cervix to similarly align in a linear manner and, due to these forces, a system of mucoid structure with similar phase separation could be maintained. Within this structure, channels of low viscosity mucus, which may then allow for directed sperm migration or perhaps propagation could be formed. As sperm have been observed to swim against the stream (Zinner et al., 1982) it would seem reasonable that cilia may establish a flow pattern within the mucus structure which could function in the orientation of migrating spermatozoa.

The importance of considering the mechanism of sperm transport as an in vivo study is expressed by Katz and Overstreet (1982). These authors suggest that careful attention must be paid not only to the forces that propel spermatozoa, but also to the resistance they must overcome. These include surface-surface interactions between spermatozoa and the mucus microstructure, as well as sperm - epithelium interactions. These forces are established as spermatozoa travel within the narrow interstices of the mucus microstructure, in close proximity to the macromolecules. Direct physical as well as hydrodynamic interactions are therefore substantial. In addition, electrostatic repulsions between the negatively charged sperm plasma membrane and acidic mucus glycoproteins are also probably involved (Katz and Overstreet, 1982).

The possible effects of sperm - epithelium interactions have been considered in greater depth by Katz et al. (1975). They found in a mathematical fluid dynamics survey that the presence of nearby walls has a significant shear influence on the propulsive velocity of "slender bodies" such as spermatozoa. This influence was found to exist when the wall separation was less than the length of the sperm.

A more complete understanding of sperm transport through the cervix must take into consideration all of these complex factors, including physical and chemical components of the reproductive fluids and how they relate to a concept of the structural anatomy of the cervix which must be more clearly defined.

MATERIALS AND METHODS

Structural Investigation

For structural and anatomical purposes, intact cervices were obtained from 11 animals representing follicular (2 cows and 2 heifers) and luteal stages (4 cows and 3 heifers). The follicular phase was determined by the presence of a mature Graafian follicle as well as estrus mucus and hyperemia of the vulvar region. For the luteal phase, animals with a mature corpus luteum were chosen.

2-1 Surface Staining

The cervix was excised from the reproductive organs within fifteen minutes following a stunning blow at slaughter and the entire length of the cervical canal was exposed with a longitudinal cut to ensure rapid fixation. Cervices were fixed in Bouin's solution for 72 h, rinsed with three changes of 70% ethanol at 24-h intervals, and stored in the final ethanol solution. Each fixed cervix was divided into halves by cutting along the base of a major longitudinal fold.

The cervical epithelium of one half of each cervix was surface-stained by total immersion in Harris' hematoxylin for one to three minutes followed by a tap-water rinse and bluing in a mild ammonia solution (2 drops concentrated ammonium hydroxide/100 ml water) to increase contrast of surface features. Portions of the other half cervix were then cross cut, perpendicular to the longitudinal axis and similarly stained by immersion in Harris' hematoxylin. This procedure allowed visualization of the "crypt" formations as they appear in cross section. Prepared tissue blocks from representative areas in three of these cervixes (2 follicular and one luteal) were photographed (while immersed in 70% ethanol to prevent glare) in the range of 3X to 6.5X using a Wild M400 Photomakroskop.

2-2 Computer-Aided Spacial Reconstruction

A computer-aided reconstruction of serially sectioned tissue from one animal was conducted to determine the presence or absence of blind crypts, tubular glands, or tunnels within the folds of the mucosa. The procedure utilized for spacial reconstruction was modified for light microscopy of serial paraffin sections from Dylewski et al. (1984), who studied the microanatomy of the Golgi apparatus using serially sectioned electron micrographs. This was accomplished by taking 25 cross sections (5 μ m) from paraffin-embedded cervical tissue at approximately 20 μ m intervals through a mid-cervical region. After staining sections with hematoxylin to differentiate the epithelial cells, photographs were made of each section at approximately 6.5X and subsequently printed as 8x10 (20.3 x 25.4 cm) enlargements for a final magnification of approximately 60X. Tracings of the epithelial conformation were made on clear 8 1/2" X 11" (21.6 x 28.0 cm) acetate sheets which were then placed in proper alignment with respect to the X and Y axes. A pair of fudicial marks was placed on each overlay in the series by driving a pin through the stack of carefully aligned sequential sections midway on the left and right margins. Using these fudicial points as a guide for proper alignment, tracings were entered into a VAX 11/780 minicomputer (Digital Equipment Corp.) with the aid of a VICOM

digital image processor. The computer was then instructed to display the reconstructed image on a video display system, and to rotate the image about the X, Y, and Z axes. Stereo pairs were generated in order to facilitate three-dimensional visualization by rotating an alternate image angle about the X axis by 7° (Dylewski et al. 1984). See Appendix (A) for mathematical specifications.

2-3 Plexiglas Model Reconstruction

An alternate method of reconstructing a cervical model using serial sections was accomplished with cervical tissue from two additional animals. This method utilized tracings from tissue obtained and sectioned as described above. However, tracings were aligned with respect to a pair of fiducial points (as described previously) between sheets of 3.18mm plexiglas (modified from Fluhmann, 1958). This technique was useful in allowing the observer to follow fine-order epithelial structures during the construction process, as well as to view the completed structure from various angles using transmitted and oblique lighting.

Histochemistry

2-4 Tissue Collection

An additional 8 animals were utilized in the histological investigation of mucoid and sperm distribution within the mucosa. Four of these (two cows and two heifers) were bred naturally at estrus to a bull which consistently delivers ejaculates of good concentration and normal sperm viability and morphology. These animals were then sacrificed and the cervixes removed 8-12

hrs post-breeding as described previously. Thus, these four animals, representing the follicular phase, were most likely in late estrus to early metestrus at the time of cervix fixation. One additional animal was sacrificed in early estrus without breeding. Of these 5 follicular animals, 3 displayed a natural estrus and 2 were induced using Lutalyse (Prostaglandin F_{2α}). Cervices from the remaining 3 animals (2 cows and 1 heifer) were obtained at diestrus (10 days following observation of estrus).

The cervix of each animal was evaluated in four sequential segments of equal size from the posterior to the anterior cervical os. Due to its larger size, the cervix from each mature cow was opened longitudinally to the lumen, and a complete longitudinal strip, approximately 1 cm wide, was then subdivided into the 4 segments. However, in the case of heifers, intact cervices were quartered for use in the same manner.

2-5 Fixation and Tissue Processing

Following a pre-fixation time of 2-3 hrs in Bouins solution, two representative tissue blocks were taken from each quarter cervix and returned to fresh fixative to complete a 72-h fixation time. This initial partial fixation reduced the mechanical deformation of the cervix resulting from finer subdivision of soft, unfixed tissue. Although the primary fixative employed in this study was Bouins solution, additional fixatives including phosphate buffered formalin, alcoholic formalin and formal saline were selected for use with some tissue samples in an attempt to improve retention of secreted mucins. These fixatives were selected based on their reported advantage in glycoprotein fixation (Romagnoli, 1985). Of these fixatives, only formal saline was found to give inconsistent staining results. Tissue samples fixed with alcoholic formalin did not preserve cellular structure as well as Bouins or phosphate buffered formalin.

Following fixation, the tissue was rinsed with three changes of 70% ethanol at 24-h intervals, dehydrated, cleared and embedded in paraffin (Fisher's Tissue Prep 11) using an Autotechnicon tissue processor. Two tissue blocks from each quarter were then sectioned at 4-5 μm using a Leitz rotary microtome. Six adjacent sections from each tissue block were mounted in preparation for the staining sequence (see Appendix B for fixative recipes used in these procedures).

2-6 Staining Procedures

Histological staining procedures were applied to each of the 4 cervical zones of all animals examined. Mucin stains were utilized in an effort to differentiate specific regions within the defined cervical structure for the production of the acidic mucins (sulfomucins and sialomucins) and neutral or unchanged mucins. Differentiation of these mucins was accomplished using the following staining techniques according to Pierce (1985): (1) Alcian blue (AB) 8GX (Sigma Chem. Co.) at pH 1.0, (2) AB-8GX at pH 2.5, (3) periodic acid Schiffs (PAS) with AB (pH 2.5), and (4) high iron diamine (HID) with AB (pH 2.5). Staining procedures included the use of AB alone in order to prevent interference resulting from a masking or pH effect due to the addition of counter stains. This was accomplished by using AB at pH 1.0 to localize sulfomucin production, and then a second time at pH 2.5 to observe a change in stain intensity (increase) due to a positive stain for sialomucins (as well as sulfomucins) at this pH (Jones and Reed, 1972). The addition of PAS to sections stained first with AB at pH 2.5 allowed the differentiation of the neutral mucins in addition to the AB positive acidic ones (sulfomucins and sialomucins). Finally, HID was utilized in combination with AB on additional sections to further differentiate sialomucins and sulfomucins by staining a combination of sulfomucins and neutral mucins black, while sialomucins remain AB positive.

Slides stained with AB at pH 1.0 and AB at pH 2.5 were evaluated at 250x magnification by estimating staining intensity on a scale of 1-5 for the following areas within each stained section: (1) apical and basal areas occurring within the deeper regions of primary and secondary folds, and (2) apical and basal areas occurring in regions lining the central cervical canal. Apical areas consist of those cells closest to the primary lumen while basal areas are composed of cells within shallow folds and grooves. These same regions were evaluated at 250x magnification for the determination of a staining pattern for AB or PAS in the PAS-AB (pH 2.5)-stained slides. In this case, however, separate estimates were made for each stain and a scale of 1-10 was used to estimate the percent cells staining positive for each stain within each region (1 = 10% to 10 = 100%). It was possible for the total % cells stained with PAS and AB to exceed 100% since some cells contained mucins positive for both stains (Fig. 25). All estimates were made using coded slides to insure an unbiased judgement.

Sections stained with HID and AB (pH-2.5) were not included in the intensity estimates due to a wide range of variability in stain intensity among samples. Also, the HID stain appeared to inhibit the stainability of AB (pH 2.5). However, HID was useful in differentiating secreted mucins held within the epithelial folds of both follicular and luteal animals, and helped to demonstrate a pattern of their release from epithelial cells. Hematoxylin and eosin (H&E) was utilized for the localization of spermatozoa within the cervix of the 4 animals bred prior to slaughter. See Appendix (C) for staining techniques used in these procedures.

Epithelial cells

2-7 Historeshin

Tissue embedded in Historeshin (LKB) allowed bright field evaluation of thinner sections than was possible using paraffin-embedded preparations. Historeshin was also useful in differentiating epithelial cells and determining sperm orientation within areas larger than those permitted by plastic-embedded preparations designed for electron microscopy. A quantitative determination of ciliated vs secretory cell ratio was also possible utilizing this technique.

Tissue preparation for Historeshin embedding involved dehydration in a graded ethanol series to 95%, followed by an overnight Historeshin infiltration time and then polymerization at room temperature in Beem (Polysciences) capsules (Appendix B). Sections (1-2 μ m) were obtained using a dry glass knife on a Porter Blum MT-2B ultramicrotome and transferred with a pair of fine forceps to a small drop of 10% acetone on a gelatin coated slide. Sections were then dried at approximately 60°C on a hot plate, stained with toluidine blue for 10-20 seconds and rinsed in distilled water. Additional sections from selected areas were mounted and stained with PAS-AB (pH 2.5) and AB alone at pH 1.0 and pH 2.5. These mucin stains were employed on Historeshin sections only to more clearly demonstrate their specificity for the mucus granules of the secretory cells.

Using bright field microscopy, toluidine blue-stained sections were examined for sperm orientation and distribution within the cervical structure, and counts were made of epithelial cells to determine the percent ciliated vs. non-ciliated cells present in each quarter of the cervix. This was accomplished by differentially counting 100 cells per zone as ciliated or non-ciliated. Separate counts were made of epithelial cells in apical regions as opposed to basal regions within

shallow folds and grooves. A total of 2,500 cells were evaluated per quarter in basal regions and 1,500 cells per quarter in apical regions, due to their relatively smaller numbers (Appendix E).

Spermatozoa Within Cervical Structure

2-8 Tissue Preparation

The distribution of spermatozoa within cervical tissue was investigated in this study using paraffin embedded tissue for observation of complete cervical cross sections (heifers) or large samples (cows). Plastic-embedded tissue was utilized for a more detailed investigation of cell structure, histochemistry and sperm orientation using light and electron microscopy. Successful observation and study of spermatozoa within the folds and grooves of fixed tissue preparations could be accomplished only in those regions where mucin preservation was also successful. The addition of 1% cetylpyridinium bromide or alcian blue to Karnovsky's fixative in preparation for plastic embedding was partially successful in reducing the loss of secreted mucins and consequently provided some improvement in the retention of spermatozoa.

2-9 Ultrastructure

Tissue samples were taken for ultrastructural examination from each of the four estrus animals bred prior to slaughter. These samples were taken from each quarter of the cervix by carefully slicing approximately 1 mm thick samples across the epithelial folds perpendicular to the longitudinal axis. These slices were then "trimmed" by removing the muscularis layers, and pre-fixed in Karnovsky's fixative (Karnovsky, 1965) for 30 minutes. Following this initial fixation time,

tissue samples were removed, divided into smaller (less than 2 mm x 1 mm) pieces, and returned to fresh Karnovsky's fixative for an additional hour. Fixed tissue samples were then rinsed over a 24-h period with three changes of phosphate buffer which was osmoregulated with sucrose. The percent sucrose required for osmoregulation was calculated according to the following formula:

$$\frac{\frac{1}{2}(\text{mOsm fixative} - \text{mOsm buffer}) + \text{mOsm buffer}}{30} = \% \text{ sucrose needed in buffer wash}$$

(Hayat, 1981). Rinsed samples were held in phosphate buffer at 4° C until dehydration and embedding. Dehydration was accomplished using a series of graded ethanol solutions followed by propylene oxide. Dehydrated samples were embedded in a mixture of Epon 812 and Araldite (Appendix B). Embedded tissue blocks were thin-sectioned (600-1000Å) using a Porter Blum MT-2B ultramicrotome. Sections were stained in uranyl acetate and lead citrate according to Venable and Coggeshall (1965) and examined with an RCA EMU-3H electron microscope.

Additional tissue samples from two cows were fixed in a Karnovsky's fixative containing either 1% AB (Behnke and Zelander, 1970) or 1% cetylpyridinium bromide (Shea, 1971). The addition of these cations, according to Behnke and Zelander, function to bind anionic sites in the mucosubstances and therefore stabilize mucus glycoproteins when used in conjunction with glutaraldehyde. In this manner, the loss of spermatozoa contained within the mucus structure could also be reduced. Of the tissue samples fixed in this manner, half were embedded as previously described in an Epoxy-Araldite mixture, and thin-sectioned for electron microscopic examination. The other half were embedded in "Historesin" (LKB) in order to permit histochemical staining on 1-2µm thick sections. A detailed account of tissue preparation and reagent recipes is presented in Appendix B.

Tissue which was thin-sectioned for electron microscopy was examined for variations in the epithelial cell structure of secretory and ciliated cells within the folds, and also for orientation

of contained spermatozoa with respect to the epithelium and direction of ciliary beat. Determination of spermatozoan orientation was based on location of acrosomal membranes or flagella on sagittal sections of the head.

RESULTS

Structure

3-1 General

Three different techniques were utilized to study the anatomical features of the bovine cervix. These included: surface staining, computer reconstruction and plexiglass reconstruction of serial sections. Anatomical differences among animals were apparent as variations in the degree of complexity in epithelial folding. The cervix of multiparous cows was found to be generally enlarged with deeper and more complex folds than was evident in nulliparous females.

3-1.1 Surface staining

Surface staining of fixed tissue blocks and entire half cervixes by immersion in hematoxylin enhanced the contrast of mucosal surface features and demonstrated the structural com-

plexity of the cervix. Based on observation of these stained preparations, the mucosal surface in each case was found to consist of a complex system of folds and grooves arranged longitudinally and simultaneously thrown into a series of two to four transverse annular ridges or rings (usually three plus that forming the external cervical os) which overlapped in a posterior direction and were arranged spirally in an interlocking manner so as to restrict the cervical canal. These annular ridges diminished in height in a cranial direction. Deep primary folds were found to maintain continuity throughout the length of the cervix, often originating in the vaginal folds of the fornix vagina and progressing into the walls of the external cervical os. In a similar manner, the folds were found to traverse each annular ridge, at which point they often reached their greatest depth and complexity. Complexity in primary folds was most often expressed with bifurcation or merging of one fold with another. Secondary folds were apparent as longitudinal branching from the primary folds producing long and sometimes very thin leaf-like projections of the mucosa. However, these secondary folds did not maintain continuity throughout the entire length of the cervix, but varied in length and depth.

Numerous shallow channels or grooves lined the walls of primary and secondary folds within all regions of the cervical structure. These grooves were observed most frequently as a series of parallel furrows which progressed longitudinally in the direction of the primary folds. They were often found to branch or vary in depth and, in isolated areas, to deviate following a course leading from the luminal surface toward the base of the primary folds. In regions nearest the cervical canal, these grooves were frequently apparent in patterns which were abrupt in their origin and/or termination, often displaying a fern-like pattern. These features are apparent in stereomicrographs of surface-stained tissue in Figures 1-3. Figure 4 presents a conceptualized illustration of a block of cervical tissue from the fornix vagina through the first annular ridge summarizing features observed in this region.

PLATE 1 Surface Stained Tissue

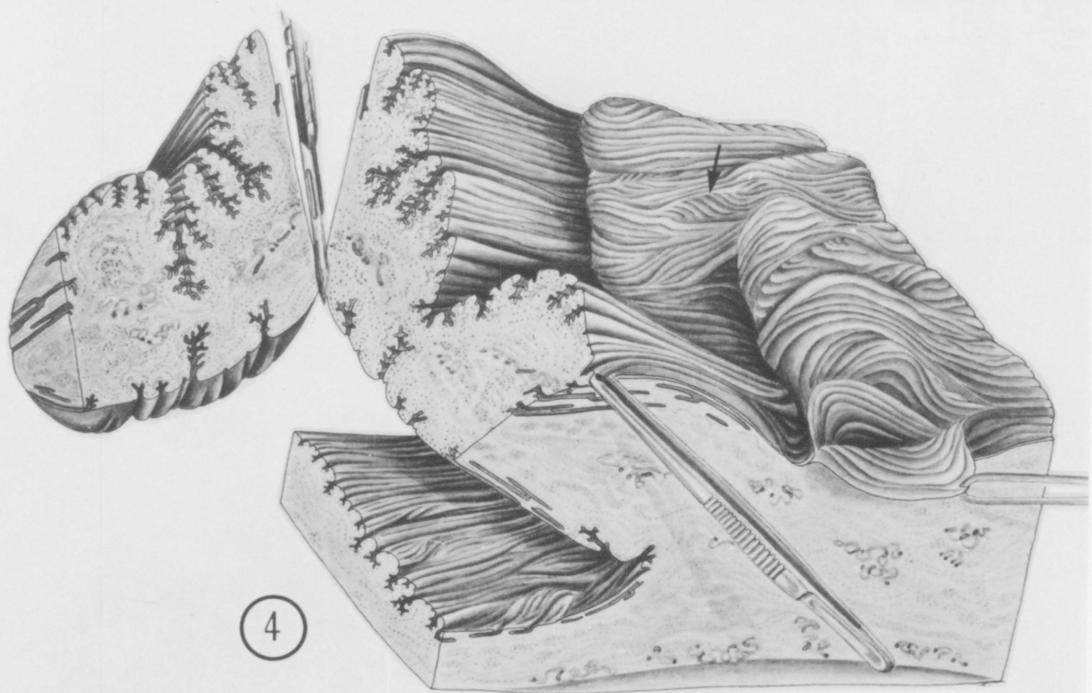
Figures 1,2, and 3. Bouins-fixed tissue surface-stained with Harris' hematoxylin.

Figure 1. A cross cut surface through deep primary folds. Note numerous shallow branches or grooves along the walls of these folds. Note also discontinuous grooves (arrows) apparent on the luminal surface. This pattern was most frequently observed on surfaces nearest the cervical canal. Grooves apparent within the base of primary folds were more continuous and most frequently paralleled the cervical canal (6.5X).

Figure 2. Secondary folds are apparent along the walls of primary folds (arrows) further subdividing the muscularis of the cervix in this cross cut surface (3.6X).

Figure 3. Exposed wall of a primary fold showing shallow longitudinal grooves of continuous parallel nature which were most typical of surfaces along the walls and base of primary and secondary folds (3.8X).

Figure 4. An illustration of a block of cervical tissue from the fornix vagina through the cervical os and first annular ring showing primary and secondary folds, subdivided by numerous shallow folds and grooves. Note the fern like discontinuous pattern of grooves in isolated areas of luminal surface (arrow) as well as the thin leaf-like projection of the mucosa (primary fold), the wall of which is exposed with forceps in lower right.



3-1.2 Computer reconstruction

A computer-generated reconstruction of 20 serial sections from a region of midcervical tissue approximately 100 μ m in length was conducted in order to verify the absence or presence of tubular or blind-ending structures within the observed epithelial folds. Examination of 18 different stereo pairs produced by the computer revealed a system of continual folds, as observed in surface-stained tissue, without evidence of blind crypts, tubular glands, or tunnels. Two of these stereo pairs (Figures 5 and 6) may be viewed with a stereoscope for a three-dimensional perspective. Figure 7 is an illustrated diagrammatic interpretation of the three-dimensional image represented in Figures 5 and 6. As may be noted from this illustration of the stereo image, the apparent tunnels or submucosal glands were a product of sectioning through portions of mucosal folds.

3-1.3 Plexiglas reconstruction

Spacial reconstructions of two additional sets of 15 serial sections (approximately 75 μ m in length) taken from areas which gave the appearance of glandular formations were produced using a plexiglas model. Construction of these models proved useful in following complex fine structures during the process of building the model as well as observing the completed structure. Figures 8 and 9 represent completed models which demonstrate a continuity within gland-like formations as they became parts of a system of branching folds. In Figure 9, these structures were found to open into a tangential section within a portion of a major fold. Figure 10 diagrammatically illustrates how glandular-appearing formations in sectioned tissue may originate from a structure which is, in fact, a winding system of open ended folds.

PLATE 2 Computer Reconstruction

Figures 5 and 6. Stereo pairs of the computer-aided reconstruction of serial sections from a mid-cervical region.

Figure 5. Boundry line reconstruction of the entire set of 20 serial sections through approximately 100u of the cervical folds. The image is displayed in diminishing shades from white to gray, where white is the closest to the viewer and gray is the farthest.

Figure 6. Solid image reconstruction of the final 10 serial sections from the series of 20. Also, as above, in diminishing shades from white to gray.

Figure 7. An illustration of the overall three- dimensional structure as interpreted from stereo pair reconstructions.

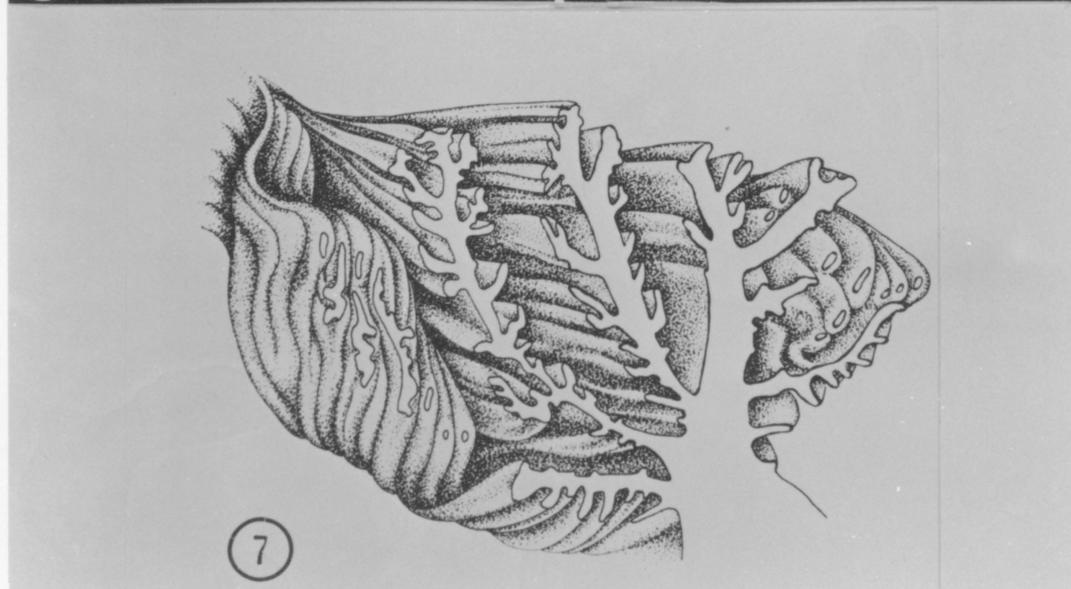
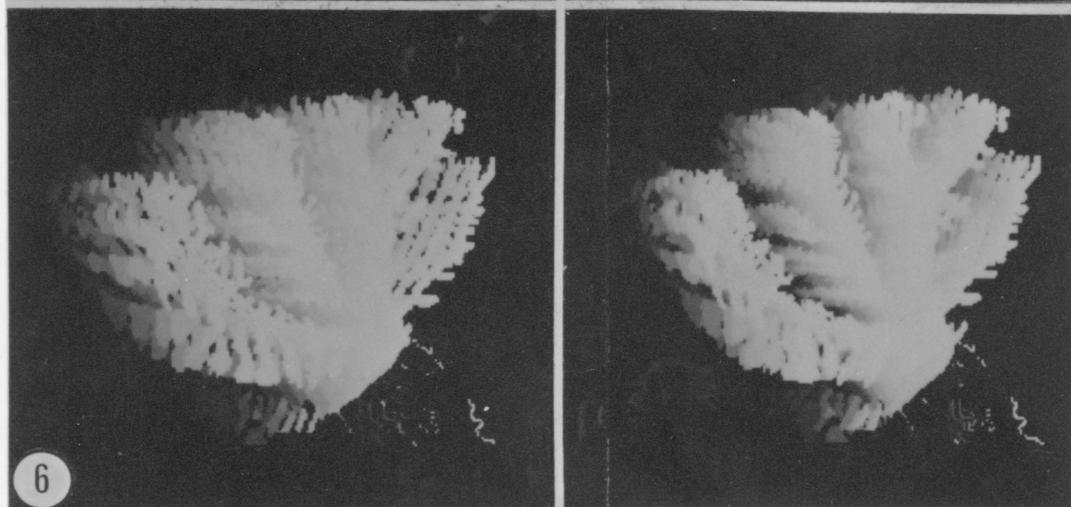
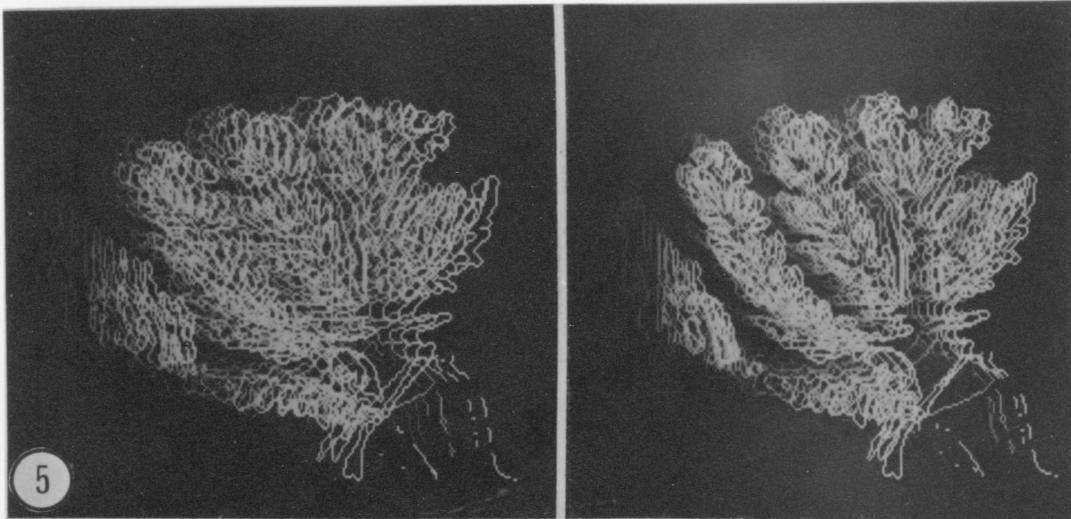


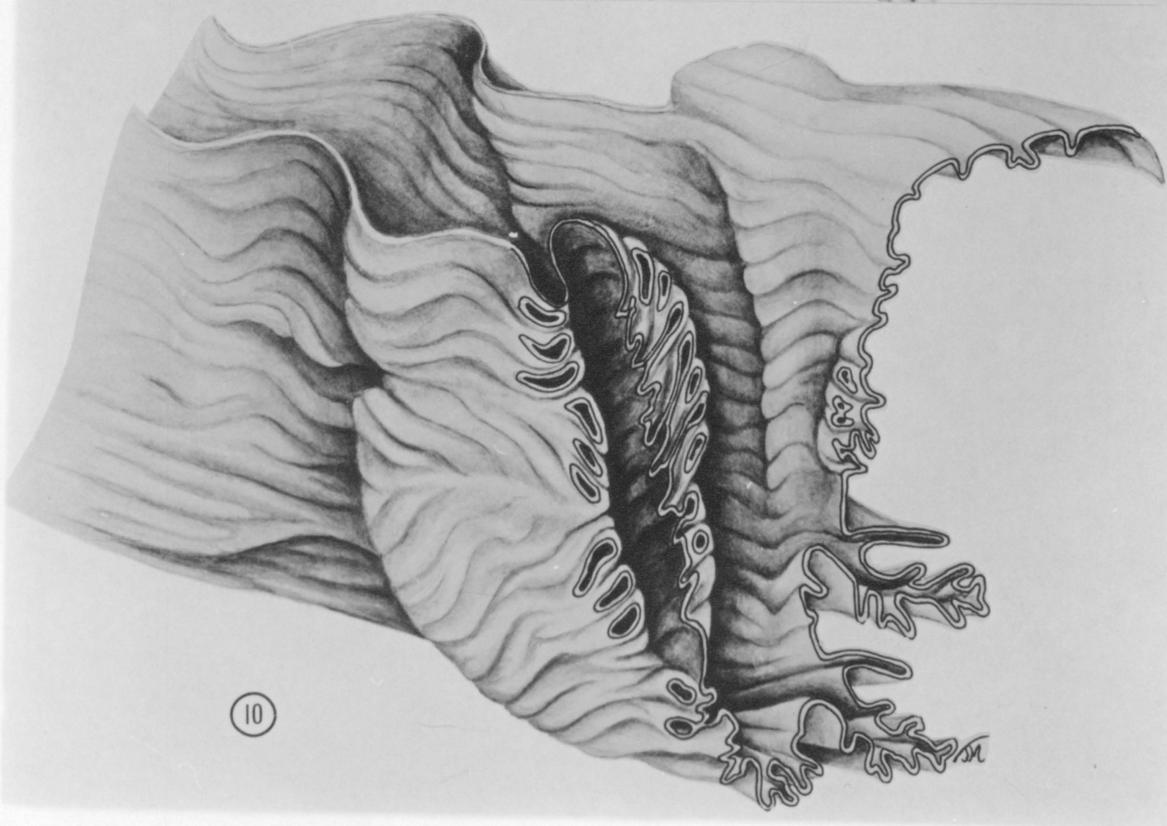
PLATE 3 Plexiglas Reconstruction

Figure 8 and 9. Spacial reconstructions of 15 serial sections through approximately 75u of cervical epithelium. Models were assembled by spacially separating clear acetate tracings of each section using 3.18mm plexiglas sheets.

Figure 8. Demonstrates a continual series of folds along a luminal surface.

Figure 9. Demonstrates an area where glandular appearing structures within sectioned tissue were found to open into a tangential section within a portion of a major fold (outlined area).

Figure 10. A diagramatic illustration demonstrating graphically how glandular-appearing formations in sectioned tissue may originate from a structure which is, in fact, a winding system of open ended folds.



3-1.4 Summary

The following features were found to be characteristic of the bovine cervical structure: 1. Deep longitudinal primary folds radiating from the central lumen were continuous throughout the cervix, often originating in the fornix vagina. 2. Secondary folds subdivided deeper regions of primary folds where they were found to branch and/or converge. Some of these secondary folds maintained continuity to the uterus, but most did not. 3. Shallow grooves with some variation in both depth and frequency were found to occur in parallel longitudinal rows along both primary and secondary folds. Grooves occurring within deeper regions of the folds appeared to maintain continuity to a greater extent than those occurring within the central region of the canal. 4. No evidence was found for blind crypts, tunnels or glands.

Histology

3-2 General

The histological and histochemical investigation of the cervix was conducted using three glycoprotein staining techniques designed to separate and identify the sites of production for sialomucins, sulfomucins and neutral mucins, while cell types and nuclei were differentiated using H&E. Although Bouins solution was the primary fixative of choice for paraffin-embedded tissue, additional fixatives, including phosphate buffered formalin, alcoholic formalin and formal saline were also employed in some samples in an attempt to better retain and preserve luminal mucins. Samples prepared for Histo-resin embedding were fixed in Karnovsky's fixative (see Sec. 2-8). A consistent and similar epithelial staining pattern was observed in all fixatives tested except

for formal saline. An inconsistency in staining pattern was found between samples preserved in this fixative, as described in more detail in Section 3-2.3.

Full cross sections of the intact cervix were obtained when the cervical diameter was sufficiently small to permit adequate fixation, allowing a reference for consistency in staining pattern from top to bottom of primary folds. This consistency was found to exist across all stains with the exception of the formal saline fixed tissue (see Sec. 3-2.3).

Based upon the described cervical structure (Sec. 3-1 to 3-1.4), evaluation for histological and histochemical features was undertaken with respect to : 1. quarter of the cervix (caudal- cranial direction, one through four), 2. central vs peripheral regions (vicinity of cervical canal vs base of primary and secondary folds distal to the cervical canal), and 3. apical vs basal epithelia with respect to the grooves (apical being those cells most proximal to the lumen and basal referring to cells distal to the lumen within grooves). These terms are set forth in Figure 11 and will be used in defining the location of histological characteristics described in this section.

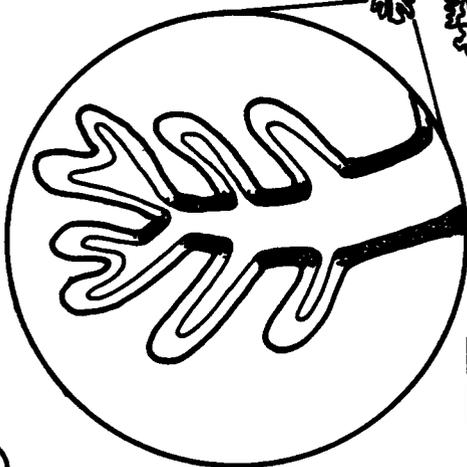
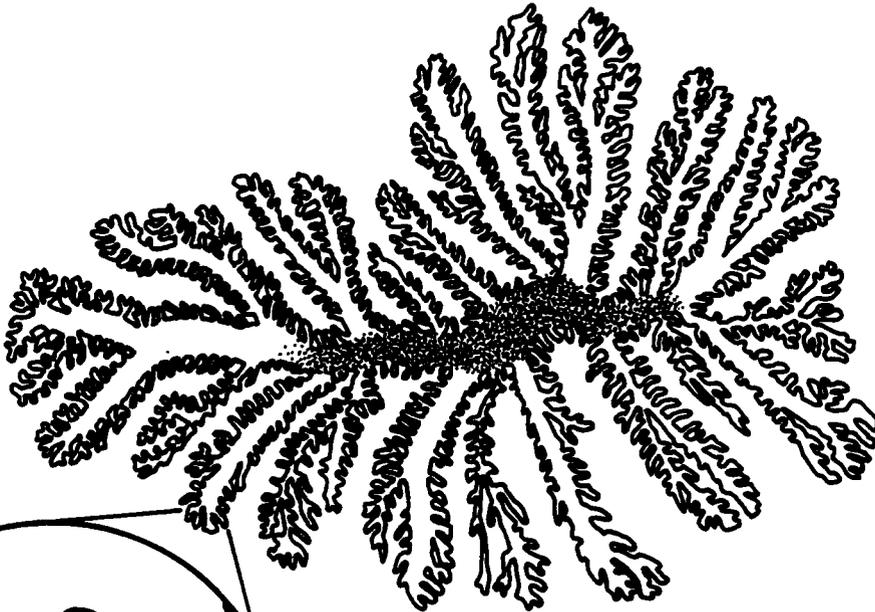
3-2.1 Staining Procedures

To evaluate mucin types, estimations for staining quality and intensity were determined for the apical and basal areas of grooves located within both central and peripheral regions of the cervix. This was carried out for the four quarters of the cervix as described in section 2.6 for AB pH1.0, AB pH2.5, and PAS-AB stains. Evaluation was made following examination of all epithelial surfaces represented on each slide, and was based on an area or group of areas which seemed to most accurately represent the entire section. Slides were coded with respect to pH, quarter of the cervix, and stage of the Estrous cycle (5 follicular phase and 3 luteal phase) from which the tissue was taken.

PLATE 4 Descriptive terminology used in this study

Figure 11. A cervical cross section showing the location of central and peripheral regions as well as apical and basal areas within each of these regions established for histological and histochemical evaluations in this study.

■ CENTRAL
□ PERIPHERAL



■ APICAL
□ BASAL

11

3-2.2 Alcian Blue

Estimates obtained from AB stained sections are presented graphically in Plate 5 (based on 5 follicular animals) and Plate 6 (based on 3 luteal animals). Comparisons were made separately for central and peripheral regions and include apical and basal intensity estimates within each quarter cervix (refer to Fig. 11). Results from follicular animals (based on plate 5) indicate the following: Tissue from follicular phase animals which was stained with AB at pH 1.0 showed a trend in most tissue samples toward slightly more intense staining in apical areas, although staining was evident in all areas of the mucosa (see Fig. 21 for photographic reference). Estimates did not vary appreciably between central (Fig. 12a) and peripheral (Fig. 12b) regions. When AB was used at pH 2.5, however, the staining pattern was reversed with a slightly deeper stain intensity now evident in basal areas of most tissue samples (see Fig. 22 for photographic reference). Comparable apical basal differences at pH 2.5 were evident (as at pH 1.0) in both central (Fig. 13a) and peripheral (Fig. 13b) regions. A comparison of differences occurring in stain intensity as a result of pH change is presented in Fig. 14 which represents an average of estimates from both central and peripheral regions. These differences demonstrate separately for apical (Fig. 14a) and basal (Fig. 14b) areas, an increase in stain intensity with AB at pH 2.5 as opposed to pH 1.0. This difference represents the increased contribution of sialomucins in the basal as opposed to apical areas of the grooves. This difference was apparent across all quarters (Fig. 14b).

Results from luteal phase animals (based on Plate 6) indicate the following: Tissue from luteal animals stained with AB at pH 1.0 showed a trend toward increased stain intensity in basal areas as opposed to apical within both central and peripheral regions (Fig. 15a & b). Tissue stained with AB at pH 2.5 similarly showed a trend toward increased stain intensity in basal areas of both central and peripheral regions (Fig. 16a & b). A comparison of the differences occurring in stain intensity as a result of pH change for the luteal animals is presented in Fig. 17a for apical areas and 17b for basal areas. These values also represent an average of central and peripheral re-

PLATE 5 Alcian Blue Staining Pattern in Follicular Phase

Cervical Tissue

Figure 12. Intensity of staining within the cervix using AB at pH 1.0. Estimates include apical and basal areas across four cervical quarters within central (12a) and peripheral (12b) regions (mean of 5 animals).

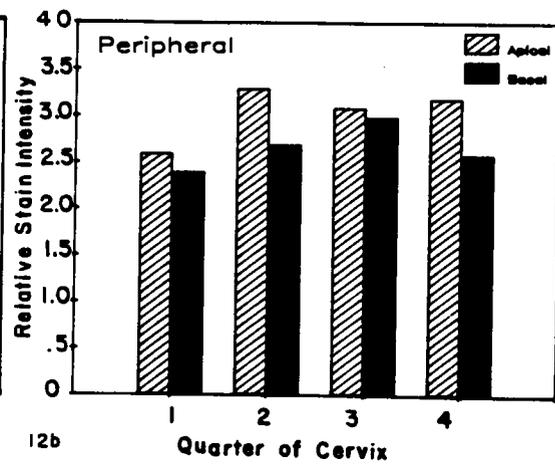
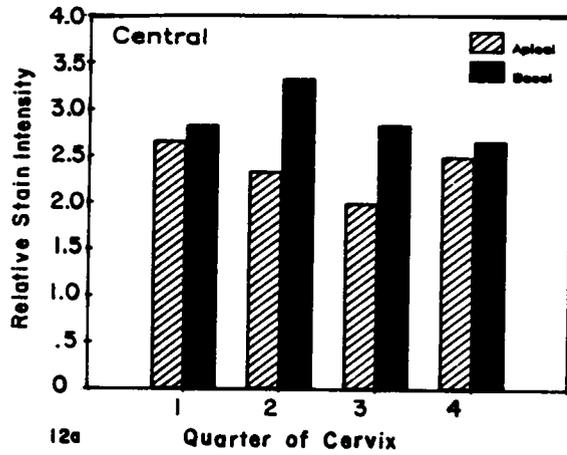
Figure 13. Intensity of staining within the cervix using AB at pH 2.5. Estimates include apical and basal areas within central (13a) and peripheral (13b) regions (mean of 5 animals).

Figure 14. A comparison of differences in stain intensity within the follicular phase cervix using AB at pH 1.0 vs AB at pH 2.5 for total values across four quarters in apical (14a) and basal (14b) areas (mean of 5 animals).

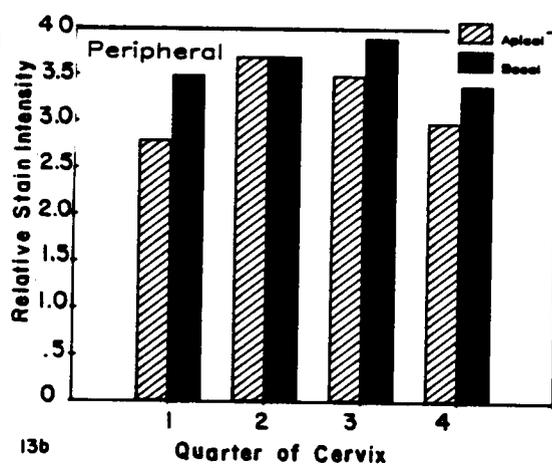
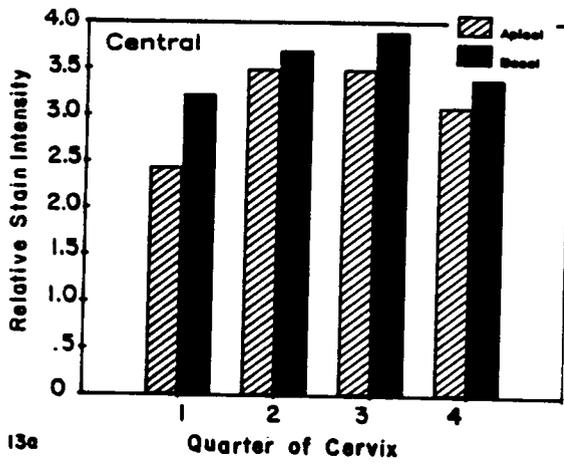
Alcian Blue Staining Pattern in Follicular Tissue.

Follicular

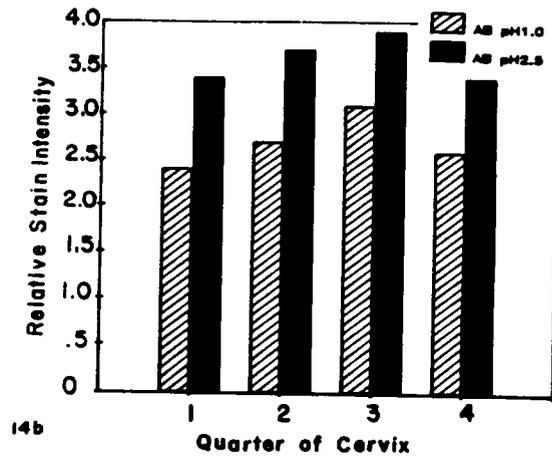
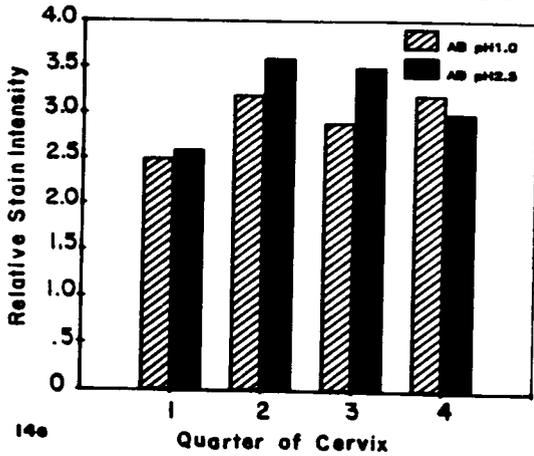
Alcian Blue pH1.0



Alcian Blue pH2.5



Alcian Blue pH1.0 vs pH2.5



gions and reveal a slight increase in stain intensity in most apical areas as a result of the pH increase. Basal areas in this tissue were not altered in stain intensity as a result of the pH change.

3-2.3 Periodic acid Schiffs - Alcian Blue

Staining with PAS was found to be slightly more intense in samples preserved in either phosphate buffered formalin or alcoholic formalin, while cell structure was found to be maintained best in either Bouins solution or phosphate buffered formalin. Overstaining with PAS was found to present a problem in the interpretation of staining patterns due to a masking effect over the relatively less intense AB, and therefore timing had to be carefully regulated when these stains were used in combination.

Estimates obtained from PAS-AB (pH 2.5) stained sections are presented graphically in Plate 7 (Figs. 18 & 19). Comparisons were again made separately for central and peripheral regions (as with AB intensity estimates) although these estimates were based on average percent staining positive for each stain per area examined (see Sec. 2-6). As some cells were observed to be mixed in their staining response, ie, contained both PAS positive mucins and AB positive mucins, these percentage estimates do not necessarily total 100%. Results from follicular phase animals (Fig. 18a & b) indicate the following: Tissue stained with PAS-AB (pH2.5) revealed a distinct trend toward a staining pattern where cells within apical areas from both central and peripheral regions stained predominantly with PAS, while cells within basal areas stained predominantly with AB across all quarters of the cervix (Fig. 22). Inconsistencies in this staining pattern were found in formal saline fixed samples from only one animal. In approximately 1 out of 4 regions within this tissue a reverse of the above staining pattern was observed, with PAS being predominant in basal areas (rather than apical) and AB predominating in apical areas (rather than basal).

PLATE 6 Alcian Blue Staining Pattern in Luteal Phase

Cervical Tissue

Figure 15. Intensity of staining within the cervix using AB at pH 1.0. Estimates include apical and basal areas across the four quarters within central (15a) and peripheral (15b) regions (mean of 3 animals).

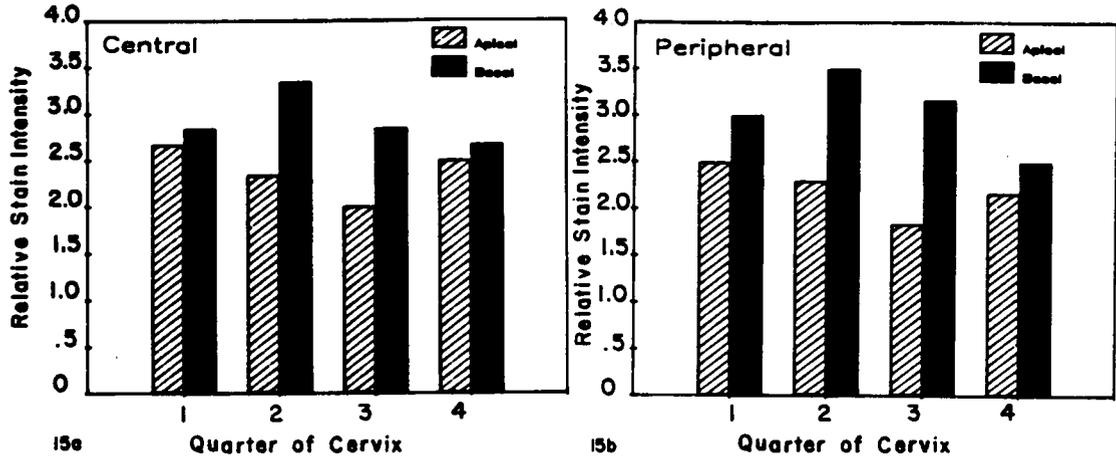
Figure 16. Intensity of staining within the cervix using AB at pH 2.5. Estimates include apical and basal areas across the four quarters within central (16a) and peripheral (16b) regions (mean of 3 animals).

Figure 17. A comparison of differences in stain intensity within the luteal phase cervix using AB at pH 1.0 vs AB at pH 2.5 for total values across four quarters in apical (17a) and basal (17b) areas (mean of 3 animals).

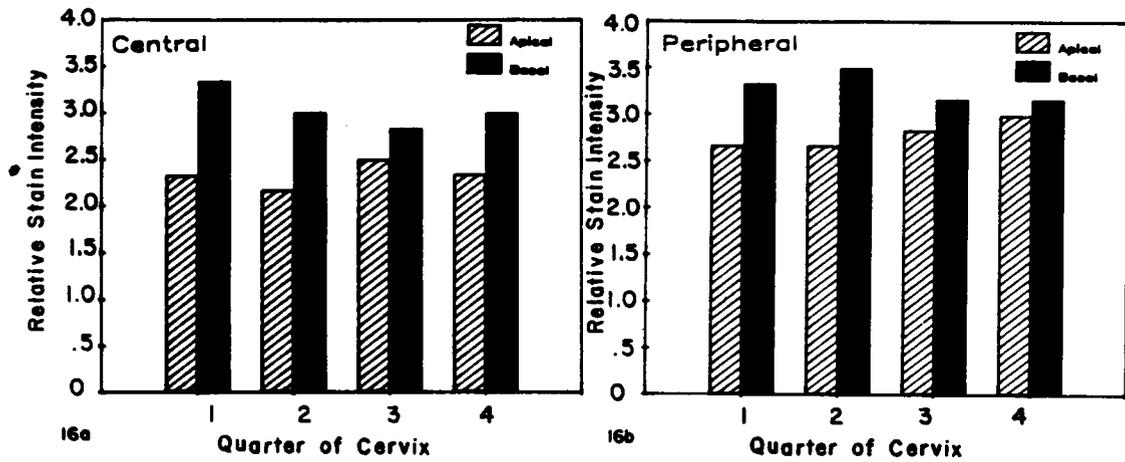
Alcian Blue Staining Pattern in Luteal Phase Cervical Tissue.

Luteal

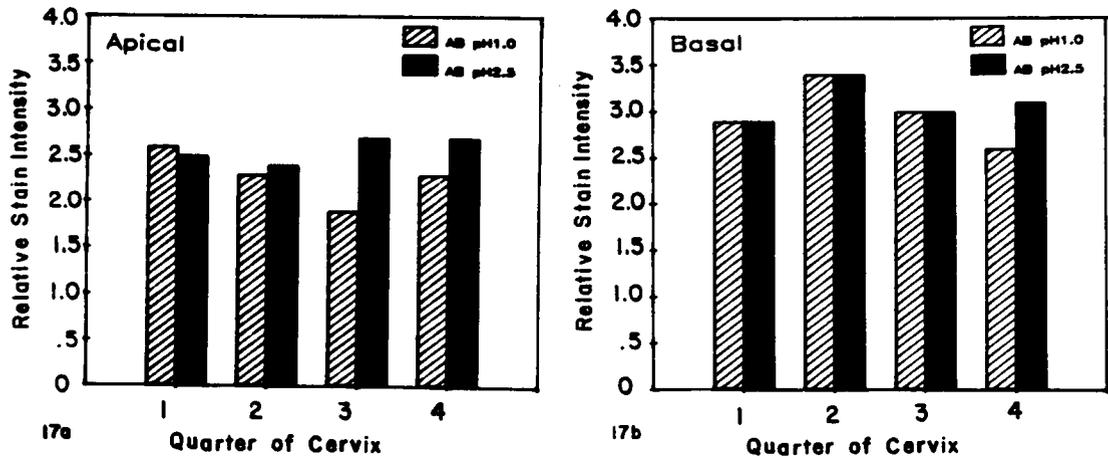
Alcian Blue pH1.0



Alcian Blue pH2.5



Alcian Blue pH1.0 vs pH2.5



Tissue from luteal animals stained with PAS-AB (pH 2.5) did not reveal the consistently distinct pattern observed across all sections of follicular phase animals. On the contrary, staining variation was evident between sections and, on occasion, within different folds in a single section. Considering these differences, estimates were determined based on a subjective averaging of staining patterns across the entire section. Graphic results of these averages from luteal phase animals are presented in Fig. 19a & b. A trend toward predominant AB staining in cells within basal areas was evident (similar to the follicular phase AB staining pattern) while PAS remained the predominant stain in apical areas of both central and peripheral regions (Fig. 23). Although a much smaller amount of mucin was present in luteal phase tissue, and a consistency in staining pattern was more erratic, cells within peripheral regions appeared to contain relatively more apical AB positive mucin than was evident in central regions. Also, a slightly higher proportion of PAS positive mucin was found to be present within basal areas in both regions of the luteal cervix as opposed to the follicular phase cervix (see Appendix D for data).

3-2.4 High Iron Diamine - Alcian Blue

Staining results with HID proved to be the most inconsistent of the techniques attempted. Fresh preparations of HID stained with insufficient intensity for adequate separation from the AB counterstain. Aged preparations of HID (one week to 2 months) stained with excessive intensity, however, and blocked the AB counterstain in spite of increased AB staining times. While a balance between these stains was inconsistently obtained across sections, Fig. 25 represents an area from a follicular cervix where both stains were properly balanced, revealing a basal predominance of AB. Due to inconsistencies in staining response, this stain was not utilized for quantitative evaluation. Where staining balance was achieved, however HID was useful in a qualitative evaluation of both intra and extracellular (or intraluminal) mucins. Extracellular mucins from follicular phase samples revealed a layered system of long-chain, HID-positive mucins with each layer emerging from an apical region within folds and extending toward the

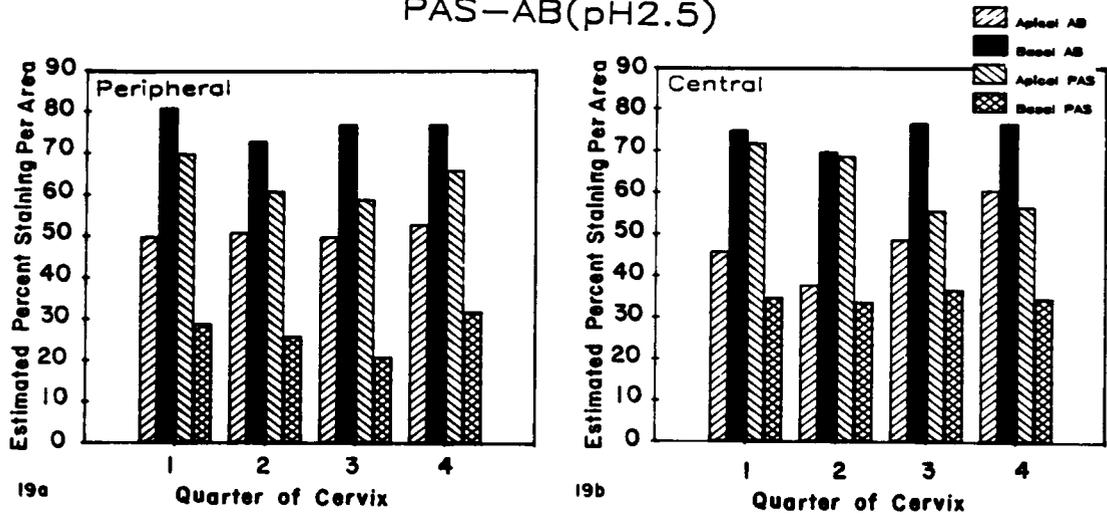
**PLATE 7 PAS - AB (pH 2.5) Staining Pattern in Follicular
and Luteal Phase Tissue**

Figure 18. A comparison of the estimated percent cells staining positive for PAS vs those staining positive for AB (pH 2.5) within apical and basal areas of the follicular phase cervix in central (18a) and peripheral (18b) regions (mean of 5 animals).

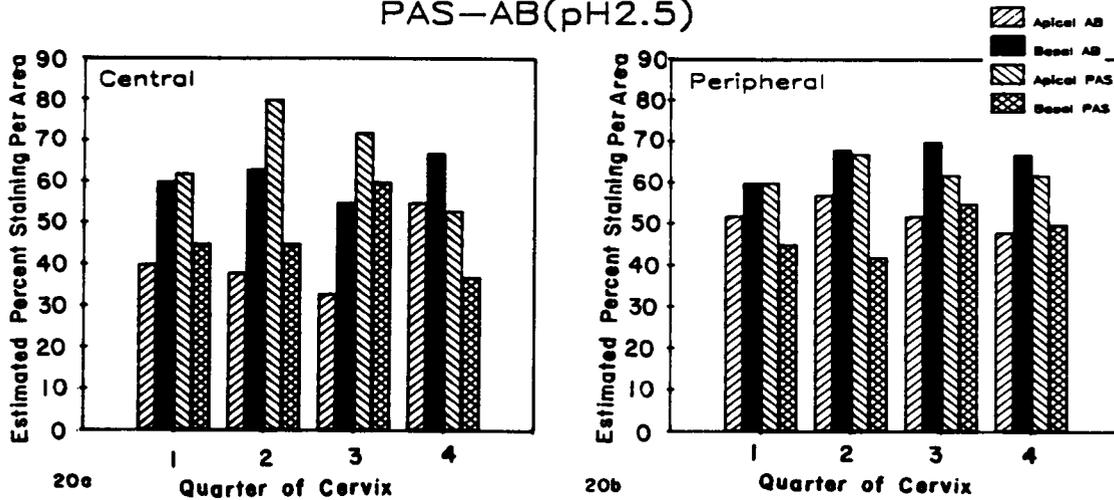
Figure 19. A comparison of the relative proportion of cells staining positive for PAS vs those staining positive for AB (pH 2.5) within apical and basal areas of the luteal phase cervix within central (19a) and peripheral (19b) regions (mean of 3 animals).

PAS-AB (pH2.5) Staining Pattern in Follicular and Luteal Phase Tissue.

Follicular
PAS-AB(pH2.5)



Luteal
PAS-AB(pH2.5)



central lumen (Fig. 26). These layers, or sheets, (presumably neutral and sulfomucins) were often observed to extend in close proximity to the epithelium in such a manner as to entrap the less densely staining AB positive mucins (presumably sialomucins) within grooves (Fig. 27). As the layers of HID positive mucins extended from the point of secretion toward the top of primary folds (central lumen) they often appeared to laminate into layers which were separated by regions of lower density mucins, which stained predominantly for AB (Fig. 28). Although the best luminal mucus retention was found to be in the early-estrus animal (non-induced), mucus was retained in some regions of the early metestrus animals, in which case a pattern of mucus release similar to that observed in the early estrus animal was observed. Cervices from luteal phase animals retained proportionally more luminal mucins, and these stained predominantly as neutral mucins with HID and PAS. In addition, layers of these neutral mucins appeared to be more tightly interconnected, forming a dense meshwork (Fig. 29). Where HID-AB stains were properly balanced in luteal animals, a reversal of the pattern evident in the epithelial cells of follicular animals was observed with HID predominant in basal areas while AB was more evident in apical areas. Figure 30 represents a graphic interpretation of mucus flow and the observed mucin staining patterns from a follicular phase cervix as it could occur in three dimensions when considering the structure as described in Section 3-1 through 3-1.3. A theoretical privileged path for sperm is also shown within cervical grooves in this diagram and will be addressed in more detail later (Sections 3-4.1, 3-4.2, 3-4.3).

PLATE 8 Histochemical Differentiation of Mucins

Figure 20. Paraffin section of the cervix obtained from a cow in follicular phase of the Estrus cycle. Section was stained with AB at pH 1.0 (sulfomucins only). Note slightly denser stain intensity in apical areas (172X).

Figure 21. Section from the same area as above but stained with AB at pH 2.5 (sulfomucins and sialomucins). Note increase in stain intensity in basal areas (as opposed to apical). This increase in staining intensity is attributed to sialomucin differentiation with AB at pH 2.5 (172X).

Figure 22. Section through a primary fold from a follicular animal stained with PAS-AB (pH 2.5) demonstrating a predominance of PAS positive (neutral) mucins in apical areas while basal areas within grooves stain predominantly AB positive (172X).

Figure 23. Luteal phase cervix stained as above (Fig. 23). Note increase in PAS positive mucins and relatively reduced AB staining in the basal areas (172X).

Figure 24. Section of Histoiresin embedded tissue stained with PAS-AB. Note appearance of both PAS and AB positive granules simultaneously within some cells (727X).

Figure 25. Paraffin section of follicular phase tissue stained with HID and AB (pH 2.5). Note the predominance of black diamine stain in apical areas indicating a combination of neutral and sulfamucins, while AB stain indicating sialomucin is predominant within basal areas (274X).

Histochemical Differentiation of Mucins.

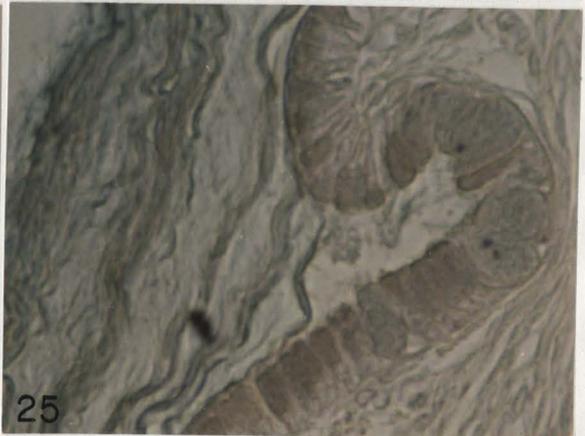
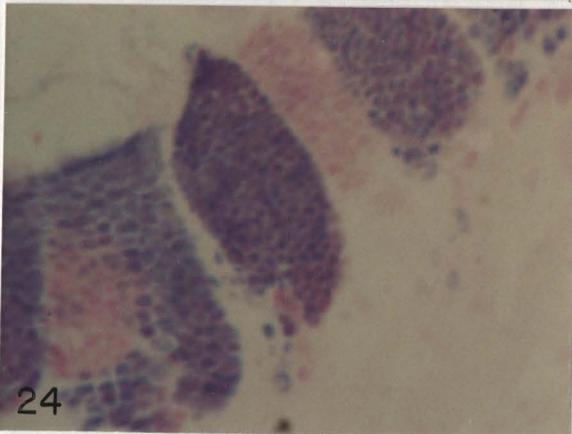
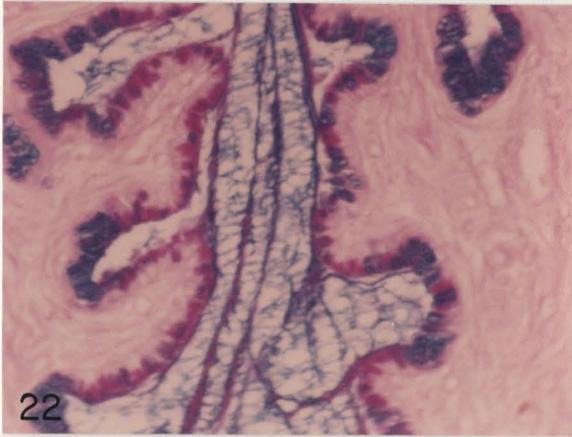


PLATE 9 High Iron Diamine Stained Luminal Mucins

Figure 26. A transverse section through a deep primary fold in paraffin embedded follicular tissue. Apparent are the densely stained layers of HID positive (neutral and sulfomucin) mucins emanating from each apical region and extending toward the central canal (arrows). Lighter staining mucins (predominantly sialomucins) are evident interspersed between these denser mucins and within grooves. (114X).

Figure 27. An area within a primary fold in follicular phase cervical tissue where AB positive sialomucins appear to be confined within grooves by denser staining HID positive neutral and sulfomucin layers emitted from adjacent apical ridges and flowing in close proximity to apical surfaces (172X).

Figure 28. A section through an area in follicular phase cervical tissue which is close to the central cervical canal. Note the laminated layers of densely HID positive mucins (x) which have concentrated into thick bands, separated by mucin layers which are more disperse and less densely staining (274X).

Figure 29. A section through a fold taken from luteal phase cervical tissue demonstrating a loss in organization of mucin layers with an increase in neutral mucins which are produced within grooves as well as in apical areas. These mucins interconnect in an intricate manner (172X).

High Iron Diamine Stained Luminal Mucins.

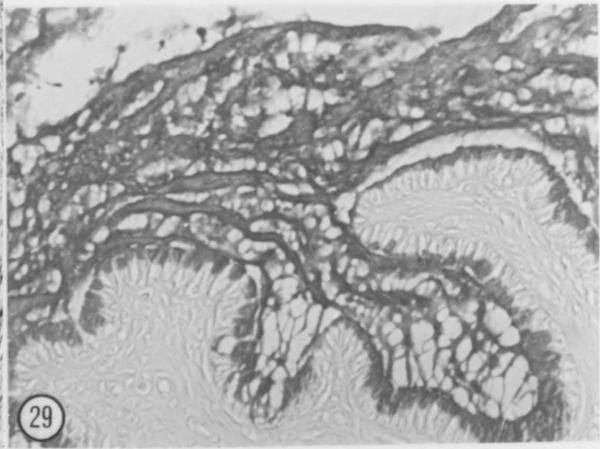
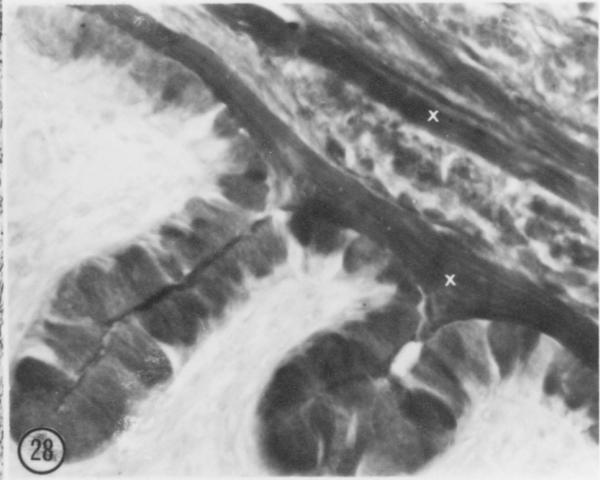
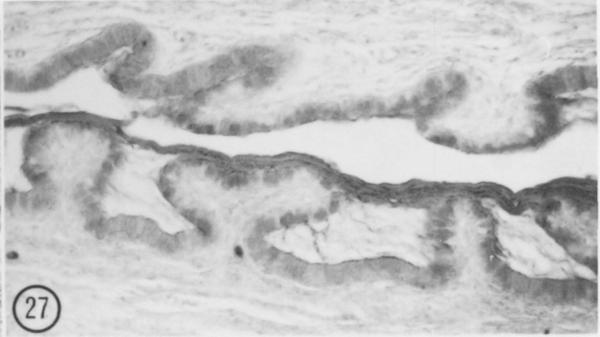
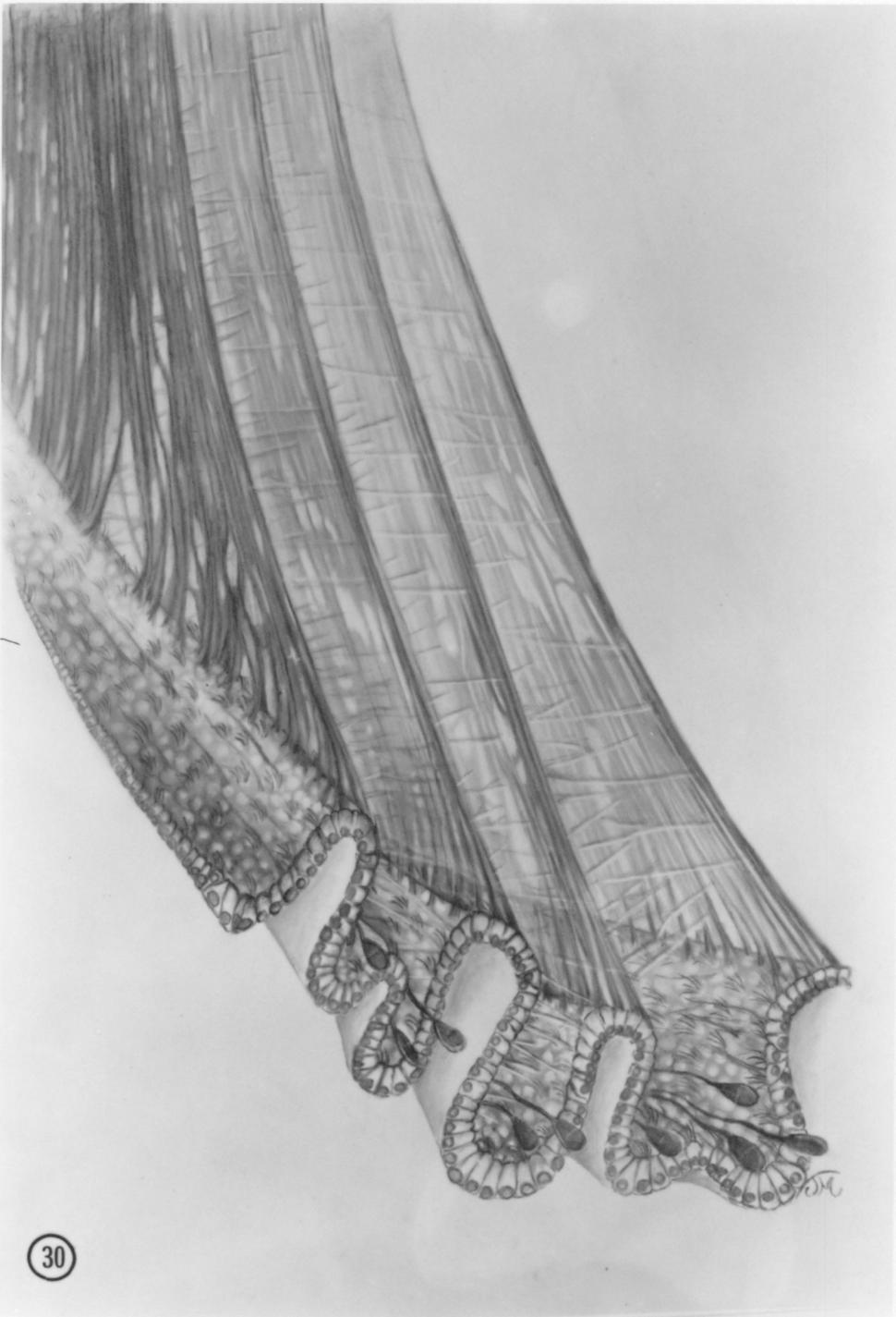


PLATE 10 Proposed Mucus Secretory Pattern

Figure 30. A diagrammatic representation of a possible secretory pattern when considering the three-dimensional structure of the follicular phase cervical mucosa. Mucin "chains" emanating from apical areas are represented here as sheets extending along apical ridges. A theoretical privaledged path for sperm in this scheme is also presented.

Proposed Mucus Secretory Pattern.



30

Epithelial cells

3-3 General

Morphology of the columnar epithelial cells was best revealed in H&E stained paraffin sections. In follicular phase animals these cells were characterized by increased height or apical swelling due to the presence of mucous granules which often crowded the nuclei toward the basement membrane (Fig. 31). In both central and peripheral regions of the cervix, there was a proportionally larger cell size (cytoplasm/nuclear ratio) in the basal areas of most grooves as opposed to the apical areas lining the lumen. Although this difference in cell size was evident in some areas of luteal tissue (particularly in peripheral regions), it was most pronounced and consistent in follicular phase tissue, undoubtedly due to the increased quantities of intra-cellular mucin which was present (Fig. 31).

Tissue preserved in Karnovsky's fixative for Histo-resin embedment revealed the presence of a conformation in many grooves whereby apical or neck areas were tightly restricted as one to three cells on opposing sides appeared joined by their apical region. The area beneath these cells, within the groove, remained open (Fig. 32). Highly vascular lamina propria and muscularis layers were evident beneath these epithelial cells throughout the cervix, and invading leucocytes were occasionally observed within sections taken from areas near the external and internal cervical os.

3-3.1 Secretory

Examination of Histoiresin sections from three follicular phase animals stained with PAS-AB (pH2.5) revealed the presence of both PAS positive granules and AB positive granules in the same secretory cell. The proportion of different staining granules varied greatly among cells (see Fig. 24). As observed in PAS-AB (pH 2.5) stained follicular phase tissue in Sec. 3-2.3, however, the relative abundance of AB staining granules was greater in cells of the basal areas while PAS positive staining granules were relatively more abundant in cells lining the apical areas. Ultrastructural distinction between these secretory cells is possible due to variation in the electron density of their secretory granules. No further distinction among secretory cells was evident in this study (Fig. 33).

3-3.2 Ciliated

Ciliated epithelial cells were generally less densely stained with toluidine blue in Histoiresin sections, presumably due to the absence of the more densely staining secretory material. These cells were found to vary greatly in their size (due to plane of section) and often appeared squeezed between secretory epithelial cells (Fig. 34).

The relative occurrence of ciliated cells appeared to vary with a gradual increase in their average numbers from the posterior to the anterior cervix. In addition, a slightly increased average percentage of ciliated cells as compared to secretory cells was present in all cases regarding apical epithelial cells as opposed to basal cells (see Fig. 35). Central vs peripheral regions were not considered here. Within the fourth quarter, or anterior cervix, isolated areas or "patches" of epithelium were encountered in which large numbers of ciliated cells were evident (40-60%) thus accounting for the large variance of this quarter (Fig. 36). Ciliary beat appeared, when plane of section permitted observation, to be in unison among cells and

unidirectional. An organized, directional beat is evident in parallel rows of cross sectioned cilia in Fig. 37. It is possible that this synchrony could be due to an artifact of tissue processing. However, it would seem unlikely due to the consistency with which this orientation was observed among sectioned samples (see Appendix E for data).

Sperm Transport

3-4.1 Paraffin Embedded Tissue

Paraffin embedded tissue from those animals which were bred 8-12 hours prior to slaughter was sectioned at 5 μ m and stained with hematoxylin and eosin for localization of spermatozoa. Spermatozoa in these sections were apparent in some isolated regions of all sections but varied greatly in number from an occasional single cell to areas of relatively heavy concentration. Areas which were heavily populated with spermatozoa were generally found in luminal regions between primary and secondary folds where long mucin chains were evident. These heavily populated mucin chains were most evident in sections taken from the cranial quarter of the cervix or the cervico-uterine junction. Cells observed within the cervical grooves were usually present in smaller numbers, and were most often observed deep within basal areas of these grooves. (Fig. 38 & 39). Spermatozoa observed within sections taken from the caudal half of the cervix, including the posterior cervical os, were most frequently found within the basal areas of grooves, as opposed to luminal areas between primary and secondary folds.

PLATE 11 Epithelial Cells of the Follicular Phase Cervix

Figure 31. A paraffin section through cervical grooves demonstrating basally located nuclei and a high cytoplasm/nuclear ratio in basal areas. (240X).

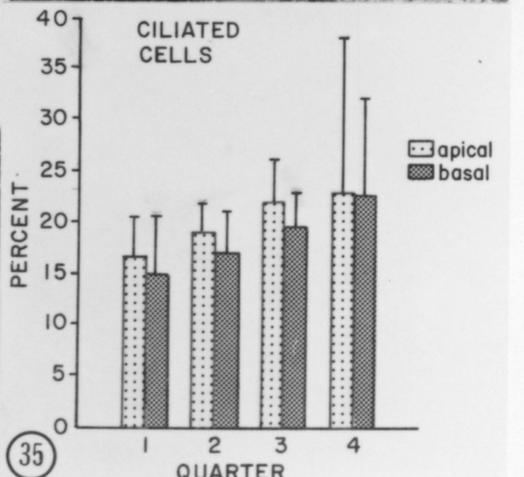
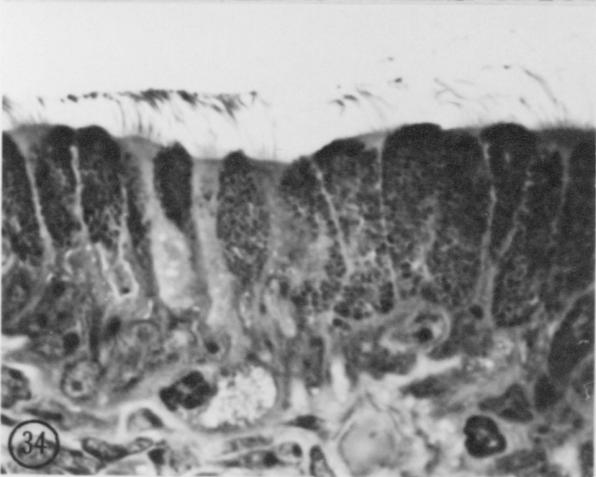
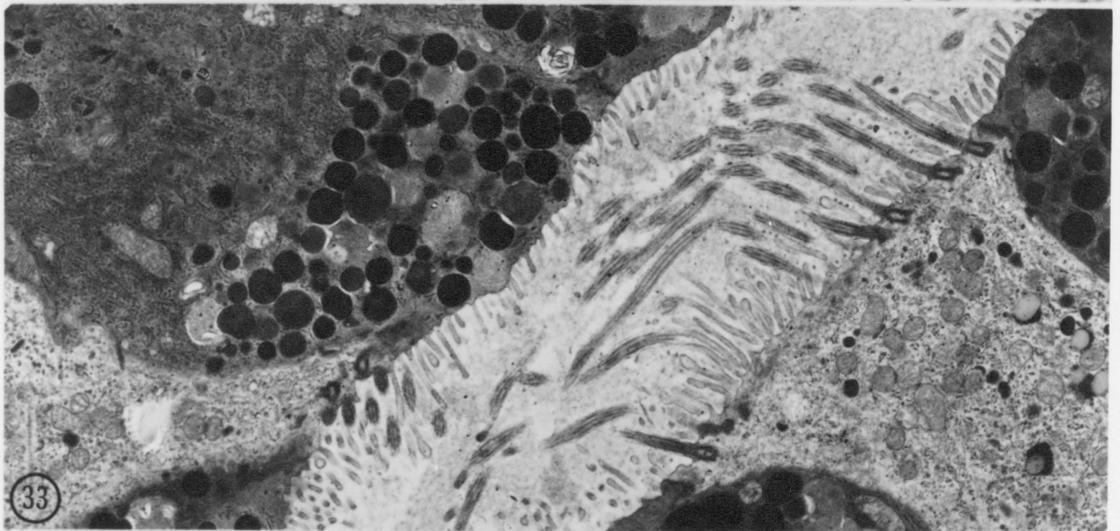
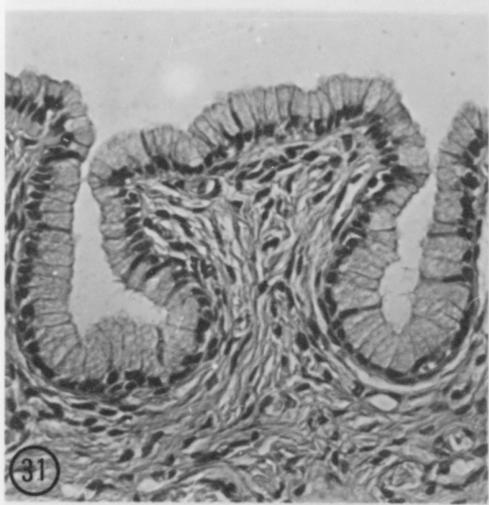
Figure 32. A Histoiresin section through cervical grooves demonstrating apical restriction of the grooves (240X).

Figure 33. An electron micrograph through a narrow epithelial fold demonstrating the presence of both secretory and ciliated cells. Note the varying density of the secretory granules and the lack of granules in the ciliated cell (3750X).

Figure 34. A Histoiresin section stained with toluidine blue which demonstrates the irregular shape of ciliated cells due to the crowding of these cells within the epithelium (240X).

Figure 35. A comparison of percent ciliated cells within the apical and basal epithelial areas across the four quarters of the cervix (mean percent + SD of 4 animals).

Epithelial Cells of the Follicular Phase Cervix.



3-4.2 Histoiresin

The embedding of Karnovsky's-fixed tissue samples in Histoiresin allowed the preparation of thin (1 to 2 μ m) sections which readily accepted histological stains. This technique, therefore, provided a clearer image of epithelial structure as well as spermatozoan structure due to reduced section thickness and improved fixation (reduced shrinkage). For general differentiation of nuclei, cell structure and mucin granules, sections were stained with toluidine blue. Evidence for a directionality of spermatozoa within the folds and grooves of the cervix was apparent in many tissue sections. In areas where longitudinal sections through a fold or groove contained sagittally sectioned spermatozoa, they were often observed to be aligned in a parallel direction above the epithelial surface and traveling in opposition to the apparent ciliary beat (Fig. 40). Sperm within cross-sectioned grooves were most often observed (as in paraffin embedded tissue) in the basal-most areas. However, sperm cells observed in a few grooves where larger sperm numbers were present appeared to be more randomly distributed, and lacked the more commonly observed unidirectional orientation (Fig. 41).

3-4.3 Ultrastructure

Cervical tissue samples fixed in Karnovsky's fixative and post-fixed in 1% osmium tetroxide were embedded in an epoxy resin for transmission electron microscopy. Areas in which good sperm retention was observed were selected for thin sectioning. Micrographs from these areas revealed, as previously noted, a directional orientation of spermatozoa in opposition to ciliary beat. This determination was based on the plane of section through either an acrosomal ridge or a portion of the flagellum (Fig. 42 & 43). A large majority of those spermatozoa in which direction could be determined, were oriented unidirectionally. An exception may be observed in Fig. 44 in which a single cell is directed opposite to other cells in the field. Closer obser-

PLATE 12 Sperm Transport and Ciliated Cells

Figure 36. A historesin section stained with toluidine blue which demonstrates an area densely populated by ciliated cells which occurred in isolated $\frac{1}{4}$ patches', particularly within the fourth (most cranial) quarter of the cervix. (727X).

Figure 37. An electron micrograph of cross sectioned cilia demonstrating the regularity of their beat pattern as cilia are aligned in straight rows (10,000X).

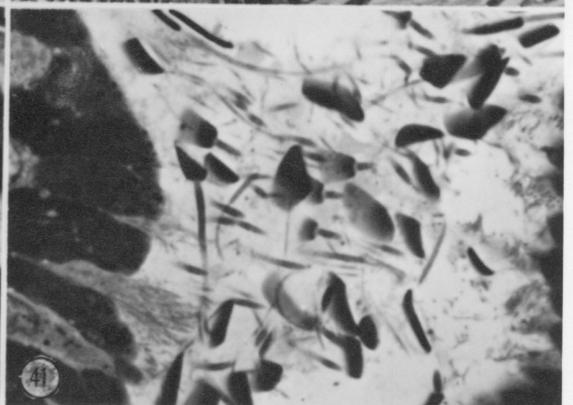
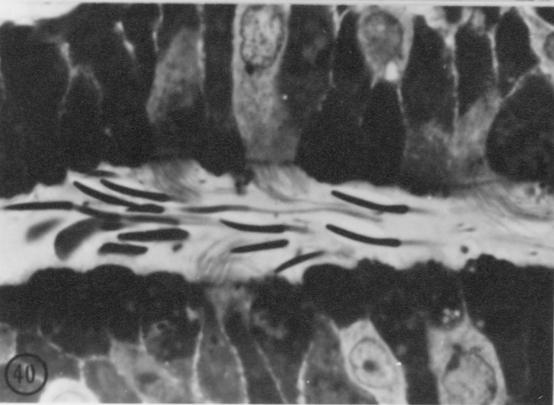
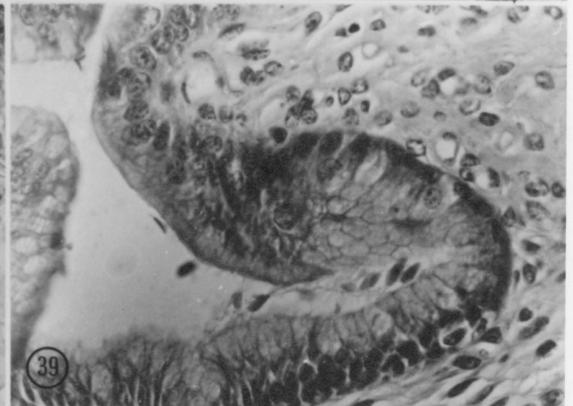
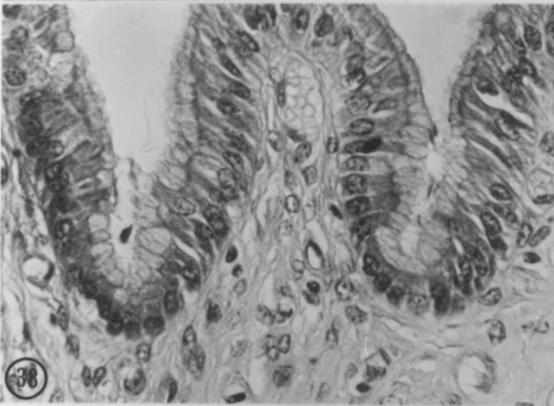
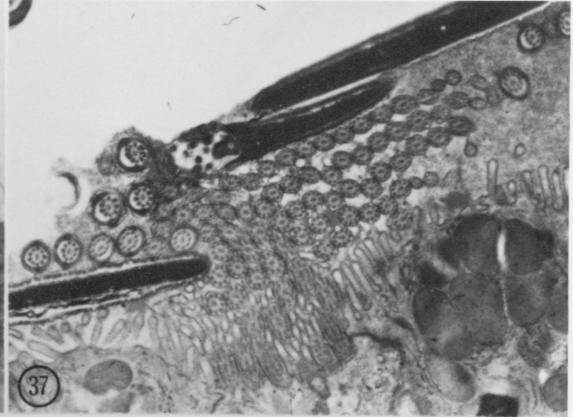
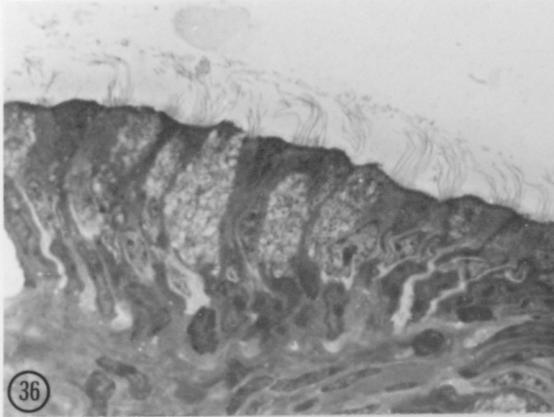
Figures 38 - 41. Cervical tissue preparations from a cow bred naturally at estrus and slaughtered 8h later.

Figures 38 and 39. Cross sections of paraffin embedded tissue through cervical grooves which have been stained with H&E demonstrate spermatozoa deep within basal areas (arrows) (290X).

Figure 40. Spermatozoa within this narrow fold in a Historesin preparation are observed swimming in a unified direction (note evidence of flagella) in opposition to the beat pattern of ciliated cells (727X).

Figure 41. A Historesin preparation showing spermatozoa evident within a cervical groove which appear to lack organization or directionality. (727X).

Sperm Transport and Ciliated Cells.



vation, however, reveals cell agglutination and acrosomal deterioration have taken place, which may have effectively prevented its intrinsic orientation.

PLATE 13 Electron Micrographs Depicting Sperm Transport

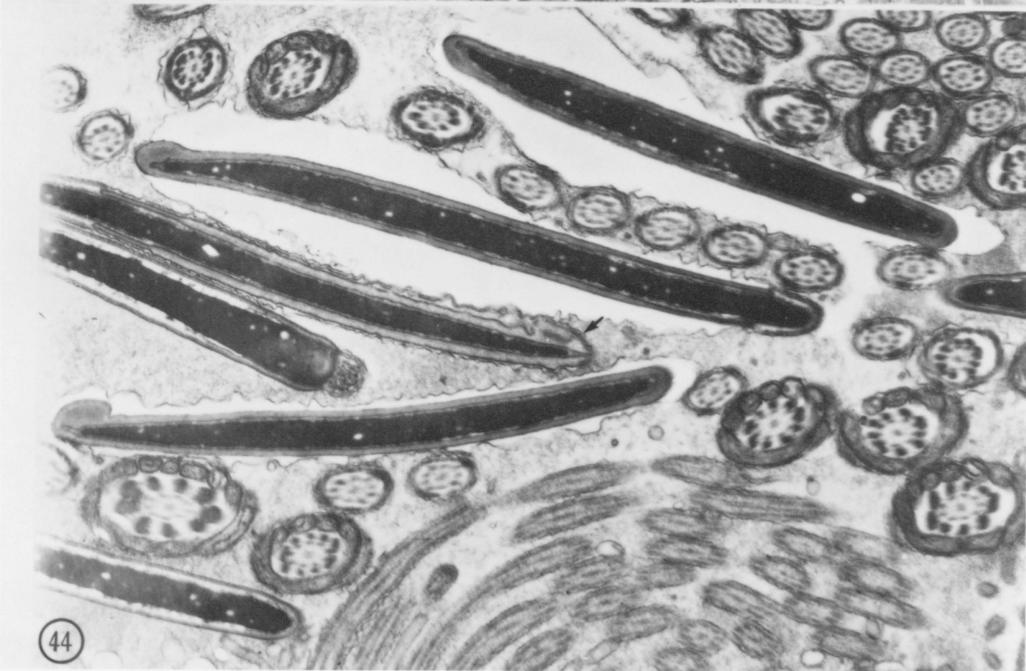
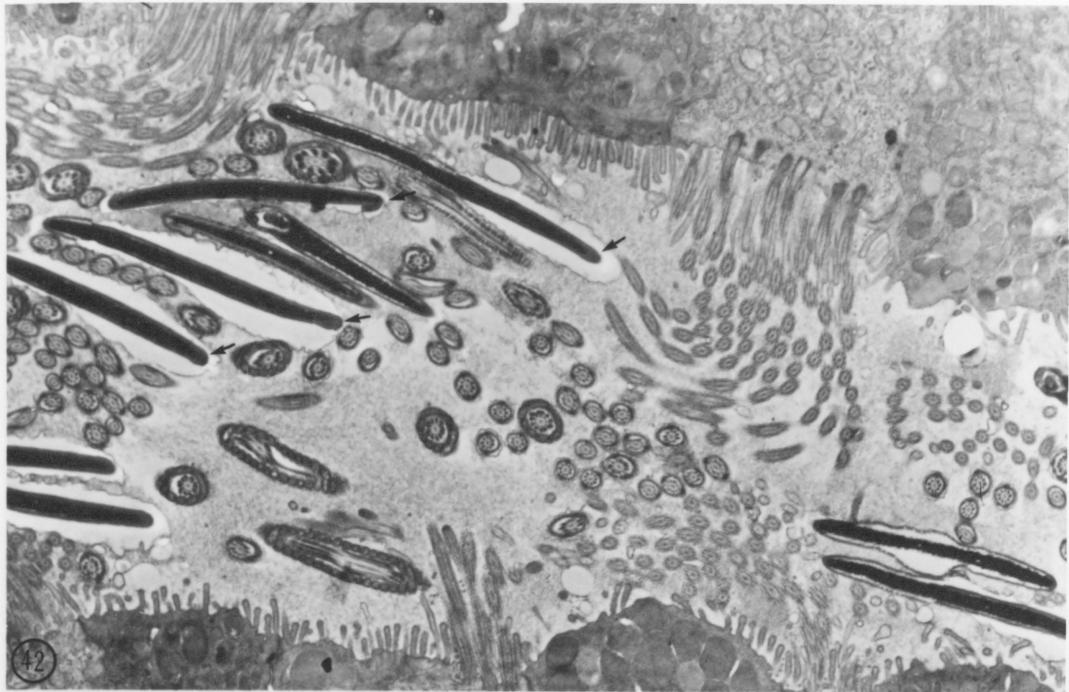
Figures 42 - 44. Transmission electron micrographs of spermatozoa traversing the cervix of a cow which was bred at estrus 8-h prior to slaughter.

Figure 42. A unified direction of travel which is in opposition to the direction of ciliary beat is apparent in sagittal sections through several spermatozoa within the field. Note: acrosomal membranes evident on the right of several cells (arrows) as well as sections through the capitular region of the flagellum on the opposite end of two additional cells (3710X).

Figure 43. As above (7258X). Spermatozoa traveling unidirectionally within a narrow fold, in opposition to ciliary beat.

Figure 44. An additional group of spermatozoa demonstrating directionality in opposition to ciliary beat, but with the exception of one cell, as evidenced by the acrosomal ridge (arrow), which is opposite in direction. However, acrosomal deterioration as well as agglutination of cell membranes with adjacent cells can be observed in this cell (12,903).

Electron Micrographs Depicting Sperm Transport.



DISCUSSION

4-1 Structure

The structure of the cervix as revealed in this study supports the concept presented by Herrick (1951) in the bovine and Fluhmann (1958) in the human. Both proposed a system of clefts and grooves as opposed to blind ending tunnels or glandular formations. The cervical canal is formed by primary and secondary longitudinal folds radiating out from a central, often flattened luminal region. These folds, some of which originated within the fornix vagina, were found to enter and traverse the cervical os and each additional annular ridge, at which points they gained their greatest depth and complexity. The numerous shallow grooves along the walls of these folds, in cross section, could suggest the formation of glandular structures which are often referred to as 'crypts'. The term 'crypt' which is used frequently when referring to cervical structure in all mucus-secreting species (Mattner, 1968; Hafez and Kanagawa, 1972) implies a definition of slender pit or small glandular cavity. Indeed, illustrations depicting cervical structure often maintain this concept. Although glandular formations of this nature were not found to be present, the winding and backward reflections of longitudinal folds and grooves (see Fig. 10) could conceivably form a functional "pocket" within complex areas. In addition, a

tunnel-like formation could exist in other grooves where the neck region was observed to be restricted due to a joining of opposing cells on either side of the apical margin (see Fig. 33). Whether this phenomenon is zonal (progressing along the apex of the entire length of the groove) or restricted to a few cells was not determined. Serial sections through a 20 -30 μ m distance in two tissue blocks, however, demonstrated apical restriction which was maintained at least through this distance. Considering the possibility that apical restriction does continue over a longer range, a functional tubular formation could be created which might be open ended. This would allow flow of mucins (due to a vaginal direction of ciliary beat) along a longitudinal path. Another possibility for the existence of a functional tubular-like formation could occur as a result of the apical production of restrictive mucin layers (as observed in Fig. 28). These mucins could similarly block the apical margin of grooves, trapping the less dense mucins secreted within the grooves as well as spermatozoa which might be traversing these areas.

4-2 Histology

Follicular phase cervical tissue which was stained with AB at pH 1.0 for the differentiation of only sulfomucins indicated that in many areas there was a slightly greater concentration of sulfated mucin within cells in apical as opposed to basal areas of the grooves. When AB was used at pH 2.5 for the differentiation of both sulfomucins and sialomucins, an increase in overall staining intensity was evident (as expected) due to the addition of sialomucin positive staining. This increase in stain intensity was, however, more evident in basal than apical areas, indicating a predominance of sialomucin production by the basal cells. These results support the conclusions of Heyden and Adams (1979), who reported apical sulfomucin and basal sialomucin production in the estrogen-stimulated cervix of domestic ruminants. It also supports the findings of Hamana et al (1969) who reported an increase in the amount of sialomucin present relative to total protein content of estrus mucins. These increased quantities of sialomucin

may then function, due to their water binding capacity, (Daunter, 1980) to aid in hydration of estrus mucus, thereby producing a predominance of the characteristic reduced-viscosity mucins within basal areas during estrus.

Similar staining in luteal phase tissue revealed a trend reversal where sulfomucins appeared to be the predominant acidic mucin in basal areas, with no appreciable sialomucin production. These results are also consistent with Heyden and Adams (1979) who reported a predominance of sulfomucin production in the absence of estrogen replacement in ovariectomized sheep.

Follicular phase tissue stained with PAS-AB revealed the production of neutral which are reportedly longer chain glycoproteins than the acidic mucins (Gallagher, 1981). Production is largely concentrated in apical areas lining the folds along with the sulfomucins already reported in these areas (as evidenced by AB pH 1.0 staining). Any structural relationship which might exist between these two mucin species has not been established. However, staining patterns evident in secreted intra-luminal mucins revealed neutral mucin layers often outlined by AB positive mucins. It is possible that sulfomucins could add to the structural integrity of neutral mucins by surrounding the non-polar, hydrophobic glycoproteins with a polar surface layer which would then be compatible with the aqueous fraction. Relatively little neutral mucin was evident in basal areas of follicular tissue which were largely filled with sialomucins (in addition to some sulfomucins). An apparent pattern of flow in secreted intra-luminal mucins revealed a laminated system of interfaces between acidic and neutral mucins and will be discussed later in this section.

Luteal phase tissue was characterized by an increase in the production of neutral mucins, particularly in basal areas where sialomucin was no longer being produced. This tissue also revealed less staining consistency than was evident in follicular phase animals. Secreted mucins within luminal areas appeared to lack the organized layers of mucus interfaces evident in follicular phase tissue (Figs. 24&30). An increase in sulfomucin production with a decrease

in sialomucin production could conceivably contribute to the intricately cross-linked system, by disulfide bonds and reduced hydration.

Contrary to the present findings, Wordinger (1972), using the PAS-AB stain technique, found no difference between apical and basal areas in follicular phase animals. This could have been due to his use of animals at day 3 of the Estrous cycle, or perhaps a result from over-staining with PAS which effectively masks AB. In addition, Heyden and Adams (1979) (also using the PAS-AB technique and animals in the follicular stage of the Estrous cycle) reported a predominance of bluish purple cells in apical regions while cells in basal areas (referred to as secondary indentations) stained red and purple. While this staining pattern is not consistent with the results of the present study and would indicate an apical production of acidic mucins and basal production of neutral mucins, their findings could be a result of formal saline fixation. Similar results were produced within some regions (approximately 1 in 4) which were fixed with formal saline in this study (see Section 3-2.3).

Satisfactory preservation of intra-luminal mucin within cervical folds proved to be difficult. However, substantial mucin preservation was achieved in one animal slaughtered at estrus, and one luteal animal, with varying degrees of successful preservation evident in all others. The secretory pattern present in luminal mucins was demonstrated in cross section by both PAS-AB and HID-AB stains, and a flow pattern within the three-dimensional structure of the cervix at estrus was apparent. Neutral mucins, accompanied by sulfomucins, emerged along the apical ridges between grooves, while sialomucins, were intermittent between neutral mucin layers and within grooves. The more densely staining long chain neutral mucins appeared to extend from apical ridges as sheets (rather than chains) of interconnected micelles, and progressed toward the central canal where they would be drawn together as they flowed toward the vagina (see Fig. 31). This pattern was most evident within deeper peripheral regions where most grooves were continual and parallel in nature as opposed to the central regions where they occasionally lacked continuity and direction. Sialomucins, which were predominant within grooves and between neutral mucin layers, are less viscous due to their hydrated nature. This would permit an increase

in flow rate either between neutral mucins layers or within grooves. It is conceivable that flow of mucins in these areas, as directed by ciliary beat, would be perpendicular to the neutral and sulfomucin layers within peripheral regions. This model of mucus structure and flow in vivo would also support the concept of a system of mucus hydration described by Tam and Verdugo (1981). They suggested that the neutral-sulfomucin layers could function as a semi-permeable membrane thereby establishing a Donnan equilibrium. In this system entrapped acidic mucins (presumably sialomucins) could generate the osmotic drive while transepithelial movement of aqueous components and electrolytes regulate hydration of the mucus structure during estrus (see Section 1.5). In addition, Dische (1963) described the existence of a reciprocal relationship in the relative proportion of neutral fucomucins and sialated mucins as they vary according to hormonal influence. This reciprocal relationship may be accomplished by an exchange of sialic acid with fucose residues at their terminal position on the glycoprotein complex (Daunter, 1984). An accompanying reduction in hydration would occur with the displacement of sialic acid (as observed in luteal mucins) which would result in a reduction of intracellular spaces. This work is consistent with the results of the present study in which the proportion of neutral mucin was observed to increase in luteal animals, while sialomucin production was reduced.

4-3 Epithelial Cells

Mucin granules present within epithelial cells contain both acidic and neutral mucins in varying proportions depending on location of the cell and stage of the Estrous cycle. These differences were evident in Histoiresin stained sections, (Fig. 25) although ultrastructural differences were not specifically defined. The distended cells in basal areas of follicular phase grooves were often less regular in shape, elongate, and with an irregular nucleus near the basement membrane. Ciliated cells interspersed among these secretory cells were found to be slightly more abundant in apical areas. This difference could be due to crowding, which was evident in basal areas. In

these instances, distended secretory cells often distorted ciliated cells into narrow bands (Fig. 35) which could have been overlooked during the counting procedure. It is also possible, however, that an increased number of ciliated cells are indeed present and are necessary in apical areas for moving heavier mucins out of the cervix, maintaining phase separation and/or providing the energy for oscillation within the mucus as suggested by Davajan (1970). Further investigation which would distinguish central vs peripheral regions would be useful and could possibly help account for some of the variability, particularly in the fourth (cranial) quarter of the cervix.

4-4 Sperm Transport

The observed alignment of spermatozoa within the cervix in opposition to the beat of ciliated cells would suggest that a second function of these cells is in directing spermatozoa toward the uterus by establishing mucus flow toward the vagina, as sperm have been observed to swim against the stream (Zinner et al., 1984). The flow of mucus toward the vagina was considered by Mattner (1966) in his proposed concept whereby privileged paths are established in the transport of spermatozoa toward the mucosa via lines of strain. A new model, which would expand his theory by considering the three dimensional structure as a series of continual grooves rather than glandular depressions, might be considered here. Spermatozoa entering the mucus structure would be compelled to follow the path of least resistance. This would be within hydrated sialomucin rich layers flowing between denser and longer chain neutral mucins. Since cervical folds were observed to originate within the fornix vagina, spermatozoa deposited in this area would have access to direct entry into these folds. They would then continue into and through the cervical structure without having to enter the central cervical canal. In a manner similar to that proposed by Mattner (1966), "privileged paths" would still lead into basal areas within mucosal grooves. Layers of neutral mucin emitted from adjacent apical areas could conceivably restrict lateral

progression of spermatozoa. Wave currents established by cilia would also encourage movement of spermatozoa toward mucosal surfaces. Spermatozoa entering the grooves could thus continue their progression toward the uterus within the confines of continuous shallow folds rather than being sequestered for storage as suggested by Mattner (1966).

This concept is supported by the distribution of intact spermatozoa observed in the present study. In cross sections of cervical tissue, spermatozoa were most frequently apparent deep within basal areas (see Figs. 39 & 40). In longitudinally sectioned tissue, spermatozoa were oriented parallel to the epithelium in opposition to the direction of ciliary beat (see Fig. 41). A three-dimensional illustration of this concept is presented in Fig. 31. In a few sections, however, exceptions to this model were observed. One such area is illustrated in Fig. 42 where spermatozoa appear to lack a unified direction. Assuming these cells were not reoriented as a result of tissue processing, it is possible that flow currents may have been disrupted in these isolated areas due to a complex twisting or reflecting of the folds, creating 'pockets' within grooves which could detain the forward progression of spermatozoa, perhaps even contributing to a storage function. Spermatozoa were also observed within the denser mucin layers of some luminal areas. It is uncertain whether these populations of cells, which were observed most often within the fourth quarter of the cervix, are indeed traversing the cervix within privileged paths, or simply trapped within neutral mucins which are being cleared to the vagina. Evidence for the latter which would suggest their loss to the vagina is presented in an investigation of the fate of sperm following uterine insemination, Mitchell et al (1985). These workers accounted for $73 \pm 3.7\%$ of the inseminate by 12-h post breeding. Accounted for in discharged mucus was $60 \pm 4.6\%$ of the spermatozoa in the inseminate. This work would indicate that a high proportion of sperm cells observed in these luminal mucins are perhaps in the process of retrograde removal.

In a study conducted by Smith, Boland and Gordon (1977), a comparison of AI conception rates in sheep was made using a deep cervical gun designed to penetrate the cervical canal to a greater distance than the normal Cassou sheath AI gun. They found that deep cervical insemination yielded lower fertility than the more shallow deposition. This would be surprising

if sperm transport was primarily through the cervical canal, but not if access to privileged paths was greatest in the fornix vagina. Other work by Fulkerson et al (1982) found that by using artificial insemination in sheep, where semen deposition was within the cervical canal, twice as many spermatozoa were required to achieve a conception rate equivalent to natural service using continually mated rams ejaculating known numbers of sperm. This would suggest that spermatozoa deposited centrally within the cervical canal are subject to removal with retrograde mucus flow and may, therefore, be in a less favorable position for cervical transport than those placed at or near the fornix vagina, where they may gain entry into basal areas of folds and grooves.

4-5 Summary

The evidence presented in this study suggest that privileged paths for the transport of viable spermatozoa are present within the cervical structure in basal areas of the grooves. These grooves offer a continual course within folds which were observed to extend from the fornix vagina to the uterus. The length of grooves within these continual folds was observed by surface staining techniques to be extensive in peripheral areas, although some variations were observed and total lengths of these fine grooves within the entire cervix could not readily be determined. Considering the model of mucus flow presented in Fig 31 and the predominance of sialomucin production in basal areas of follicular phase tissue, sperm entry into these areas would be facilitated and maintained as the course of least resistance. In addition, since spermatozoa were observed to oppose the direction of ciliary beat (Figs. 42, 43, & 44) the wave action of the cilia may serve to establish a flow current, or a system of propagating waves as suggested by Odeblad (1962). This could serve to maintain a progressive directionality among sufficiently viable sperm.

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Appendix A. Computer-Aided Reconstruction of Serial Sections

Stereo pairs were generated by taking the y coordinate for corresponding points to be identical. Given an image with a boundary point having x-coordinate x_1 and a 3-D-depth z , the corresponding point on the second image has x-coordinate x_2 where

$$x_2 = x_1 \cos(A) \cos(B)$$

$$B = \tan^{-1} z x_1$$

$$A = B + \Delta\Theta$$

The angle $\Delta\Theta$ was set to be 7° . Stereo pairs were photographed directly from the display screen with a Matrix Instruments color graphics camera using Polaroid SX - 70 film.

Appendix B. Tissue Fixation

Bouins Fixative (Lillie, 1965):

picric acid, saturated aqueous	225 mls
37-40% formaldehyde solution	75 mls
glacial acetic acid	15 mls
Fixation time - 72 hrs.	
Rinse and store in 70% ethanol.	

Phosphate Buffered Formalin:

Buffered Formaldehyde - Fresh (10%) from Fisher Scientific.

Alcoholic Formalin (Lillie, 1965):

37-40% formaldehyde solution	100 mls
95% alcohol	900 mls

Formal Saline (Lillie, 1965):

37-40% formaldehyde solution	100 mls
sodium chloride	8.5 gms
distilled water	900 mls

Karnovsky's Fixative: 1.5% gluteraldehyde and 1% paraformaldehyde (Karnovsky, 1965):

1. Dissolve .53 gms paraformaldehyde in 49.4 mls 60-70°C water, and clear with 2 drops 1N NaOH.
2. Cool to room temp. and add 5.6 mls 25% gluteraldehyde solution (Sigma).
3. Adjust pH to 7.2.
4. Add .2M phosphate buffer (23mls soln A and 77mls soln B) 1:1 with fixative as prepared above.

Phosphate buffer:

Stock solution A: .2M NaH₂PO₄

Stock solution B: .2M Na₂HPO₄

5. Obtain osmolarity reading.
6. Prepare 0.1M buffer rinse.
23mls solution A
77mls solution B
99mls distilled water
1ml of 1% CaCl₂ added slowly to above solution while stirring.
7. Obtain osmometer reading of prepared 0.1M buffer rinse and adjust by the addition of sucrose according to the following formula (Hayat, 1981):

$$\frac{\frac{1}{2}(\text{mOsm fixative} - \text{mOsm buffer}) + \text{mOsm buffer}}{30} = \% \text{ sucrose needed in buffer wash}$$

8. Following a fixation time of 1 to 1 ½h, rinse in three changes of prepared phosphate buffer and store until embeddment.

Karnovsky's Fixative: 4% gluteraldehyde and 2% paraformaldehyde (Karnovsky, 1965)

1. Dissolve .62g paraformaldehyde in 21 mls 60 to 70°C water and clear with several drops 1 N NaOH solution (Sigma).

2. Adjust pH to 7.2.
3. Add 31 mls .2 M phosphate buffer (see step 4 above)
4. Repeat steps 5 - 8 as above.

Embedding procedure for Electron microscopy

1. Dehydration of fixed and rinsed tissue samples as follows:

70% Ethanol	10 min
95% Ethanol	10 min
95% Ethanol	10 min
100% Ethanol	10 min
100% Ethanol	10 min
Propylene oxide	10 min
Propylene oxide	10 min
Propylene oxide	10 min

2. Infiltrate dehydrated tissue in 1:1 propylene oxide and epoxy araldite mixture for 4 h or overnight.
3. Heat at 60°C for 1 h or until reduced by 50% in volume to drive off propylene oxide.
4. Embed infiltrated tissue in freshly prepared Epoxy- Araldite resin. Heat at 60°C for 1 to 3 days to polymerize.

Preparation of Epoxy-Araldite Resin (Polysciences, Inc)

Nadic Methyl Anhydride (NMA)	6.0 g
Araldite Epoxy Resin 502	8.0 g
mix thoroughly and add:	
Dodecenylsuccinic Anhydride (DDSA)	10.8 g
Poly Bed 812 Embedding Media	9.6 g

mix thoroughly with magnetic stir or by hand for about 5 min., then add accelerator:

Tri(Dimethyl Amino Methyl) Phenol (DMP 30) .48 ml

mix completed resin thoroughly and seal to prevent hydration until use.

Preparation of Histo-resin (LKB)

1. Dehydration of fixed and rinsed tissue samples as follows:

70% Ethanol 10 min

70% Ethanol 10 min

95% Ethanol 10 min

95% Ethanol 10 min

2. Infiltrate tissue with a 1:1 solution of 95% ethanol and "infiltration solution" (prepared as directed in Histo-resin kit) for two or more hours. Transfer to 100% infiltration solution for an additional 4 h or overnight.

3. Embed infiltrated tissue in the following embedding media:

Infiltration solution 15 mls

activator 1 ml

mix thoroughly

The embedding media will begin to polymerize within 10 to 15 min of the accelerator addition.

Tissue blocks will be hard enough for sectioning within 24 h.

Appendix C. Staining Procedures

Alcian Blue pH 1.0 procedure (Pearse, 1985)

Method:

1. Bring sections to water.
2. Stain for 30min in 1% Alcian Blue 8GX (Sigma Chem. Co.) in 0.1N HCl (pH 1.0).
3. Blot dry with fine filter paper (or bibulous paper), and rinse in running water for 5 min.
4. Dehydrate in alcohols, clear in xylene and mount.

Result:

Sulfated mucosubstances, only, stain blue.

Alcian Blue pH 2.5 procedure (Pearse, 1985)

Method:

1. Bring sections to water.
2. Stain for 30 min. in freshly filtered 1% Alcian Blue 8GX in 3% acetic acid (pH 2.5).
3. Wash in running water for 5 min.
4. Dehydrate in alcohols, clear in xylene and mount.

Result:

Weakly acidic sulphated mucosubstances, hyaluronic acids and sialomucins, dark blue.
Strongly acidic sulphated mucins are stained weakly.

Alcian Blue-PAS Procedure (modified from Pearse, 1985)

Method:

1. Bring sections to water.
2. Rinse briefly in 3% aqueous acetic acid.
3. Stain for 1h in 1% Alcian blue 8GX in 3% acetic acid (pH 2.5).
4. Rinse briefly in water and then in 3% acetic acid, running water and distilled water.
5. Oxidize for 10 min. in freshly prepared 1% periodic acid (aqueous) at room temp.
6. Wash in running water for 5 min.
7. Immerse in Schiff's reagent for 1 min.
8. Wash in running water 2 min.
9. Rinse in 2 changes of 0.5% sodium bisulphite, 1 min each.
10. Wash in running water for 5 min.
11. Dehydrate, clear and mount.

Result:

Acid mucosubstances including hyaluronic acid, sialomucins and all but the most strongly acidic sulphated mucosubstances stain blue or bluish-purple, periodate- reactive neutral mucosubstances stain red.

High Iron Diamine-Alcian Blue Stain (HID) procedure (Pearse,1985)

Prepare a fresh solution containing 120mg N,N-dimethyl-m-phenylenediamine dihydrochloride and 20mg N,N-dimethyl-p-phenylenediamine hydrochloride in 50 ml distilled water. Add this solution to a Coplin jar containing 1.4 ml 10% FeCl₃.

Method:

1. Bring sections to water.
2. Oxidise for 10 min in 1% aqueous periodic acid (to demonstrate neutral as well as sulfated mucosubstances).
3. Rinse for 5 min in running water.
4. Stain for 18 h in the high iron diamine solution.
5. Rinse rapidly in water and stain in 1% alcian blue in 3% acidic acid for 30 min.
6. Dehydrate, clear and mount.

Result:

Sulphated and neutral mucosubstances, purple-black. Sialomucins unstained (blue after Alcian blue).

Hematoxylin and Eosin (modified from Lillie, 1965)

Method:

1. Bring sections to water.
2. Stain in Harris' hematoxylin for 2 min.
3. Rinse for 5 min in running water.
4. Blue in mild ammonia solution (2 drops concentrated ammonium hydroxide per 100 ml water) and rinse briefly in water.
5. Bring sections to 70% alcohol.
6. Stain in alcoholic Eosin-Phloxine for 30 sec.
7. Dehydrate and mount.

Result:

Nuclei stain blue, cytoplasm pink to orange.

Uranyl Acetate and Lead Citrate Staining Procedure for Copper Grids (Venable and Coggeshall, 1965)

Method:

1. Submerge grid in uranyl acetate for 3.5 min.
2. Remove and rinse thoroughly with distilled water.
3. Flood with lead citrate and stain, covered, for 1 to 2 min.
4. Rinse very thoroughly with distilled water.
5. Allow 30 min drying time before viewing in electron microscope.

Stain Recipes

Shiffs Reagent:

1. Dissolve 1gm basic fuchsin in 200 ml boiling distilled water.
2. Add 2 g Potassium metabisulfite and 10 ml 1N HCl.
3. Let mixture stand for at least 24 h in refrigerator.
4. Add 0.5 g activated carbon, shake well and filter. Filtrate should be colorless.

Harris' Hematoxylin:

Hematoxylin crystals	5.0 g
Ethanol, absolute	50.0 ml
Aluminum ammonium sulfate	100.0 g
Distilled water	1000 ml

Mercuric oxide

2.5 g

Dissolve the hematoxylin in the alcohol and set aside. Dissolve the alum in the water while gently heating. Add the alcohol solution to the alum and rapidly bring to a boil. Remove from heat and immediately but slowly add mercuric oxide crystals. Reheat until solution returns to a rapid boil. Remove from flame and cool vessel in a basin of cold water. Add 2 ml of glacial acetic acid per 100 ml of solution and filter before use.

Eosin Phloxine:

Eosin Y	4.0 g
Phloxine B	1.0 g
Ethanol, 80%	2000 ml

Toluidine blue:

1% toluidine blue in 1% borate

Uranyl Acetate:

0.9g uranyl acetate
30 ml double distilled water

Mix with magnetic stirrer until dissolved (about 1 h). Spin down at 12100 x g for 20 min immediately before use.

Lead citrate:

0.09 g lead citrate
30 ml double distilled water
0.3 ml 10N NaOH

Add .3 ml 10N NaOH to 30 ml water. Add lead citrate crystals to solution and mix gently until dissolved. Spin down at 12100 x g for 20 min immediately before use.

Appendix D. Histology Data Values

The following tables contain values obtained from stain intensity estimates which were compiled and presented graphically in Figures 12 - 19 (Plates 5, 6 and 7).

Estimates were obtained in duplicate from two different tissue blocks within each quarter cervix and are represented in these tables as 'a' and 'b' data points for each of 5 follicular animals and each of 3 luteal animals. Data points presented for Cow 1 (a and b) in the follicular values (Tables I, II, and IV) were obtained from an animal sacrificed at the peak of Estrus. The remaining animals included in the follicular data were sacrificed in early metestrus, 8 to 12 h post breeding. All data values are presented separately, as indicated in the tables, for apical (top) and basal (bottom) areas within the central (left) and peripheral (right) regions of the cervix.

I
FOLLICULAR
ALCIAN BLUE pH 1.0

		CENTRAL				PERIPHERAL			
	Cow	Q ₁	Q ₂	Q ₃	Q ₄	Q ₁	Q ₂	Q ₃	Q ₄
APICAL	1a	4	3	2	2	3	3	3	1
	1b	1	2	2	2	1	2	2	2
	2a	1	3	4	2	1	3	3	3
	2b	4	5	2	5	4	5	3	5
	3a	-	4	3	4	5	4	5	4
	3b	3	4	3	4	3	5	3	4
	4a	1	2	4	2	3	2	4	3
	4b	3	2	2	3	2	3	3	3
	5a	2	3	3	4	2	3	3	4
	5b	3	2	2	3	2	3	2	3
AVERAGE:		2.40	3.00	2.70	3.10	2.60	3.30	3.10	3.20
BASAL	1a	3	2	3	2	2	2	4	2
	1b	1	2	3	2	1	4	3	2
	2a	2	2	4	2	2	2	2	2
	2b	4	4	3	4	4	4	2	4
	3a	-	3	4	4	4	3	4	3
	3b	3	4	4	3	2	4	4	3
	4a	1	2	3	2	2	1	3	3
	4b	2	2	3	3	2	2	3	2
	5a	2	3	3	3	2	3	3	3
	5b	3	3	2	2	3	2	2	2
AVERAGE:		2.33	2.70	3.20	2.70	2.40	2.70	3.00	2.60

II
FOLLICULAR
ALCIAN BLUE pH 2.5

		CENTRAL				PERIPHERAL			
	Cow	Q ₁	Q ₂	Q ₃	Q ₄	Q ₁	Q ₂	Q ₃	Q ₄
APICAL	1a	4	2	3	2	3	3	2	1
	1b	1	2	2	2	1	2	2	1
	2a	1	4	5	3	1	4	5	3
	2b	5	5	3	5	5	5	3	5
	3a	-	4	3	5	4	4	3	4
	3b	2	3	3	3	3	3	3	3
	4a	1	4	5	2	3	4	5	3
	4b	2	3	4	3	3	4	5	4
	5a	2	4	3	3	2	4	3	3
	5b	4	4	4	3	3	4	4	3
AVERAGE:		2.44	3.50	3.50	3.10	2.80	3.70	3.50	3.00
BASAL	1a	4	3	4	3	4	3	4	3
	1b	3	3	3	3	3	3	3	3
	2a	2	4	5	4	2	3	5	3
	2b	5	5	4	4	5	5	4	4
	3a	-	4	4	5	4	4	4	5
	3b	3	4	3	3	4	4	3	4
	4a	2	4	4	3	3	4	4	3
	4b	3	3	5	3	3	4	5	3
	5a	3	4	3	3	3	4	3	3
	5b	4	3	4	3	4	3	4	3
AVERAGE:		3.22	3.70	3.90	3.40	3.50	3.70	3.90	3.40

III
LUTEAL
ALCIAN BLUE pH 1.0

		CENTRAL				PERIPHERAL			
	Cow	Q ₁	Q ₂	Q ₃	Q ₄	Q ₁	Q ₂	Q ₃	Q ₄
APICAL	1a	2	3	3	1	2	4	2	2
	1b	3	3	2	2	3	2	1	1
	2a	1	2	2	3	1	2	3	3
	2b	3	2	3	4	3	2	3	4
	3a	3	3	1	4	3	3	1	2
	3b	4	1	1	1	3	1	1	1
AVERAGE:		2.67	2.34	2.00	2.50	2.50	2.30	1.84	2.17
BASAL	1a	2	4	3	2	2	4	3	2
	1b	4	3	3	0	4	4	3	0
	2a	2	3	3	3	2	3	3	3
	2b	3	3	4	4	3	3	4	4
	3a	3	4	2	4	3	4	3	3
	3b	4	3	2	2	4	3	3	3
AVERAGE:		2.84	3.34	2.84	2.87	3.00	3.50	3.17	2.50

ALCIAN BLUE pH 2.5

APICAL	1a	3	2	3	2	4	3	3	4
	1b	2	3	2	1	3	4	2	1
	2a	3	1	3	3	3	1	4	4
	2b	4	2	3	2	4	3	4	4
	3a	1	3	2	4	1	3	2	3
	3b	1	2	2	2	2	2	2	2
AVERAGE:		2.33	2.17	2.50	2.34	2.67	2.67	2.84	3.00
BASAL	1a	4	2	3	3	4	3	3	3
	1b	3	3	2	1	3	4	2	1
	2a	3	3	4	4	3	3	4	4
	2b	5	2	3	3	4	3	4	4
	3a	2	4	3	4	3	4	3	4
	3b	3	4	2	3	3	4	3	3
AVERAGE:		3.38	3.00	2.83	3.00	3.33	3.5	3.17	3.17

V

LUTEAL

Periodic Acid Schiff's - Alcian Blue pH 2.5

Cow	CENTRAL								PERIPHERAL								
	Q ₁		Q ₂		Q ₃		Q ₄		Q ₁		Q ₂		Q ₃		Q ₄		
	AR	PAS															
APICAL																	
1a	5	8	4	8	2	8	5	7	4	8	8	6	5	7	2	7	
1b	3	8	3	8	5	8	6	5	4	8	6	5	7	8	7	6	
2a	5	5	4	8	6	4	8	5	5	5	4	8	4	6	8	5	
2b	3	6	7	8	3	7	5	6	7	5	6	4	7	4	8	5	
3a	3	7	3	7	2	8	4	6	5	5	7	3	5	5	3	7	
3b	5	5	2	8	2	8	7	3	8	4	3	7	3	7	3	7	
AVERAGE:	4.0	6.2	3.8	6.0	3.3	7.2	5.5	5.3	5.2	6.0	5.7	6.7	5.2	6.2	4.8	6.2	
BASAL																	
1a	7	6	7	4	8	5	7	3	6	5	8	6	8	5	7	5	
1b	7	3	8	5	7	8	6	4	6	7	7	4	7	8	7	6	
2a	5	5	4	8	4	8	7	4	2	8	5	6	7	7	7	7	
2b	6	4	7	4	7	4	8	5	7	5	6	5	7	5	6	5	
3a	5	5	6	4	3	7	8	2	7	3	8	2	7	4	7	3	
3b	6	4	8	2	6	4	6	4	8	2	7	3	8	4	6	4	
AVERAGE:	6.0	4.5	6.3	4.5	5.5	6.0	6.7	3.7	6.0	4.5	6.8	4.2	7.0	5.5	6.7	5.0	

IV

FOLLICULAR

Periodic Acid Schiff's - Alcian Blue pH 2.5

Cow	CENTRAL								PERIPHERAL								
	Q ₁		Q ₂		Q ₃		Q ₄		Q ₁		Q ₂		Q ₃		Q ₄		
	AB	PAS															
APICAL																	
1a	7	8	8	8	4	7	8	7	8	7	2	8	8	7	4	8	
1b	1	8	2	8	4	1	7	4	2	8	3	8	3	8	4	8	
2a	8	4	2	8	8	8	4	8	9	4	5	8	4	7	8	8	
2b	4	8	4	7	3	7	5	7	8	7	4	8	3	7	5	7	
3a	4	7	5	5	5	8	4	7	4	7	8	4	8	5	4	8	
3b	5	8	7	8	7	3	8	8	8	8	8	8	7	3	7	7	
4a	8	8	3	7	8	8	5	8	8	4	7	3	7	7	7	4	
4b	4	7	5	7	4	8	8	4	8	7	7	7	7	8	8	5	
5a	8	7	3	7	5	5	7	3	2	8	8	4	5	5	5	5	
5b	3	7	4	8	5	5	7	3	2	8	3	7	3	5	4	8	
AVERAGE:	4.8	7.2	3.8	6.8	4.8	5.8	6.1	5.7	5.0	7.0	6.1	6.1	5.0	5.8	5.3	6.6	
BASAL																	
1a	8	2	8	5	7	4	8	3	8	2	7	3	8	2	8	2	
1b	8	2	8	2	8	8	8	3	8	2	8	2	8	1	8	3	
2a	8	4	8	2	8	2	8	2	8	4	8	3	8	2	8	2	
2b	7	4	7	3	8	2	7	4	8	3	8	3	8	1	7	3	
3a	7	3	7	3	8	3	7	4	7	3	7	3	8	2	8	3	
3b	8	4	8	1	8	1	8	6	8	3	8	1	8	1	7	7	
4a	8	5	7	3	8	8	7	4	8	4	7	3	7	3	7	4	
4b	7	4	7	5	7	4	8	4	7	3	7	3	8	2	8	3	
5a	8	4	7	3	8	2	8	2	7	3	8	1	8	2	8	2	
5b	7	3	5	5	5	5	7	3	8	2	8	4	5	5	7	3	
AVERAGE:	7.5	3.5	7.0	3.4	7.7	3.7	7.7	3.5	8.1	2.8	7.3	2.6	7.7	2.1	7.7	3.2	

Appendix E. Percent Ciliated Cells

PERCENT CILIATED CELLS								
	Q_1		Q_2		Q_3		Q_4	
	<u>APICAL</u>	<u>BASAL</u>	<u>APICAL</u>	<u>BASAL</u>	<u>APICAL</u>	<u>BASAL</u>	<u>APICAL</u>	<u>BASAL</u>
	12	13	24	17	21	14	20	16
	19	11	20	18	26	19	17	12
	15	10	23	21	22	21	10	21
	21	10	18	20	27	16	63	18
	18	8	18	17	27	29	15	46
	12	14	17	21	23	21	17	43
	19	20	17	16	24	17	25	21
	20	26	22	20	24	21	25	17
	12	22	16	16	24	16	20	11
	19	13	14	18	17	17	21	18
	14	16	20	20	14	21	23	18
	17	15	23	17	18	23	25	45
	14	13	17	15	17	21	22	39
	21	19	21	13	25	24	29	16
	19	11	18	8	23	23	25	16
	—	6	—	15	—	25	—	17
		8		15		15		28
		12		17		20		19
		14		20		19		20
		18		13		21		18
		25		24		20		23
		21		16		16		24
		23		12		19		26
		17		17		18		23
		11		21		18		20
Average	16.80	15.04	19.2	17.08	22.13	19.76	23.1	22.84
SD	3.2	5.3	2.8	3.39	3.83	3.38	15.57	9.58

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