THE EARLY EFFECTS OF ESTRADIOL STIMULATION *IN VIVO* ON THE VAGINAL EPITHELIUM OF PREPUBERTAL BALB/cCRGL MICE

by

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To

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INTRODUCTION

Cyclical cytomorphological alterations in the mouse genital tract were first described in detail by Edgar Allen in 1922 (1). This description of the changes during the estrus cycle of the mature mouse codified and unified the many, prior investigations into a comprehensive picture. Snell (79) enlarged on Allen's findings and added both a review of the interim literature and his considerable experience gained at the Roscoe B. Jackson Memorial Laboratory. Working with colchicine-treated rat genital tract, Bertalanffy and Lau (9) contributed a comprehensive study of mitotic rates, cell renewal, and tissue cytodynamics at the light microscopic level. That these changes are produced by estrogens, primarily estradiol (55, 81), has been established by the work of many authors, including Allen and Doisy (2), Biggers (11), Perotta (64), Husbands and Walker (49), and Epifanova (24).

The changes occurring in rodent vaginal epithelium from diestrus to estrus include both cell proliferation and differentiation (9, 12, 30, 40). With the exception of immature and ovariectomized mice, estrogen-induced proliferation occurs only in the basal cell layer (5, 9, 30, 40). Cell differentiation occurs under the influence of estrogen and/or progesterone (43) with formation of either mucus or keratin at the surface (4, 11, 17). Recent evidence from autoradiographic studies indicates that the potential for differentiation of any cell is unidirectional after it leaves the stratum germinativum. Although hormones affect the expression of this potential in cells above the basal layer, they do not determine the type of differentiation (5, 28, 40). The basal cells, therefore, are the only vaginal cells with the potential for

multidirectional differentiation. As further proof of this, the vaginal epithelium of both immature and ovariectomized mice demonstrates mitoses in the superficial cell layer after estrogen stimulation; however, daughter mucous cells will not redifferentiate into keratinizing cells, with estrogen stimulation, nor will daughter stratum granulosum cells, which synthesize prekeratin fibrils, redifferentiate into mucous cells under estrogen-progesterone stimulation (5, 30, 40).

In order to obtain a research model in which reproductive hormones could be controlled and monitored effectively, most investigators have chosen to ovariectomize mature, female mice (3, 12, 43, 66, 71). With the exception of contributions by Forsberg and Olivecrana (30) and Cooper, Cardiff, and Wellings (17) all the aforementioned observations of exogenous hormonal effects on vaginal epithelium describe research on ovariectomized mice.

An alternate research model is the prepubertal female mouse. The primary requirement for the use of this model is that the hormonally manipulated animal has not reached the stage of endogenous reproductive hormone secretion.

J.-G. Forsberg *et al.* have described the early development of vaginal epithelium from 15 days gestation, where the vaginal anlage consists of paired Mullerian ducts and a caudal urogenital sinus, to completion of the vaginal canal and epithelium from cervix to introitus at 5 days postpartum (27, 28, 30). In addition to documenting the normal cytomorphologic alterations in the Mullerian and urogenital sinus epithelium, Forsberg *et al.* have described histochemical alterations in glycogen and mucus content (30). Glycogen is spread diffusely

throughout both the sinus epithelium and cranial, Mullerian epithelium at seventeen days gestation. In the newborn (approximately 20-21 days gestation) glycogen content and distribution are unchanged; however, with amylase digestion a thin layer of PAS-positive material remains on the surface of the Mullerian epithelium. At 5 days postpartum the Mullerian epithelium and sinus epithelium form one completely epithelialized vagina with two distinct zones, the basal and superficial layers. The superficial zone is heavily glycogenated; the basal zone is very slightly glycogenated. "After amylase treatment a thin layer of PAS positive substance remains on the surface of the epithelium and a diffuse staining can also be seen in the superficial cells" (30). While the author agrees with the presence of a superficial layer of PAS-positive, amylase resistant material, no diffuse staining of the superficial cytoplasm can be seen in the photomicrographs presented. By the 14th day postpartum the glycogen is limited to the superficial layer and the PAS-positive, amylase resistant material on the surface and in the most superficial part of the epithelium has increased. At 21 days postpartum the same conditions maintain (30).

When newborn mice are stimulated with 0.1 µgm to 5 µgm of estradiol, both mucification and squamification with keratinization occur promptly, thus demonstrating that neonatal mice have the ability to respond to estrogen stimulation by cytodifferentiation (28).

Forsberg *et al.* have shown that prepubertal mice are functionally inactive until approximately 12-13 days postpartum. Thus they should be equivalent to ovariectomized mice in their response to estrogens, without the morbidity of surgical intervention and without alteration of

physiologic homeostasis. In view of the fact that, as Cooper *et al.* have observed (17), "...these products, mucin and keratin, appear *de novo* during the first exposure of the vaginal epithelium of the immature animal to estrogen...", the non-stimulated prepubertal animal should provide an excellent model for the sequential study of the development of the mucin and keratin producing cytoplasmic systems.

With the many light microscopic studies of mouse vaginal epithelium (*vide supra*) and the numerous investigations utilizing mouse vaginal epithelium *in vivo* (45, 46, 47, 66, 82, 83, 85) and *in vitro* (13, 18, 44, 53), Cooper *et al.* (17) were surprised to find that no definitive study of mouse vaginal ultrastructure existed. The principal, ultrastructural studies of rodent vagina were limited to Burgos and Wislocki (14), Roig De Vargas-Linares and Burgos (72, 73), and Merker (54). Burgos *et al.* had focused their interest on the cyclic changes of the guinea pig and hamster genital tracts during estrus-diestrus cycling, while Merker was concerned with the effects of estrogen on the nuclei of rat vaginal epithelium.

Cooper *et al.* (17), therefore, documented the cytomorphologic events in keratin formation and at the same time provided an ultrastructural foundation for pre-existent light microscopic data on vaginal keratinization and for studies using vaginal epithelium as a model system (*vide supra*). Expanding Forsberg's observations, they stated, "The epithelium undergoes a progressive, orderly squamous transformation...(which)...experimentally recapitulates the approximate time sequence of diestrus to estrus changes in the intact adult mouse..." in response to estradiol stimulation (17).

In the study by Cooper *et al.* (17), the earliest observations of estrogen stimulated epithelium were recorded at 24 hr post-stimulation. At this time, a secretory product was already well established within the superficial layer of cells. This secretory product in the immature mouse under estrogen stimulation has been shown to be PAS-positive, amylase resistant and consistent with the histochemical characteristics of mucus (11, 30). Kamell and Atkinson had identified this product in ovariectomized mice stimulated by combined estrogen and progesterone much earlier in the literature (43). During the late diestrus and proestrus of normal mice, the superficial layer is composed of abundant mucous cells (79). Similar mucous cells are seen in guinea pig vaginal mucosa in late diestrus (14). In the mouse these cells are the offspring of basal cell proliferation which took place at the same point in the preceding estrous cycle, late diestrus and proestrus (88). It is apparent then that by using prepubertal mice, one may, by appropriate hormonal manipulation, initiate mucus formation in cells which have neither been previously coded by prior estrogen stimulation (postpubertal animal) nor possibly affected by surgical manipulation (ovariectomized animal).

Mucus formation has been studied for over 100 years. As early as 1837, Henle associated mucous secretion with the goblet cell of the intestine (36). Since then numerous investigators have attempted to define the cytomorphologic events in mucus formation and secretion at the light and ultrastructural levels (10, 19, 20, 26, 31, 34, 38, 39, 52, 56, 69, 75, 77, 84). The intestinal goblet cell is a merocrine cell and is held to continually form mucus from the time of proliferation in

the intestinal crypts until degeneration and slough at the villous tip (60). Recent work has concentrated on the fine structure and precursor autoradiography of the intestinal goblet cell during secretory activity (32, 60). Although it is known that the Golgi apparatus, and the smooth and rough endoplasmic reticulum participate in mucus formation, any description of the sequential cytomorphological events in mucus formation in intestinal goblet cells has, by necessity, to be based on comparisons of cells, whose relative states of activity are assumed.

The similarities between the intestinal goblet cell and the vaginal mucous cell are quite striking. By light microscopy, both demonstrate apical mucus accumulation and parabasal nuclei. At the electron microscopic level, the presence of microvilli and granular, membrane-bound aggregates of mucus are similar (17, 32). Both Freeman in intestinal goblet cells (32) and Cooper *et al.* in vaginal mucous cells (17) have noted that the rough-surfaced endoplasmic reticulum plays an important role in the process of mucus formation. With a continuously active cell population, such as the intestinal goblet cell, it is difficult to trace biochemical and cytomorphological pathways since substrate, precursors, and end product coexist in different cells at any point in time and isolation is extremely complicated. With the vaginal mucosa of rodents, the vaginal mucous cell derives from an "undifferentiated" cuboidal cell and begins mucus production at some point within the first 24 hr of differentiation (17). These cuboidal cells have been shown, in non-estrogen stimulated immature epithelia, to be totally devoid of mucus production. In addition, they are holocrine cells which slough shortly after cytoplasmic mucus synthesis is completed. Using these cells, one

should be able to study the temporal relationships in the formation and activation of the organelles involved in mucus production. It can additionally be assumed that only precursors exist in the early stages of differentiation and only end product prior to sloughing.

In light of the possible biochemical and cytomorphological advantages of vaginal mucous cells over intestinal goblet cells as a model for the study of mucus synthesis, a light and electron microscopic investigation of the early cytomorphological changes induced by estrogen in prepubertal mouse vaginal epithelium was undertaken.

The intent of this project is to define the *in vivo* effects of estradiol stimulation on the vaginal epithelium of prepubertal BALB/cCRGL mice during the initial 24 hr following stimulation. Since the techniques and materials are nearly identical to those used by Cooper *et al.* (17), the results represent an extension of and are directly comparable to their study. Special emphasis in this study, however, is placed on the early, temporal cytomorphological differentiation of the mucus secreting cells and their organelles. With this work as a morphological baseline, future histochemical, autoradiographic, and biochemical study of precursors and products should be facilitated and should aid in definition of the complex interrelationships between structure and function during mucus and keratin synthesis.

MATERIALS AND METHODS

ANIMALS

A breeding colony of BALB/cCRGL mice, originally obtained from the Cancer Research Genetics Laboratory, University of California, Berkeley, California, was maintained in transparent plastic cages. The bedding consisted of San-I-Cel^{R} (Paxton Processing Co., Paxton, Illinois) beneath a layer of aromatic red cedar shavings. The mice were fed Purina Laboratory Chow (Ralston Purina Co., Checkerboard Square, St. Louis, Missouri) and tap water *ad lib*.

The quarters were windowless and artificially illuminated from 7:30 a.m. to 4:30 p.m. each day.

BREEDING

Females and males were placed in cages in the ratio of two females per one male. As females became visibly pregnant (approximately 12th to 14th day of gestation), they were moved to individual cages. Beginning at the 18-19th day, the gravid females were observed for evidence of parturition at 12 midnight and again at approximately 2 hour intervals from 6:00 a.m. to 6:00 p.m. Thus, the birth date and time of birth were known within ± 6 hr at most.

On the seventh postpartum day, the litters were sexed, and the males removed. All litters born within ± 6 hr of any given time were combined randomly into one experimental grouping. Females in the same experimental groups were distributed so that there were never more than 6 newborn females per nursing mother.

EXPERIMENTAL ANIMALS

The mice were injected subcutaneously with 0.1 µgm of estradiol in 0.1 ml of distilled water. The estradiol was prepared by dilution of an original aqueous suspension of 0.25 mgm estradiol per ml with 0.5% phenol added as preservative (Progynon^{R}, Schering Corp., Bloomfield, New Jersey). The estradiol was administered subcutaneously between the scapulae using 1.25 inch, 25 gauge needles and disposable tuberculin syringes. The injection tracts were purposely made long in order to avoid leakage, the site of needle puncture being over the sacrum. Estradiol was used in order to maintain materials consistent with the investigation of Cooper *et al.* (17).

The injected animals were sacrificed by 240 hr postpartum so that no mouse exceeded 10 days ± 6 hr of age at the end of the investigation. This procedure was enforced to insure that no endogenous estrogen effect could insert an uncontrolled variable into the experiment. Cooper *et al.* (17) had noted early estrogen effects in occasional control mice at 13 days of age.

CONTROL ANIMALS

Using the identical estradiol product, dose and route of injection as well as earlier members of the same BALB/cCRGL inbred colony, Cooper *et al.* (17) demonstrated that animals inoculated with dry needles, 0.1 ml of distilled water, or 0.1 ml of distilled water containing 0.002 mg of phenol responded no differently than non-injected control animals. Therefore, one or two randomly selected females from each experimental

group were either injected with a dry needle or left uninjected. These mice were sacrificed at the beginning and end of each experiment as controls. Table 1 compares experimental animal weights to control animal weights and indicates the random character of the selection of the controls.

PREPARATION OF TISSUES

The experimental and control animals were sacrificed by cervical dislocation at time intervals varying from 3 hr to 24 hr after estradiol injection. In each experimental group, two animals were used as controls, and two mice were sacrificed at one or more of the established time intervals. Each mouse was weighed, sacrificed, and pinned to a cork board with its ventral surface exposed. The abdomen was opened, and the pubis removed. Vaginal length (cervix to introitus) was measured *in situ* by compass with the vagina under sufficient tension to render it straight. The vagina and uterus were mobilized from attached rectum and fat by grasping the bladder with forceps and stripping the material away with small dissecting scissors. The bladder, urethra, uterus, and vagina were removed *en bloc* by cutting the oviducts and ureters bilaterally and the vagina at the introitus. The organs were placed in two drops of ice-cold fixative. Using a razor blade to cut across the vagina immediately below the cervix, the bladder and uterus were discarded. Bisecting the vagina transversely, the caudal half was discarded. The cranial (Müllerian) half was bisected sagitally, and the area underlying the urethra was removed and discarded. The remaining strips of vagina were further reduced to 1 mm squares and placed in 1/2 to 1 ml of

TABLE

COMPARATIVE MEAN WEIGHTS AND VAGINAL LENGTHS OF EXPERIMENTAL AND NON-INJECTED CONTROL GROUPS*

Hours Post-Injection	Experimental			Control		
	No. Animals (N_e)	$\overline{X}_e$ Total Weight (gm)	$\overline{X}_e$ Vaginal Length (mm)[2]	No. Animals (N_c)	$\overline{X}_c$ Total Weight (gm)	$\overline{X}_c$ Vaginal Length (mm)
6	7	5.25 (4.75-6.00)[1]	4.43 (4.00-5.00)	6	4.71 (4.00-5.00)	4.08 (4.00-5.00)
12	13	6.54 (4.75-7.75)	4.38 (4.00-5.00)	10	5.70 (4.00-7.50)	4.23 (4.00-5.00)
18	21	6.45 (4.50-7.50)	4.31 (4.00-5.00)	15	6.27 (4.00-9.25)	4.20 (4.00-5.00)
24	8	7.50 (7.00-8.00)	4.63 (4.00-5.00)	7	6.82 (5.25-8.25)	4.57 (4.00-5.00)

1. The numbers within the brackets indicate the range of the individual values.

2. The vaginal lengths in this table are not directly comparable with those of Cooper *et al.* (17) due to a difference in measurement technique.

ice-cold fixative.

Three methods of fixation were used in an attempt to better demonstrate the cellular alterations.

Type A:

The tissue blocks were fixed in 1% osmium tetroxide in veronal-acetate buffer (pH 7.4) with 0.045 gm per ml sucrose added for 2 hr at 0° C (16).

Type B:

The tissue was fixed in 1.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 2 hr and post-fixed in 1% osmium tetroxide prepared as above for 2 hr, both 0° C (74).

Type C:

The tissue was fixed in a mixture of 1.25% glutaraldehyde and 1% osmium tetroxide in phosphate buffer (pH 7.4) at 0° C for 30 to 40 min. It was then post-fixed in 0.5% uranyl acetate at 0° C for 25 min (37, 51).

Immediately following fixation, the epithelial blocks were rapidly dehydrated in graded (50%, 70%, 95%, 100%) ethanols (5 min in each concentration at 0° C). The tissue was usually stored overnight in 100% ethanol at 0° C.

Swiss araldite (Fluka A G, Chemische Fabrik, Buchs S G) embedding medium was used (50). 500 to 700 Å thick sections were cut on an LKB ultrotome equipped with a Pyrex^{R} glass knife, mounted on 2% parlodion (Mallinckrodt) coated, carbon layered grids (Ernst Fullam Co.) and triple stained 5 min in lead citrate, 5 min in uranyl acetate, and 5 min in lead citrate (68, 89). The sections were viewed in a RCA EMU-3G electron

microscope.

One to 2 micron sections for light microscopy were cut from the same blocks, mounted on glass slides, and stained by Richardson's method (70).

RESULTS

LIGHT MICROSCOPY

Controls.

The cytomorphology of the vaginal epithelium from control mice, regardless of dry-needle injection or non-injection, was constant. The vaginae of control mice sacrificed at time zero were identical with those sacrificed 24 hrs later.

The epithelium consisted of three to four layers of cuboidal cells separated from an underlying, loose fibrovascular stroma by a well-defined basement membrane (Fig. 1). The basal and intermediate cells had large, central nuclei, containing multiple irregular chromatin clumps in a moderately dense karyoplasm. The cytoplasm was dense, homogeneous, and finely granular.

Abundant, pale, moderately granular cytoplasm surrounded oval to round, central nuclei in cells of the superficial layer. The nuclei contained at least one, prominent, dark, eccentric nucleolus. Nearly every cell, regardless of layer, had one or more chromatin clumps of variable size located randomly along its nuclear membrane. Except for the luminal cell membrane and the basement membrane, the cytoplasmic membranes were indistinct.

Experimental:

6 and 12 hr after estradiol injection:

Although reported in other studies, identifiable changes in either nucleolar size and appearance or other cellular features were not present in specimens taken 6 hr after stimulation. The cellular

alterations which occurred seemed to be both gradual and variable.

At 12 hr post-injection, alterations which were consistent from animal to animal were accompanied by marked variability in cell size and epithelial thickness (Figs. 2 and 3). Due to poor cytoplasmic membrane definition, cell size could only be estimated as ranging from four to ten microns in width and nine to twenty microns in height. The greatest variation in height and width occurred in the basal cell layer. The total epithelial thickness varied from three to five cells and was variable from area to area in the same animal.

Consistent findings in 12 hr epithelia included multiple, round to oval, chromatin clumps in the superficial nuclei, vertical orientation of elongate basal and parabasal cells, and the presence of mitotic figures in the basal layer (Fig. 2). The intermediate cell layer, one to two cells in thickness, remained cuboidal. The superficial cells ranged from cuboidal to columnar, the latter with basal nuclei and increased apical cytoplasm. Cytoplasmic staining became less intense as the cells approached the surface; however, the cytoplasm was coarsely granular throughout. Although intercellular cytoplasmic membranes remained indistinct, intercellular spaces among the intermediate cells increased in both size and number.

18 hr after estradiol injection:

The variability of the epithelial response in a single vagina (Figs. 4 and 5) was almost as great as the range of difference noted between epithelia from various time periods during the first 18 hrs post-injection. In the most responsive areas, the basal cells maintained an elongate orientation perpendicular to an indistinct basement membrane.

The nuclei of the basal cells conformed to the cell shape and were large with round to rod-shaped nucleoli. At this time, the intermediate cells first demonstrated vertical compression with horizontal flattening. Despite the difference in polarity, basal and intermediate cell cytoplasm had similar intensity of staining and granularity and intermediate cell nuclei were vesicular. The superficial layer, one to two cells in thickness, continued to stain less intensely than the other layers and was composed of two distinct cell types. The majority of columnar superficial cells had homogeneous, finely granular cytoplasm and flat luminal cytoplasmic membranes. However, smaller numbers of cells, usually grouped in two's and three's, demonstrated vesiculation of the supranuclear cytoplasm. Some of these clear, round spaces contained dense droplets near their centers (Fig. 4). Although not consistently present, the luminal cytoplasmic membranes of these cells were modified by occasional cytoplasmic outpouchings projecting into the vaginal lumen. These thin-stalked blebs were never seen on non-secretory surface cells.

In less responsive areas in the 18 hr stimulated epithelia, the above described changes were less frequent and more variable from cell to cell in the same layer. Even in areas of poor response, however, the features which identified this as stimulated epithelium included scattered, isolated superficial columnar cells with vacuolated apical cytoplasm containing dense droplets in clear, vacuolar spaces, increasingly granular cytoplasm, and the presence of polymorphonuclear leukocytes in the subjacent fibrovascular stroma. In rare foci, no alterations existed which served to distinguish them from the control specimens.

21 hr after estradiol injection:

While variation between specimens and within the same specimen was still existent, more advanced alterations were present in the most responsive areas (Fig. 6). The lower epithelial layers were composed of both basal and parabasal cells having deeply stained, coarsely granular cytoplasm. The intermediate layer was one to two cells in thickness and cytoplasmic staining was intermediate in intensity between that of the basal and superficial cells. Most of the cells were horizontally flattened. The superficial layer was one to two cells in thickness and was composed of the two cell types described at 18 hr. However, the ratio of vacuolated cells to finely granular cells was reversed, so that the majority of columnar cells had prominent mucous droplets in the apical cytoplasm. Additionally, disintegrating cells were scattered randomly among the superficial cells.

24 hr post-injection:

By 24 hr all sections showed definite estradiol effects. Figures 7 and 8 illustrate the variations present. In the least responsive areas the elongate, perpendicular basal cells, cuboidal parabasal cells and flattened or randomly oriented intermediate cells had irregularly granular, coarse, darkly stained cytoplasm (Fig. 7). The superficial cells were more consistently columnar with basal nuclei and apical cytoplasm. Only an occasional cell contained mucous droplets (md).

In the most responsive epithelia (Fig. 8) the epithelium was up to eight cells in thickness. The basal cells were typically vertically elongate and their cytoplasm was homogeneous and dark. The nuclei

contained chromatin masses of variable size. The intermediate layer was two to five cells in thickness and had two distinct sublayers. The deeper sublayer, adjacent to the basal cells, was two to four cells thick, deeply stained, and horizontally oriented. The dense, flattened cells tapered at both ends and had vesicular central nuclei which usually contained one large oval to rod-shaped nucleus. Numerous intercellular bridges (desmosomes) were apparent at all surfaces. Superficial to this sublayer, a single thickness of less densely stained, cuboidal to slightly flattened cells formed a transition zone between the intermediate and superficial layers. Although the vesicular nuclei and prominent nucleoli resembled those of the deep intermediate sublayer, they were less frequently visualized. The cytoplasm was coarsely granular. On the superficial aspect of these cells, intercellular spaces and bridges were indistinct, while on their deep aspect, they were prominent. With more advanced squamification (17) these intermediate sublayers form the *stratum spinosum* and *stratum granulosum* respectively of keratinizing vaginal epithelium.

Both cuboidal and columnar cells in variable proportion formed the single celled superficial layer. The large, pale nuclei contained small oval to rod-shaped nucleoli frequently located adjacent to the nuclear membranes. In most cells, the cytoplasm was finely granular with occasional dense, round bodies in the cytoplasm. A few cells, either individually or in groups of two or three, had markedly vesicular cytoplasm with small, compressed, dark basal nuclei. The vacuoles contained numerous, dense, round bodies, one per vacuole. The shape and size of these cells varied depending on the quantity of mucus present.

Polymorphonuclear leukocytes were present in many specimens 24 hours after estradiol administration. In animals which were 11 days of age at the time of sacrifice, they were present in both the epithelium and stroma, while in those that were 10 days of age at sacrifice, they were confined to the stroma.

ELECTRON MICROSCOPY

Control Animals.

Superficial cells:

The cuboidal cells had a nuclear:cytoplasmic ratio of approximately 2:1 with central nuclei (Fig. 9). On the luminal aspect of the cell membrane there were moderate numbers of microvilli, approximately 0.5 micron in height, surrounded by a small amount of glycocalyx. Where adjacent cell membranes met at the surface, both zonulae occludentes and terminal bars (desmosomes) were noted. Sparse nuclear chromatin aggregates, approximately 200 to 1000 Å, and occasional dense chromatin clumps along the nuclear membrane characterized the nuclei. Many contained randomly located single nucleoli, approximately 2 microns in diameter. The cytoplasm contained empty, non-dilated, rough-surfaced endoplasmic reticulum, 200 to 1000 Å in cross-section, and scattered polyribosomes. Small Golgi apparatus, < 1 micron in longest dimension, were present primarily in the apical cytoplasm (Fig. 10). Ovoid mitochondria, with maximum dimensions of approximately 750 Å, were sparsely spread throughout the cytoplasm. Dense, osmophilic, lipid droplets were seen randomly in occasional cells.

Intermediate cells:

The intermediate cell nuclear:cytoplasmic ratio was 4:1 to 5:1. As in the superficial cell layer the cytoplasm contained Golgi apparatus, mitochondria, ribosomes and endoplasmic reticulum. Additionally, perinuclear collections of pale staining granules measuring approximately 250 Å in diameter (glycogen) (Fig. 11) and small desmosomes with sparse fibrils (filament groups) (Fig. 12) were present. Individual filaments were approximately 70 Å in diameter. The cytoplasmic membranes were closely apposed with infrequent, open intercellular spaces. These spaces were empty and modified focally by villiform cytoplasmic projections. The nuclei, except for the paucity of nucleoli, resembled those in the superficial layer.

In one cell a unique, perinuclear, elongate organelle was interpreted as a heterotopic cilium (Fig. 13).

Basal cells:

These cuboidal cells, with a nuclear:cytoplasmic ratio similar to the intermediate cells, rested on a distinct, continuous 350 Å basement membrane, from which they were separated by a clear zone approximately 700 Å in width (Fig. 14). Occasional hemidesmosomes (basal attachment plates) were located along the basal aspect of the cell membranes. The ribosomal concentration varied from sparse, as in the surface cells, to moderately dense (Fig. 14). Non-dilated, empty endoplasmic reticulum and ovoid mitochondria were present randomly in the cytoplasm. A well-defined Golgi apparatus was not identified. Nuclei had numerous irregular chromatin aggregates.

The cytoplasmic membranes of most cells were regular and straight with scant desmosomes and inapparent intercellular spaces. Between occasional cells there were foci of villiform cell membrane interdigitation (Fig. 14) and increased numbers of desmosomes with prominent, elongate cytoplasmic fibrils (Fig. 15).

Experimental Animals.

Unlike the control epithelia, the variation in findings among cells in a single layer, at any specific time, made a representative composite description difficult. In certain cells no significant changes, as compared to previous time periods, were identified. In order to avoid redundancy, only the most advanced changes in each cell layer, and those which represent alterations first noted at that particular time interval, are described.

6 hour experimental:

Superficial cells--Many of the cells exhibited increased chromatin and ribosomal density. Associated with the increase in ribosomes, the endoplasmic reticulum was more prominent, although generally non-dilated. In a few cells the endoplasmic reticulum at the cell apex was markedly dilated, up to 5000 Å in width, and contained amorphous material of low density (Fig. 16). Round to oval juxtanuclear multivesicular bodies, approximately 0.45 micron in diameter, were observed. The mitochondria were more elongate but were not demonstrably increased in size or numbers. The cell membrane, even in the most differentiated cells, was adesmosomal, and the numbers of microvilli at the luminal surface were unchanged. Golgi apparatus were enlarged to approximately 1.5 microns in greatest dimension, although the vesicles were non-dilated.

Intermediate cells--The intermediate layer was divided into a number of cell types. The upper cells had greater numbers of ribosomes than did the controls, and their Golgi apparatus were enlarged to greater than 1.5 microns in greatest dimension (Fig. 19). Desmosomes were increased in numbers, and more prominent intracellular fibrils were observed to connect adjacent desmosomes. Randomly placed, shorter filament groups were also noted in the cytoplasm. Intercellular spaces had increased in prominence.

The deeper intermediate cells had many similarities to the basal layer (Fig. 18). The ribosomal density was not increased over the controls. The endoplasmic reticulum was less prominent than in the upper intermediate cells, but the cisternae were more electron dense than the surrounding cytoplasm. Intercellular spaces were less prominent than in the superficial intermediate cells, and desmosomes, 2500 to 5000 Å in length, were scanty and had shorter fibril groups. Free intracytoplasmic filaments were fewer in number.

Basal cells--As compared with controls, the cells were more rectangular with their long axes perpendicular to the basement membrane (Fig. 17). Nuclear shape was modified to conform to the cellular elongation. Hemidesmosomes were slightly increased in number, but other cellular features were unchanged from controls.

12 hour experimental:

Superficial cells--Changes qualitatively similar to those at 6 hours were occurring in a greater percentage of surface cells. Quantitative changes were not identified.

Intermediate cells--There was considerable variation among the intermediate cells dependent upon their position (Figs. 20 and 21). Occasional cells in the uppermost intermediate layer had enlarged Golgi apparatus, approximately 2 microns in greatest dimension, with juxtaposed, dilated, though seemingly empty, rough-surfaced endoplasmic reticulum (approximately 1000-2500 Å) (Fig. 20). Occasional short fibrils (< 0.5 micron in length) were scattered among moderately dense ribosomal aggregates. Desmosomes were inapparent and small, approximately 1300 Å. Intercellular spaces were non-dilated with only scattered larger spaces containing villiform cytoplasmic projections.

The deeper intermediate cells (Fig. 21) had more prominent ribosomal aggregates outlining distinct, but non-dilated (≈ 500 Å in cross-section) endoplasmic reticulum. Small non-dilated perinuclear Golgi apparatus were noted in the majority of the cells. Prominent fibrils, up to 3 microns in length and 2500 Å in width coursed through the cytoplasm. Desmosomes were present in greater numbers along the cell membranes and were up to 2500 Å in length. A few large intercellular spaces, equal in size to the upper intercellular spaces, were noted.

Basal cells--Polyribosomes and mitochondria had increased in number (Fig. 22). The cytoplasmic membranes were separated by occasional large intercellular spaces with complex interdigitations. There were scattered small desmosomes, approximately 1500 Å in length, along the lateral and superficial aspects of the cell. The basement membrane was unaltered, although hemidesmosomes appeared more frequently.

15-17 hour experimental:

Superficial cells--Many of the cells had assumed a conical shape with prominent free borders (Fig. 23). Irregular surface microvilli had a prominent glycocalyx. The cytoplasm was filled by numerous polyribosomes and a rough-surfaced endoplasmic reticulum which contained granular material of medium density (Figs. 23 and 24). In the apical, perinuclear cytoplasm of many cells, the mitochondria had increased markedly in number, but not in size. Cytoplasmic vacuoles were moderately numerous.

The Golgi apparatus present in the majority of surface cells had enlarged to greater than 2 microns in longest dimension and consisted of dilated, though empty appearing, cisternae (1800-2500 Å in cross-section).

Intermediate and basal cells--Changes were minimal at this time interval.

18 hour experimental:

Superficial cells--The surface cells had become increasingly conical and goblet cell-like (Fig. 25). They projected irregularly into the lumen and in many instances one cell would stand above its neighbor by one-half of its total height. Along the microvillous luminal surface, cytoplasmic fragments from disintegrating cells frequently overlay viable cells. Dilated, rough-surfaced endoplasmic reticulum containing moderately dense granular material occurred randomly in the polyribosome-dense, apical cytoplasm. In some cells, the apical cytoplasm contained membrane-bound, mucus-containing vacuoles having a granular to reticular pattern. At one point in Figure 26, the rough-surfaced endoplasmic reticulum, with its less dense granular aggregates, appeared

to communicate with one such vacuole. The Golgi apparatus were prominent, enlarged to greater than 3 microns in greatest dimension, and had prominent vesicles at their concave surface. These small, smooth-surfaced membrane-bound vacuoles contained amorphous material of moderately osmophilia (Fig. 28).

Intermediate cells--Except for the uppermost cells (*vide supra*), there was an increase in fibril numbers, desmosomes, and polyribosomes. Other cytoplasmic organelles, including mitochondria, endoplasmic reticulum, and Golgi apparatus were decreased in amount or prominence. Glycogen aggregates were present in the perinuclear area (Fig. 27). Nuclei throughout the intermediate layer had large nucleoli and dense chromatin lining the nuclear membrane.

Basal cells--The basal cells had become more vertically elongate. Polyribosomal concentrations, as well as mitochondrial numbers, were increased. A rare, small Golgi apparatus was seen in the apical perinuclear cytoplasm. Desmosomes and areas of complex membrane interdigitation were more numerous. The ovoid, elongate basal cell nuclei had more numerous and smaller chromatin masses.

21 hour experimental:

Superficial cells--The luminal surfaces of all mucus-forming cells were modified by numerous well-defined microvilli having a hair-like glycocalyx (Figs. 29 and 30). Their apical cytoplasm was very dense and filled with large to small vacuoles. In some micrographs (Fig. 29), the mucous content of the vacuoles varied from a lightly stained, finely granular network, in the area immediately adjacent to the nucleus, to a more dense material, with a decrease in vacuole

diameter, near the luminal surface. Near the surface membrane, extremely dense mucus filled ovoid vacuoles, approximately 0.5 to 0.9 micron in greatest dimension (Fig. 29); while the dilated rough-surfaced endoplasmic reticulum appeared almost empty (Fig. 30).

Intermediate cells--The upper intermediate cells resembled the non-secretory superficial cells. Their Golgi apparatus was rudimentary, and their scanty rough-surfaced endoplasmic reticulum was non-dilated. Desmosomes, with short dense fibril attachments were more numerous on the deep aspect of the cell membrane. Numerous short, discontinuous, thin fibrils were admixed in the cytoplasm with abundant polyribosomes.

The lower intermediate cells were horizontally elongate (Fig. 31). The cell membranes demonstrated numerous desmosomal modifications. Fibrils, ranging in thickness from less than 100 Å to 1500 Å, coursed through the cytoplasm. Their multiple branchings and fusions produced a complicated network which seemed to interconnect the desmosomes one with another. Between desmosomal pairs, dilated intercellular spaces were either empty or contained complex membranous interdigitations. A dense polyribosomal population occasionally outlined indistinct ovoid cytoplasmic bodies, resembling degenerating mitochondria.

Basal cells--The findings were similar to those of basal cells at 18 hours post-estrogen.

24 hour experimental:

Superficial cells--Approximately 50% of the surface cells contained mucous vacuoles in their apical cytoplasm. The remaining cells lacked both a large dilated Golgi apparatus and mucous vacuoles, but did have dilated, rough-surfaced endoplasmic reticulum (approximately 1000 Å

in cross-sectional diameter) (Fig. 33). Their dilated lumina contained moderately dense, amorphous material. Microvilli, invested with a moderately dense glycocalyx, were equal in number and similar in size on the free surface of both cell types. The bristle-like projections of the glycocalyx were oriented perpendicular to the surface of the microvilli (Fig. 32). Dilated intercellular spaces were present at many sites along the lateral and deep cytoplasmic membrane. As at 21 hours, the mucus containing vacuoles were more dense and smaller near the cell surface (Fig. 33). The apparent sequence in mucus secretion, subsequent to processing in the Golgi apparatus, is illustrated in Figure 35. The Golgi apparatus seemed to pinch off small mucus containing vacuoles from its inner cisternae into the concave mature face. These small vacuoles coalesced into vacuoles measuring up to 1 micron in diameter. The larger vacuoles were displaced from the region of the nuclear apex toward the luminal surface. Mucus in vacuoles near the cell surface was condensed into dense, central, spherical masses attached to the vacuolar membrane by dense, linear spokes. This finding is felt to be all artefact secondary to osmium fixation and probably accounts for the light microscopic finding of "mucous droplets surrounded by clear, vacuolar areas." Mitochondria were plentiful in the cytoplasm but were smaller than the mucous vacuoles (5000-7000 Å) and more difficult to visualize.

Intermediate cells--The horizontally oriented intermediate cells had an occasional, poorly developed Golgi apparatus and a moderate amount of non-dilated endoplasmic reticulum (Fig. 34). Mitochondria demonstrated low contrast. Desmosomes were numerous along the entire length of the cell membrane. Dilated intercellular spaces and villiform

modifications of the cell membrane were also numerous. Polyribosomes were densely concentrated in the cytoplasm of the intermediate cells. Cytoplasmic fibrils varied from short lengths (< 1μ) containing only a few filaments to long, thick bundles (Fig. 31).

Basal cells--These vertically elongate cells with their irregularly shaped bodies formed a pseudostratified layer. Each basal cell made contact with the basement membrane, although new basal cells developing just above the basement membrane appeared to force some cells into parabasal position. Such cells could occasionally be demonstrated to maintain limited contact with the basement membrane (Fig. 34). Loss of basement membrane contact seemed to be associated with assumption of a more horizontal orientation and the development of intermediate cell characteristics.

True basal cells were very irregular in size and shape. While desmosomes were variable in numbers along the lateral aspects of cell membranes of basal cells, many more desmosomes were present when the basal cell membranes were juxtaposed with those of the intermediate cells (Fig. 34). Intercellular spaces were smaller and membrane interdigitations were less complex than in the intermediate cell layer. Mitochondria were numerous and randomly distributed in the cytoplasm. Up to 50% of basal cells were noted to contain a rudimentary Golgi apparatus and scanty fibrils lateral to a large, oval nucleus. Dense polyribosomes outlined sparse, random segments of non-dilated, rough-surfaced endoplasmic reticulum. In randomly distributed cells, constituting 1 to 5% of the total basal cell population, mitotic figures were identified.

DISCUSSION

Even though Husbands and Walker (40) have shown that mouse vaginal epithelium is a homogeneous cell population in its response to estrogen stimulation, a finding supported by Ladinsky and Peckham (49) in rats, the findings in the present study indicate that the degree of cellular response during the first 24 hours following estradiol stimulation is extremely variable in mouse vaginal epithelium. The stage of differentiation reached in one area of epithelium at 24 hours may have been reached by more responsive cells in the same animal at 18 hours. Moreover, at the ultrastructural level, changes noted in one cell at 6 hours (Fig. 16) may not occur in another cell until 24 hours post-stimulation (Fig. 33). More important, however, is the fact that although quantitatively variable, the cytomorphologic alterations during cellular differentiation are qualitatively predictable and reproducible.

The non-stimulated vaginal epithelium of prepubertal mice, as also described by Cooper, Cardiff, and Wellings (17), is a simple, three layer, cuboidal epithelium similar to that of ovariectomized animals (11, 43). The large central nuclei have inapparent nucleoli and variable granular and diffuse chromatin. The cytoplasm, most abundant in the supranuclear region of the surface cells, contains minimal numbers of polyribosomes, rough-surfaced endoplasmic reticulum, and mitochondria. Only the intermediate and superficial cells contain small Golgi apparatus which have smooth-surfaced vesicles and cisternae. The intermediate cells further contain a moderate amount of glycogen and occasional, short cytoplasmic fibrils (filament bundles). The superficial cells have microvillous modifications of the luminal portion of

the cytoplasmic membrane. Each microvillus has its individual glycocalyceal investment (antennulae microvillares) (6, 67). Other features which set the superficial cells apart from the other two layers include areas of cell membrane fusion (approximately 3000 Å in length) at the luminal aspect of adjacent cell membranes (zonulae occludentes) and subjacent terminal bars (desmosomes) (Fig. 10). The remainder of the superficial cell membrane is free of desmosomes. The intermediate and basal cells have only a few small desmosomes (approximately 1500 Å in length) located randomly along their cell borders. Hemidesmosomes (basal attachment plates) located along the basement membrane are limited to three or four per cell.

With intravaginal estradiol application, the nucleoli of basal cells increase in size and develop densely staining intranucleolar bodies as early as one hour after application with maximum change in 2 to 5 hours (66). Using subcutaneous injections of 0.1 µgm estradiol, light micrographs of the most advanced epithelia reveal the earliest significant changes at 6 to 12 hours. These include vertical elongation of basal cells with occasional mitotic figures, an increase of up to 5 cells in epithelial thickness, and an increase in apical cytoplasm of the superficial cells. The nuclei may have slightly enlarged, more intensely stained nucleoli on a quantitative (statistical) basis, but qualitative changes are not striking. Ultrastructurally, some stimulated cells at 6 hours demonstrate cytomorphologic alterations. The polyribosomal concentration in the superficial and upper intermediate cells is increased, and a few superficial cells have dilated rough-surfaced endoplasmic reticulum containing an amorphous, moderately

electron dense material. Multivesicular bodies are present; and, in the upper cell layers, Golgi apparatus, consisting of non-dilated cisternae and vesicles, have lengthened to approximately 1.5 microns in longest dimension. At 6 hours desmosomes in the lower intermediate and basal cells have increased in length (3000 versus 1500 Å) as have their cytoplasmic fibrils, composed of 70 Å filaments. These features, along with increasing numbers of ribosomes, produce a progressively more squamous appearance in the intermediate cells over the first 24 hours.

The superficial cells are so variable in their individual rate of response that some of the cells do not become fully mucified even after 48 hours of estradiol stimulation (17). Thus at 12 hours, in this study, the primary change, as compared to 6 hours, was an increase in the numbers of cells showing early secretory development and a decrease in non-responsive cells. From 12 to 24 hours post-stimulation the superficial cells assume first a conical and than a goblet cell-like shape with basally placed nuclei and prominent apical cytoplasm. Polyribosomes and mitochondria become increasingly abundant in the perinuclear area by 18 hours, which coincides temporally with biochemical studies indicating increased RNA production and metabolic activity in these cells (33, 57, 58). The rough-surfaced endoplasmic reticulum, frequently dilated and containing moderately dense, amorphous material in its lumina, appears in close proximity to the enlarging Golgi apparatus from 6 through 24 hours.

By 18 to 24 hours the Golgi apparatus, which is usually present in the apical perinuclear cytoplasm, has enlarged to greater than 3 microns in greatest dimension. The smooth-membrane lumina are dilated from 2 to

3 times their original 500 to 1000 Å cross-sectional width. A fine, reticular network of dense material is noted within the lumina of the inner cisternae, and coalescing vesicles are present at the mature, concave face by 18 hours. Also by 18 hours, vacuoles, up to 1 micron in diameter and containing reticular material have been displaced into the apical cytoplasm. The accumulating vacuoles contain a secretory product which has previously been identified as mucus by Kamell and Atkinson (43) and Biggers (11).

Mucus formation in vaginal epithelial cells has not been extensively studied. The superficial similarities between these cells and intestinal goblet cells, which have been extensively studied, were previously noted. The subsequent discussion will compare the two cells, so that the literature concerning the goblet cell may be used to better understand vaginal mucus formation.

In 1958, Burgos and Wislocki (14), describing the cyclical changes in genital mucosa of the guinea pig, noted the accumulation of an amorphous, flocculent, low density material inside saccular dilatations of the endoplasmic reticulum which they identified as mucus. Drawings indicate that the endoplasmic reticulum was rough-surfaced. Freeman (31), working with mouse ileum goblet cells in 1962, presented evidence favoring the hypothesis that the ergastoplasm (rough-surfaced endoplasmic reticulum) creates mucus and the Golgi apparatus concentrates it. He noted support for this hypothesis from two prior studies. A comparative study of Golgi apparatus by Dalton and Felix (21) suggested that the Golgi apparatus was functional in removing water from secretions, while electron micrographs by Palade (62) demonstrated that continuity existed

between the rough-surfaced endoplasmic reticulum and the smooth-surfaced Golgi vesicles.

In a more recent study Freeman (32) has modified his hypothesis. Study of the goblet cell in man, rat, and guinea pig has led to the observation that a moderately electron-dense material within the rough-surfaced endoplasmic reticulum is associated with the Golgi apparatus but "...recognizable granules of mucin within the lamellae of the reticulum have not been demonstrated."

Mucus is composed of a glycoprotein moiety (48, 61, 80) and a mucopolysaccharide moiety (11, 61, 78) containing both neutral and acid mucopolysaccharides (23). The mucopolysaccharide component of mucus utilizes glucose in its formation (80). Peterson, Neutra, and Leblond (60, 65), using glucose-H^3, attributed the formation of mucopolysaccharide to the Golgi apparatus in rat goblet cells. Earlier Jennings and Florey (42), using S^{35}, also placed the sulfation of mucus in the Golgi apparatus.

Both Freeman (32) and Cooper *et al.* (17) suggest that the rough-surfaced endoplasmic reticulum is involved in mucus production. Figure 26 illustrates amorphous, electron-dense material in the lumen of the rough-surfaced endoplasmic reticulum, in close apposition to the finely granular reticular network of mucus in a vacuole. Even though it appears that the two lumina communicate, the morphological dissimilarity between the two materials raises the possibility, if not probability, that the communication is artefactual. What, then, is the material within the endoplasmic reticulum?

Rough-surfaced endoplasmic reticulum has been shown to produce secretory proteins which then migrate to the Golgi apparatus and are packaged into secretory granules or vesicles (15, 59, 87). Additionally, it is known that mucus is partially composed of glycoproteins (*vide supra*). Based on these observations it is hypothesized that the glycoprotein moiety is synthesized in the endoplasmic reticulum, migrates to the Golgi apparatus, and is modified by the addition of the mucopolysaccharide moiety in the Golgi apparatus. Final proof of this hypothesis awaits autoradiographic studies using labeled glycoprotein precursors and labeled glucose after the fashion of Neutra and Leblond (60, 61).

The cytomorphological events in mucus secretion in mouse vaginal epithelium are best seen in Figure 35. These findings closely resemble the morphologic descriptions of mucigen formation in the goblet cells of man, rat, and guinea pig (32).

The cup-shaped Golgi apparatus (complex) with numerous rough-surfaced vesicles at its convex forming face (Fig. 20) is composed of multiple layers of parallel flattened cisternae. The inner cisternae, closest to the concave mature face of the apparatus, contain a moderately dense, reticular material thought to be the complex carbohydrate, mucus (7, 60). The inner cisternae pinch off small vesicles, filled with mucus, into the concave mature face of the Golgi apparatus (Figs. 28 and 35). The small vesicles coalesce into larger smooth-membrane bound vacuoles up to 1 micron in diameter. The vacuoles now in the supranuclear region are displaced toward the luminal cytoplasmic membrane.

In certain of the micrographs (Figs. 29 and 33), the mucous vacuoles become smaller and increasingly denser as they near the surface. Two possible explanations for this finding are suggested:

1. The vacuoles containing the mucus, concentrate it by removing water as the vacuole moves toward the surface, or

2. The increased density and decreased vacuole size are artefacts caused by the glutaraldehyde-osmium tetroxide mixture used for fixation.

The first explanation cannot be ruled out completely as the vacuolar system is known to function in a wide range of processes (22). However, in the micrographs by Cooper *et al.* (17) and in micrographs of spontaneous estrogen effects in control mice, not included in this study, it was noted that mucus, when fixed by buffered osmium tetroxide alone, does not change morphologically as it is displaced toward the surface. Occasionally, even with buffered osmium tetroxide alone, artefactual concentration of mucus into central, dense droplets occurs (Fig. 35). The partially empty vacuoles, however, retain their full size. The second explanation, therefore, appears the more likely. It would seem that as the concentration gradient of the fixatives decrease, the artefactual condensation of mucus also decreases.

Although the cytoplasm is never completely filled with mucus by 24 hours, Cooper *et al.* (17) have shown that after 48 hours of estradiol stimulation the superficial cells are tightly packed with mucus. The progressive formation of mucous vacuoles causes basal displacement and flattening of the nucleus as well as swelling of the cytoplasm with ovoid to round cellular outlines. When, at some time between 48 and 96 hours, the cytoplasm is maximally secretory, the cell ruptures and is

shed into the vaginal lumen (17).

Assuming that the Golgi apparatus has, as its primary function, the formation and secretion of mucus, its occurrence as a small, but fully developed structure in the superficial cells of non-stimulated prepubertal mice is of interest. In the absence of exogenous estrogen stimulation, mucus is not formed prior to the 12th post-natal day (17, 30). If the mere presence of the Golgi apparatus implies function, a second secretory product should be present.

Forsberg and Olivecrona (30) have noted that an amylase-resistant, PAS-positive, thin surface layer is seen in the vaginal epithelium of mice from birth. Although Forsberg *et al.* chose to interpret all PAS-positive, amylase-resistant material as mucin, the present study indicates that it is not and suggests that the thin surface layer is indeed the glycocalyceal coating of the microvilli. Ultrastructurally, a glycocalyx has been shown, in this study, to be present on the surface of the microvilli of control epithelium and persists throughout the 24 hour experimental period.

The glycocalyx (cell coat) of many different epithelial cells is known to contain both glycoprotein and mucopolysaccharide moieties (67). Recent work by Berlin (8) indicates that the Golgi apparatus in the absorptive cells of rat intestine, following irradiation, contains increased amounts of acid mucopolysaccharides which later appear at the cell surface as "glycocalyx". In addition, within 15 minutes of the administration of S^{35} and tritiated glucose, Ito and Revel (41) demonstrated the presence of these components in the Golgi region of cat intestinal mucosa and in the surface coat at 2 to 3 hours.

In view of the above it seems probable that the Golgi apparatus of mouse vaginal epithelium is responsible for the secretion of two, very similar, complex carbohydrates, glycocalyx and mucus, production of the former being estrogen independent and of the latter being estrogen dependent.

SUMMARY AND CONCLUSIONS

Prepubertal mouse vaginal epithelium, when stimulated by 0.1 µgm of estradiol, responds by both cell proliferation and differentiation. In the 24 hours following injection, the simple cuboidal superficial cells become mucified and goblet-cell-like, while the intermediate cells assume early squamous characteristics. Evidence of increased metabolic activity and cell division is seen as early as 6 hours post-injection in the basal cell layer. The degree of cellular differentiation varies from cell to cell in the same layer, but the cytomorphological sequence is predictable.

Light and electron microscopic comparison of the differentiating vaginal mucous cell and the intestinal goblet cell of the rat, as described by Freeman (32), indicates that the vaginal mucous cell has a holocrine secretory mechanism, while the goblet cell has a merocrine or, possibly, apocrine mechanism. Both cells, however, utilize the rough-surfaced endoplasmic reticulum, Golgi apparatus, and vesicles for mucus production and intracellular transport. Recent histochemical and autoradiographic studies on the intestinal goblet cell and other secreting cells indicate that the rough-surfaced endoplasmic reticulum produces a protein moiety which is converted into mucin, by the addition of glucose molecules, in the Golgi apparatus.

Although not directly observable, evidence is presented which suggests that the Golgi apparatus participates in the production of the glycocalyx which invests the surface microvilli of these and other glandular cells. This secretory activity is estrogen independent and

may account for the presence of a small Golgi apparatus in the simple, cuboidal surface cells of the non-stimulated prepubertal epithelium.

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LIGHT MICROSCOPY

Figures 1, 2, 4, 5, and 8:

The tissue was fixed in 1% osmium tetroxide for 2 hr and then serially dehydrated in 50%, 70%, 95%, and 100% ethanol (5 min at each concentration). The tissue was stored in 100% ethanol. (See Materials and Methods for details and references.)

Figures 3 and 6:

The tissue was fixed in 1.5% glutaraldehyde for 2 hr and post-fixed in 1% osmium tetroxide for 2 hr. It was then dehydrated in serial ethanol solutions as above (see Materials and Methods).

Figure 7:

The tissue was fixed in 1.25% glutaraldehyde + 1% osmium tetroxide in phosphate buffer for 30 to 40 min. It was then post-fixed in 0.5% uranyl acetate for 25 min and dehydrated as above (see Materials and Methods).

Figure 1 (0 hr control):

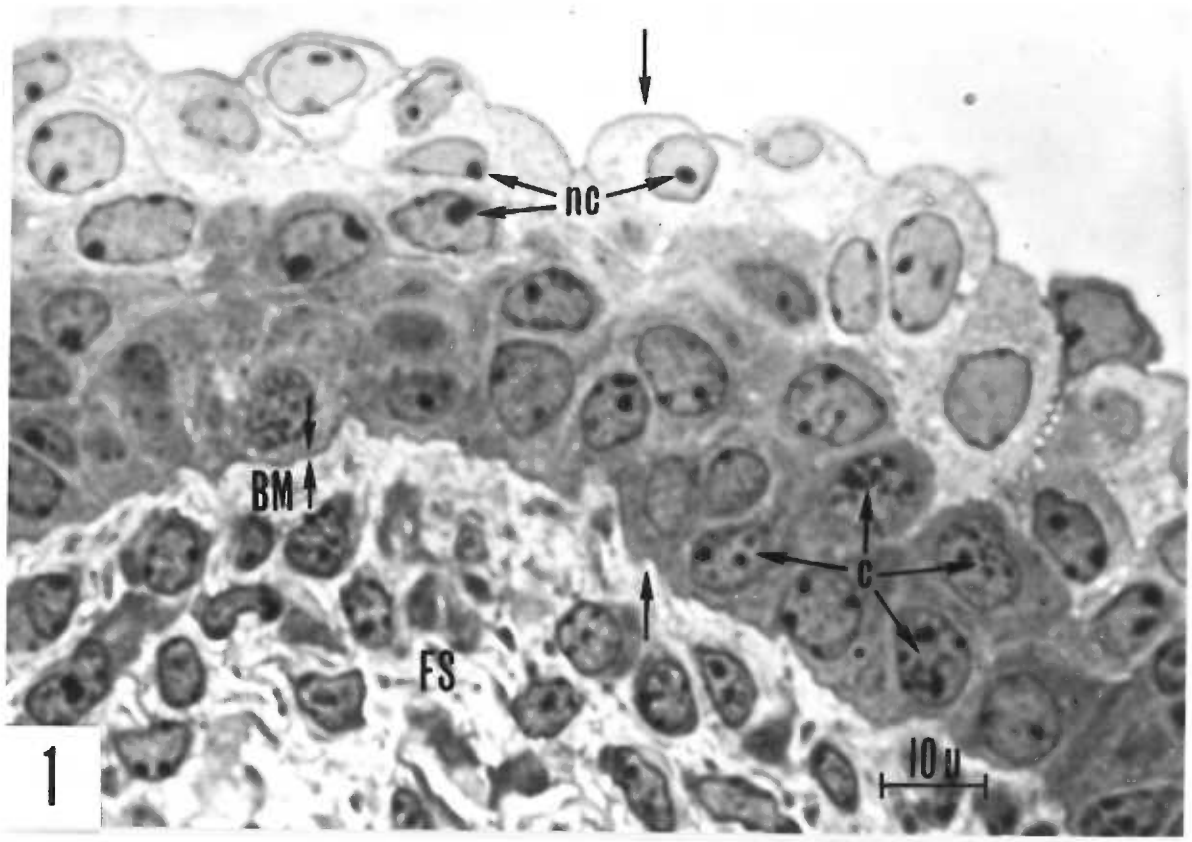
The epithelium (↕) consists of 3 to 4 layers of undifferentiated cuboidal cells overlying a fibrovascular stroma (FS). The basal and intermediate cell cytoplasm is homogeneous and stains with medium intensity. The superficial cell cytoplasm is moderately granular and stains lightly. The cell borders are poorly defined. The nuclei have well defined membranes. Prominent nucleoli (nc) are present in the superficial layer, while basal and intermediate cell nuclei have multiple chromocenters (c). The basement membrane (BM ↕) is prominent.

990X.

Figure 2 (12 hr stimulated):

The basal layer of the epithelium is a single cell in thickness. These elongate, rectangular cells are oriented perpendicular to the poorly defined basement membrane. The basal nuclei have become more elongate and conform to the alteration in cell shape. A metaphase plate (M) is noted in the basal layer, and a degenerating nucleus (dn) is present in the surface layer. Intercellular spaces (IS) of the superficial and intermediate layers are prominent. Cell borders are not well defined.

990X.



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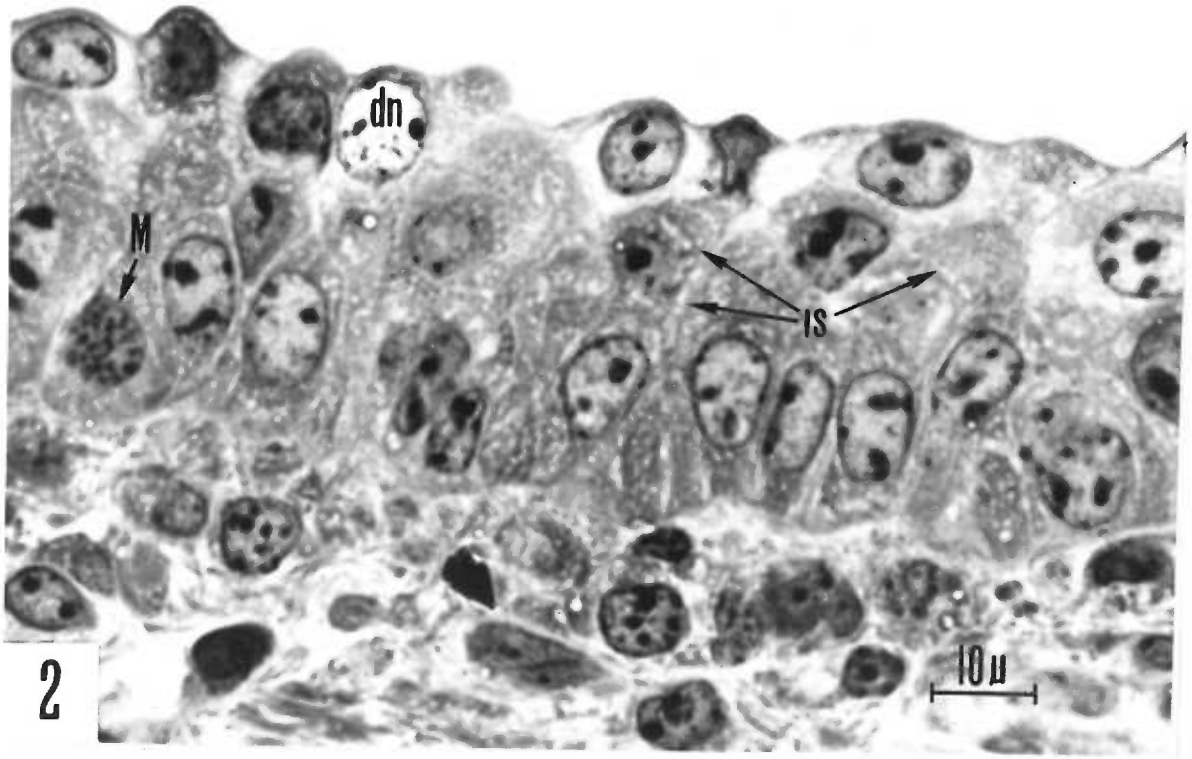


2

Figure 3 (12 hr stimulated):

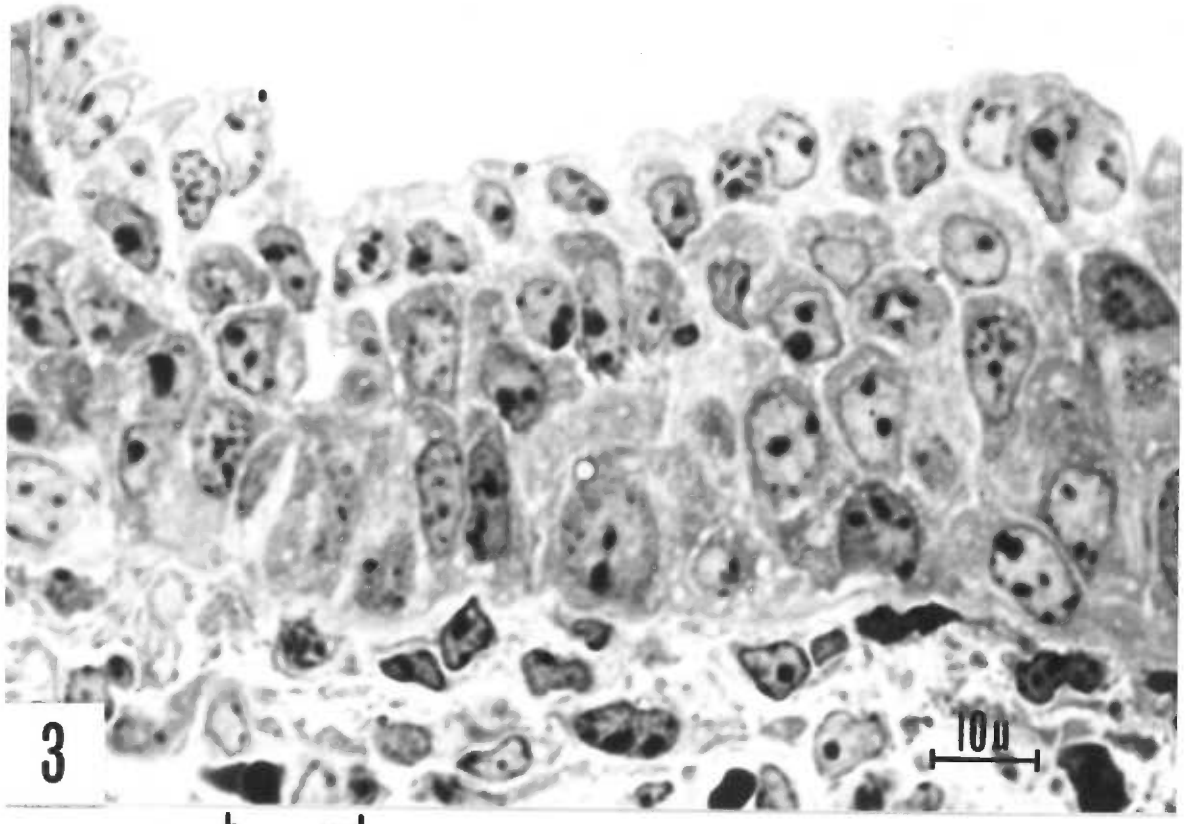
In contrast to the three cell layered epithelium of the previous 12 hr stimulated specimen, the epithelium of this 12 hr stimulated specimen has increased to approximately 5 cells in average thickness, and individual cells are smaller in size. The basal layer contains both cuboidal and rectangular cells in a semi-stratified arrangement. The intermediate layer is pseudostratified with a vertical orientation of nuclei. Superficial cells, while generally similar to intermediate cells, exhibit less intense cytoplasmic staining. Intercellular spaces are prominent in the intermediate and superficial layers.

990X.

Figure 4 (18 hr stimulated):

The basal cells are oriented perpendicular to the basement membrane and are moderately elongate. The intermediate cells are beginning to demonstrate compression and flattening. Irregular cytoplasmic content and staining characteristics, with variably granular and homogeneous cells, are typical of the superficial layer. Blebs (b) of cytoplasm and dense, rounded droplets within clear vacuolar spaces (md) are present in the apical cytoplasm of the surface cells. The superficial layer is 1 to 2 cells in thickness.

990X.



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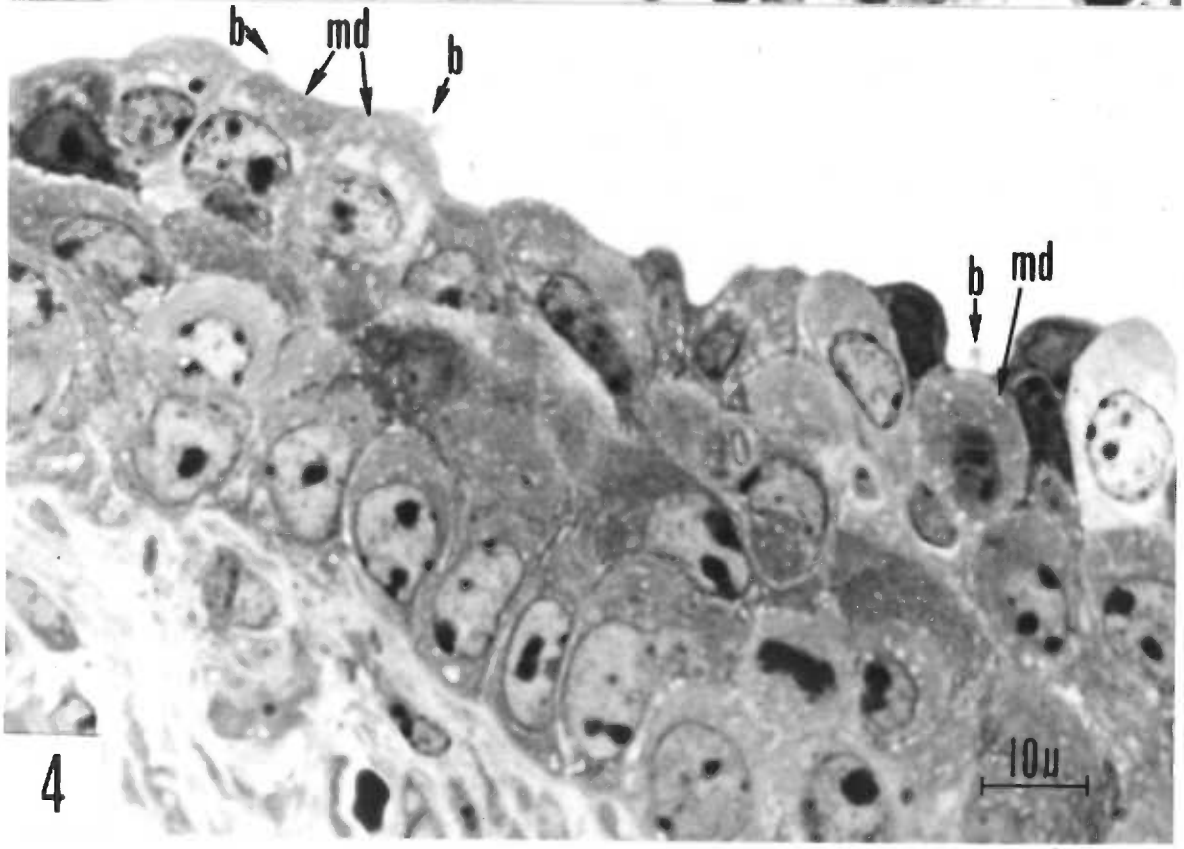

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Figure 5 (18 hr stimulated):

In this specimen the basal and intermediate layers (↕) are composed of cuboidal cells without specific orientation. Their cytoplasm is homogeneous to irregularly granular. The pale cytoplasm of the superficial layer is finely granular and one cell contains dense, rounded droplets in clear vacuolar spaces (md). The perinuclear clear areas without membranes are considered to be artefactual. Chromocenters are most numerous in the basal nuclei. The cell borders are indistinct. The basement membrane is easily identified, and one polymorphonuclear leukocyte (PMN) is noted in the fibrovascular stroma.

990X.

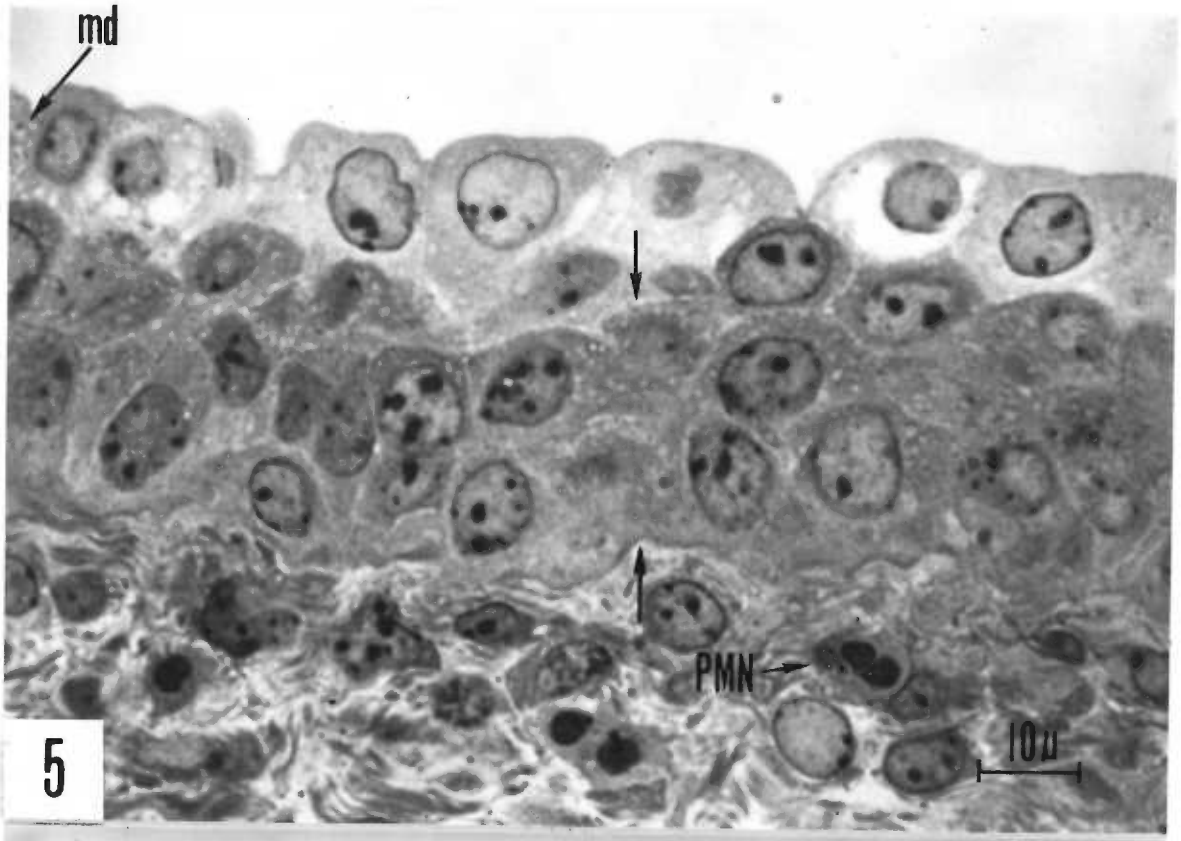
Figure 6 (21 hr stimulated):

The basal cells have the usual perpendicular orientation to the basement membrane. The juxtaposed, parabasal cells are arranged such that nuclei of neighboring cells are located directly above and below one another (↕). The single cells of the intermediate layer are partially horizontal in orientation without prominent intercellular spaces or desmosomes. The staining intensity of cell cytoplasm decreases from the basal layer to the superficial layer.

The superficial layer is two or more cells in thickness with an irregular surface and occasional disintegrating cells (dc). The cytoplasm of the surface cells contains increased numbers of droplets in clear spaces (md).

A few scattered cells in the superficial layer have very heavily stained cytoplasm and inapparent nuclei.

990X.



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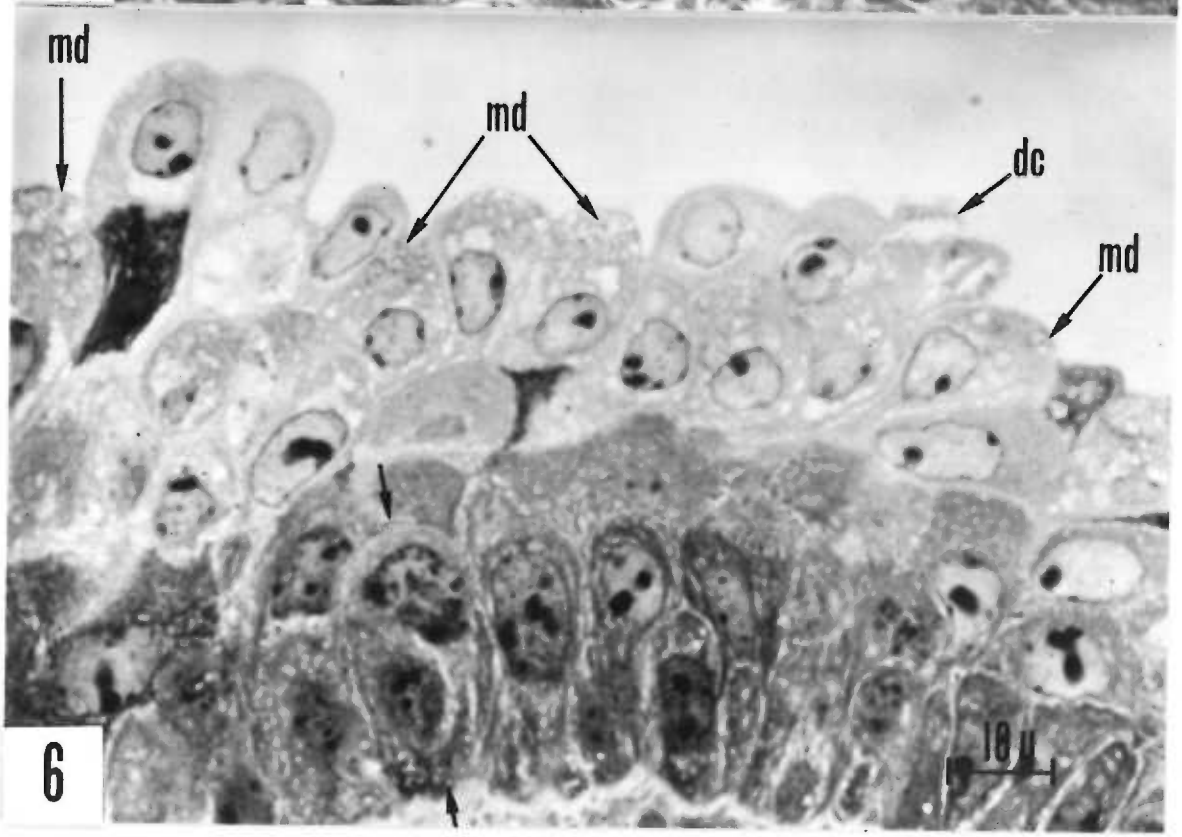


6

Figure 7 (24 hr stimulated):

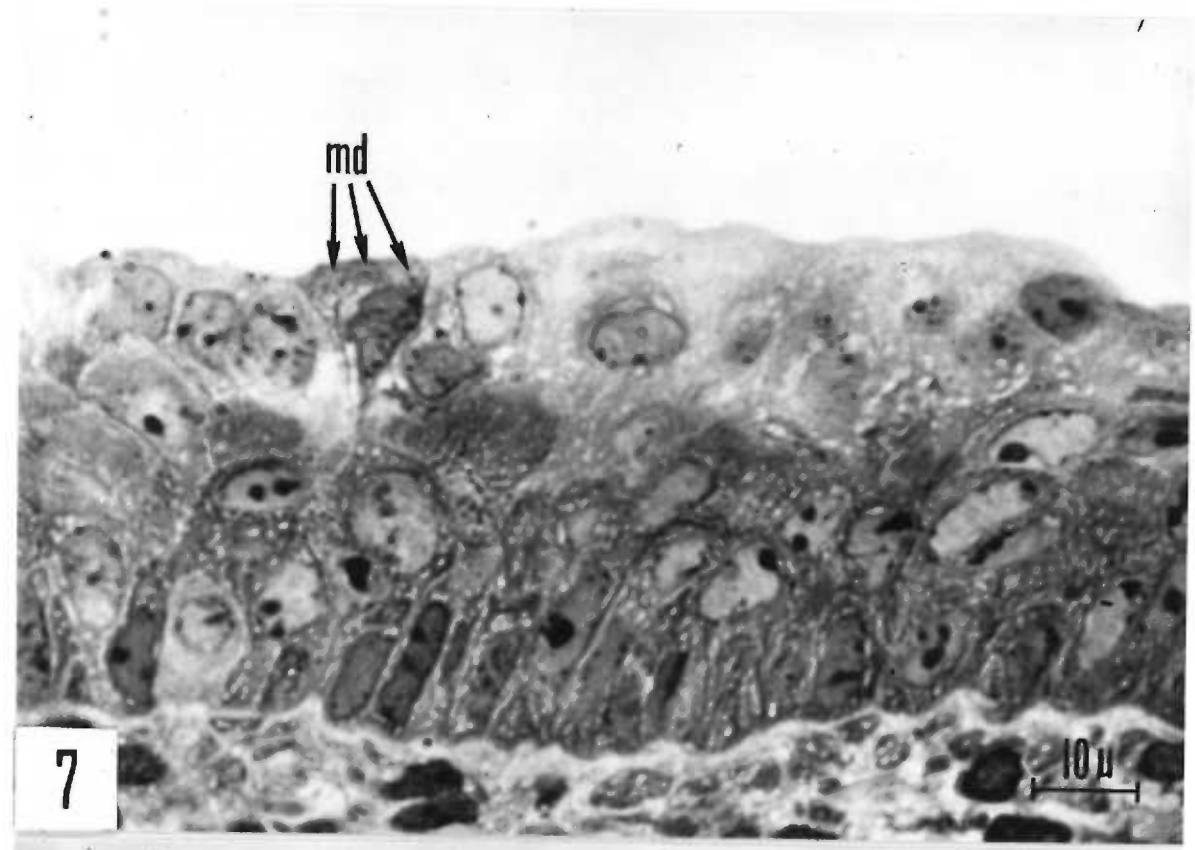
In this specimen the basal cells are tall, rectangular, and oriented perpendicular to the well-defined basement membrane. They have oval, elongate, narrow nuclei. The intermediate layer is 1 to 2 cells thick, and the nuclei are irregularly disposed. Intercellular spaces are focally prominent and are interrupted by desmosomes (intercellular bridges). The superficial layer is 1 to 2 cells thick. One cell demonstrates prominent, dense, rounded droplets within clear, vacuolar spaces (md).

990X.

Figure 8 (24 hr stimulated):

The basal cells are similar to previous specimens and the basement membrane is prominent. The intermediate layer (IM ↕) in this specimen is 2 to 5 cells thick. The cells are flattened and oriented horizontally with trapezoidal cells resembling the *stratum spinosum* of keratinizing epithelium. Numerous desmosomes are apparent between the cells of the intermediate layer, including the apical portion of the basal cells, but are absent along the juxtaposed membranes of the superficial cells. The superficial layer is one cell thick, very lightly stained. One cell demonstrates numerous dense, rounded cytoplasmic droplets (md) and vacuoles. Polymorphonuclear leukocytes (PMN) are noted in the fibrovascular stroma.

990X.



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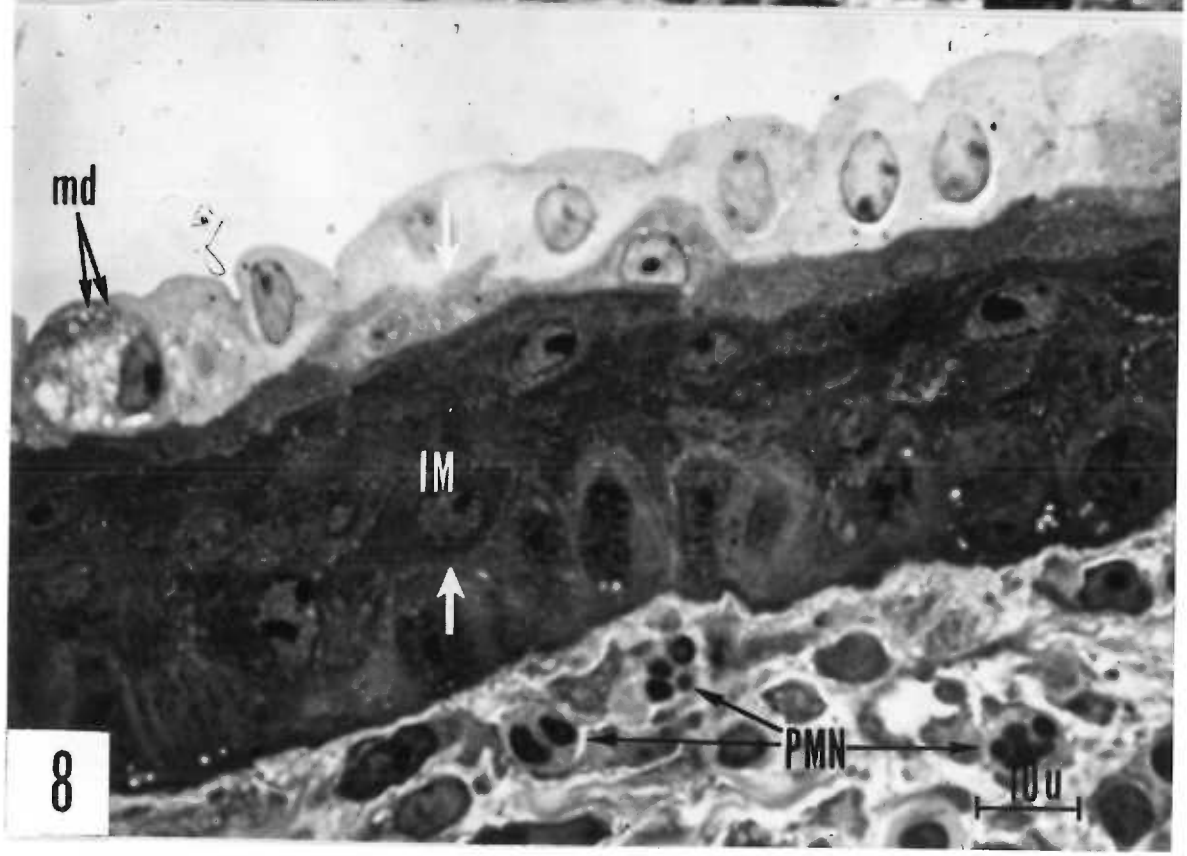


8

Figure 9 (0 hr control - type C fixation) (for details of fixation in this and subsequent micrographs please see the section on Materials and Methods):

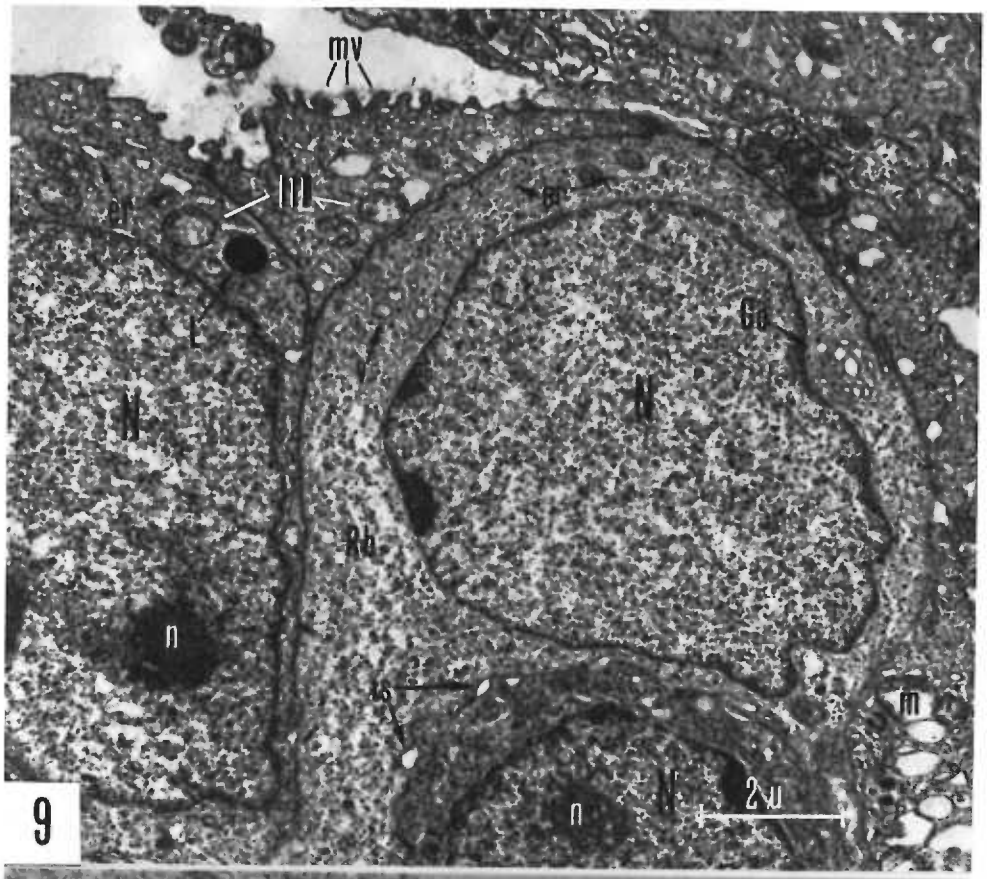
Superficial Cell. These cuboidal cells have prominent, central nuclei (N), diffuse aggregates of chromatin, and small, dense nucleoli (n). At their luminal surface, prominent microvilli (mv), approximately 250 mμ in height, are invested with a moderately dense, reticular material. The cytoplasm contains sparse, granular aggregates (Rb) from < 200 Å to > 1000 Å in diameter, and numerous organelles. Ovoid mitochondria (m) are scattered in small numbers throughout the cytoplasm. Rough-surfaced endoplasmic reticulum (er), some of which is slightly dilated, is located primarily in the apical regions of the cells. A small Golgi apparatus (Go), less than 1 μ in diameter, and small, circular vesicles (v) lie lateral to the nucleus, usually in the apical hemisphere. Occasional intercellular spaces (IS) may be seen between cells of the superficial and intermediate layers. Rare dense, spherical, osmophilic bodies (L), interpreted as lipid, are present randomly in the cytoplasm.

X8,400.

Figure 10 (0 hr control - type C fixation):

Superficial Cells. At this magnification, the major cellular features are demonstrated. They include a lightly stained microvillous investment (gc), mitochondria (M), rough surfaced endoplasmic reticulum (er), a small Golgi apparatus (Go) with distinctive longitudinal and spherical vesicles, and slightly dilated, rough-surfaced endoplasmic reticulum (er) located immediately inferior to the smooth vesicles. Adjacent cell membranes, at their juxtaluminal aspect, are modified by a zona occludens (ZO) and a terminal bar (desmosome) (TB). Elsewhere along the cell membrane, desmosomes between the superficial cells are extremely rare.

X22,800.



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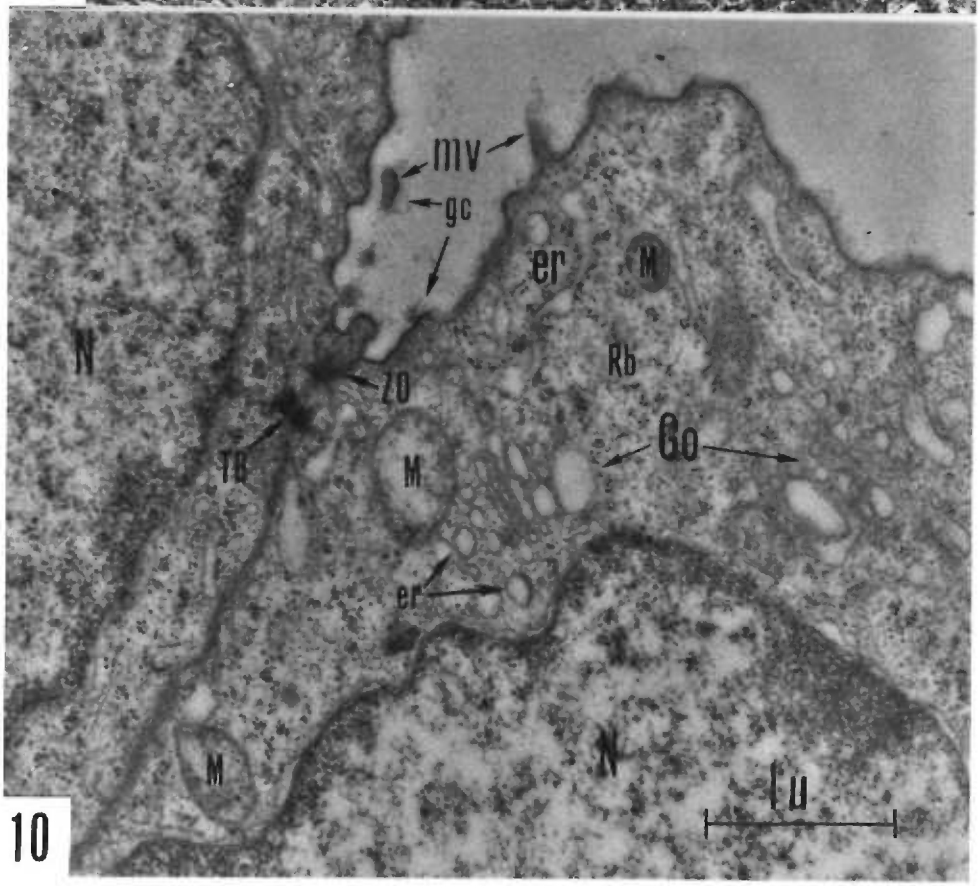


10

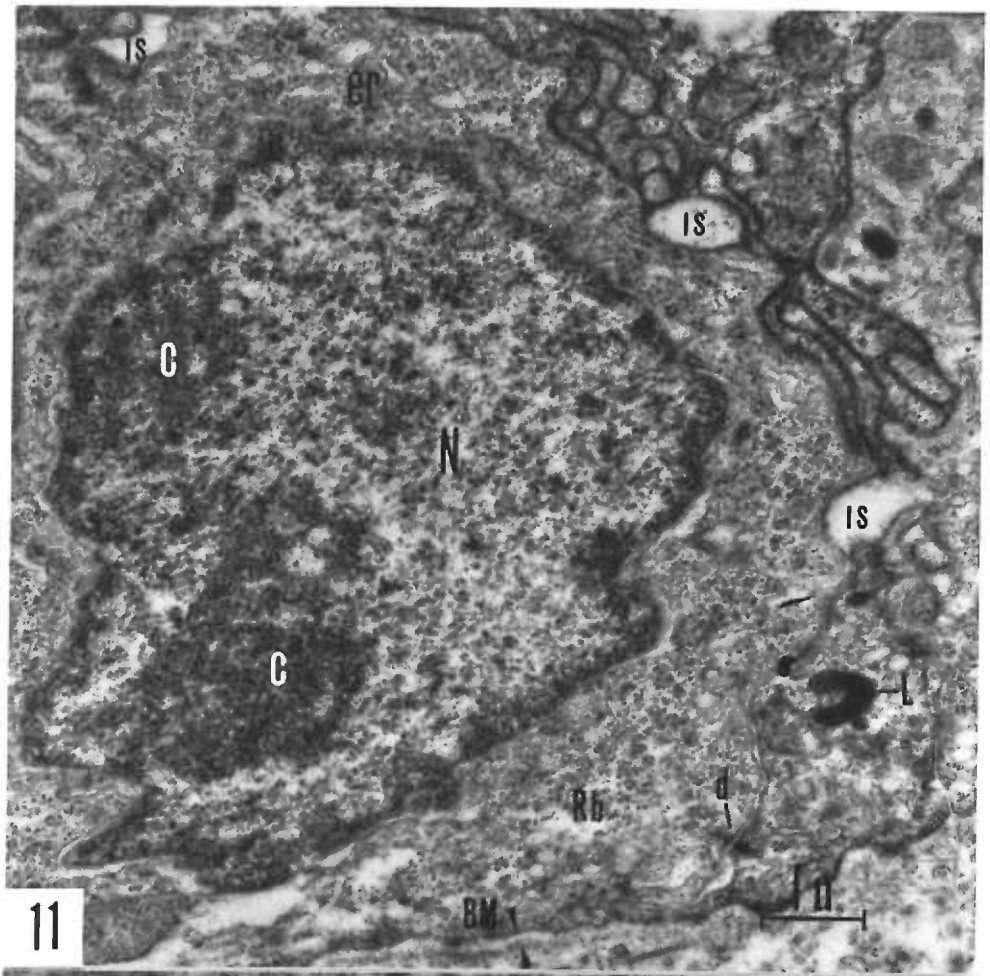
Figure 11 (0 hr control - type C fixation):

Basal Cells. The cell is cuboidal with a central, large nucleus (N) and two, moderately dense, chromocenters (C). The cell borders form complex interdigitations. Intercellular spaces (IS) between adjacent basal and intermediate cells are prominent. An occasional desmosome (d) is noted. Ribosomal aggregates (Rb) are uniformly distributed in the cytoplasm. The endoplasmic reticulum (er) is slightly dilated in the apical area. One U-shaped, osmophilic body (L) is noted. The basement membrane (BM ↓↑) is approximately 600-700 Å in width and, in this section, has no hemidesmosomes. X11,600.

Figure 12 (0 hr control - type C fixation):

Basal Cell. By comparison with Figure 11, which is more representative of basal cells in control specimens, these basal cells have larger numbers of desmosomes (d) with short but distinct fibril bundles (f). Two hemidesmosomes (h) modify the basement membrane. Mitochondria appear more prominent in both number and size near the cell borders.

X22,800.



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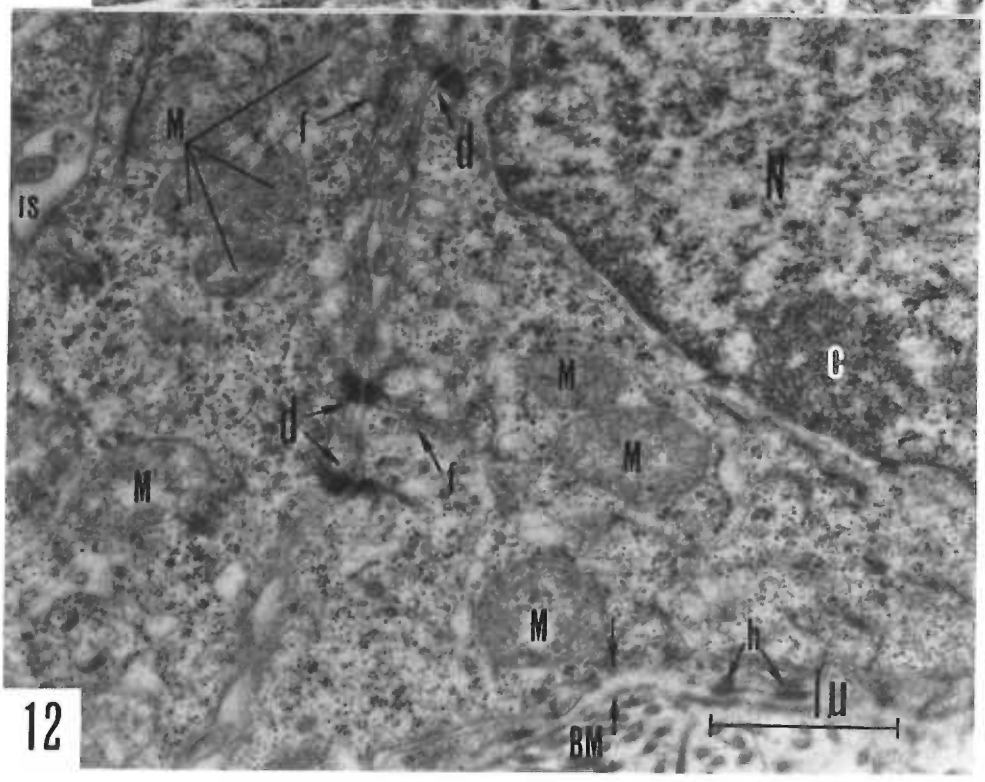

12

Figure 13 (0 hr control - type C fixation):

Intermediate Cell. Glycogen granules (Gly) are frequently noted in the apical perinuclear cytoplasm. Individual granules are larger in size (∿ 250 vs 70 Å in diameter) and are less osmophilic than the ribosomes.

X22,800.

Figure 14 (0 hr control - type C fixation):

Intermediate Cell. One small Golgi apparatus (Go), 1 μ in diameter, segments of slightly dilated endoplasmic reticulum (er), a few fibrils (F), and two desmosomes (d) are present.

X22,800.

Figure 15 (0 hr control - type C fixation):

Parabasal (lower intermediate) Cell. In addition to mitochondria (M), Golgi apparatus (Go), and a nucleus (N), an elongate, membrane-lined space, 0.3 μ by 1.9 μ in length, contains an osmophilic column (Ci), 0.13 μ by 1.4 μ, with numerous longitudinal striations. This unusual organelle is interpreted as a normal, but heterotopic, cilium.

X16,400.

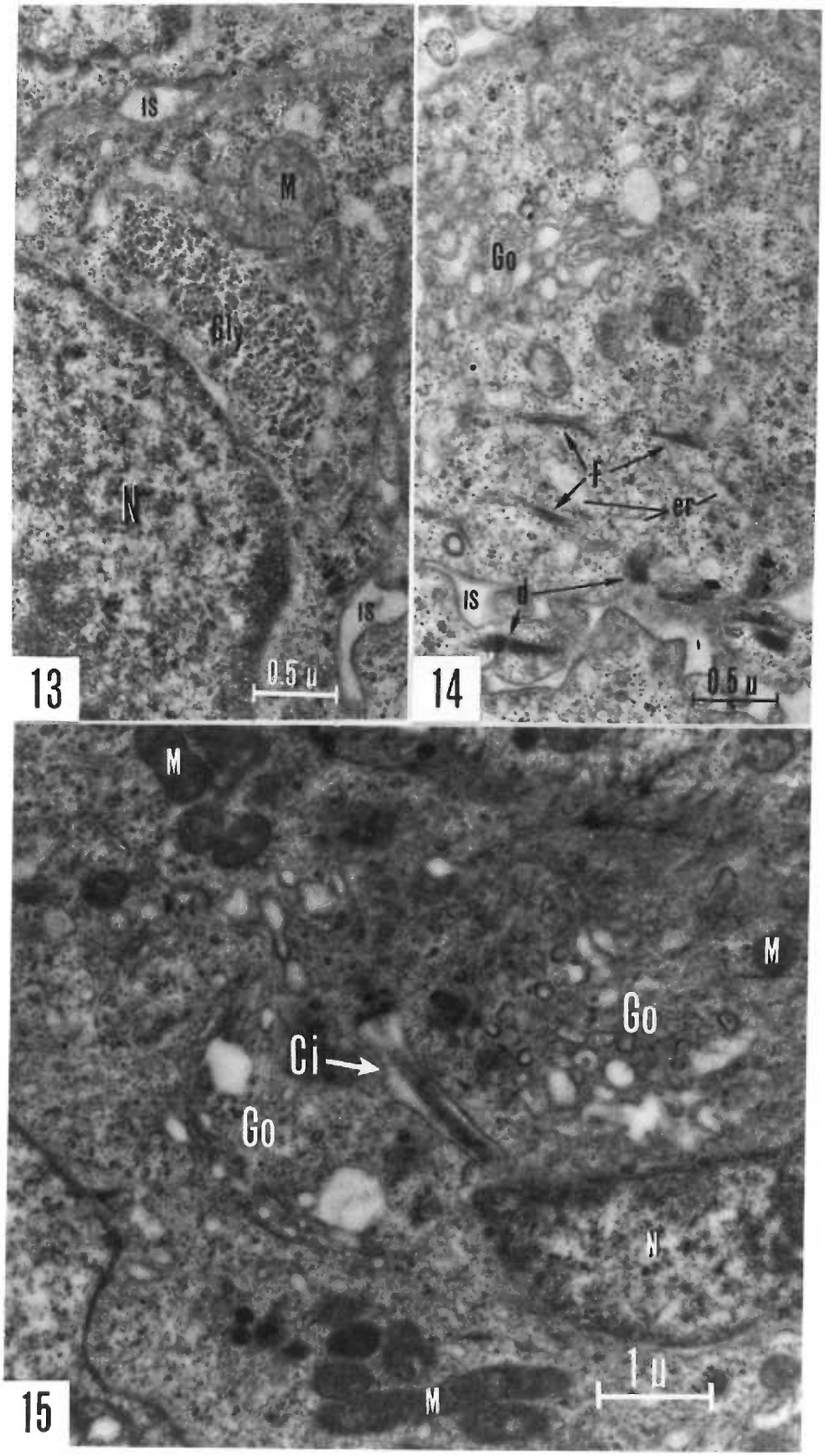
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Figure 16 (6 hr experimental - type A fixation):

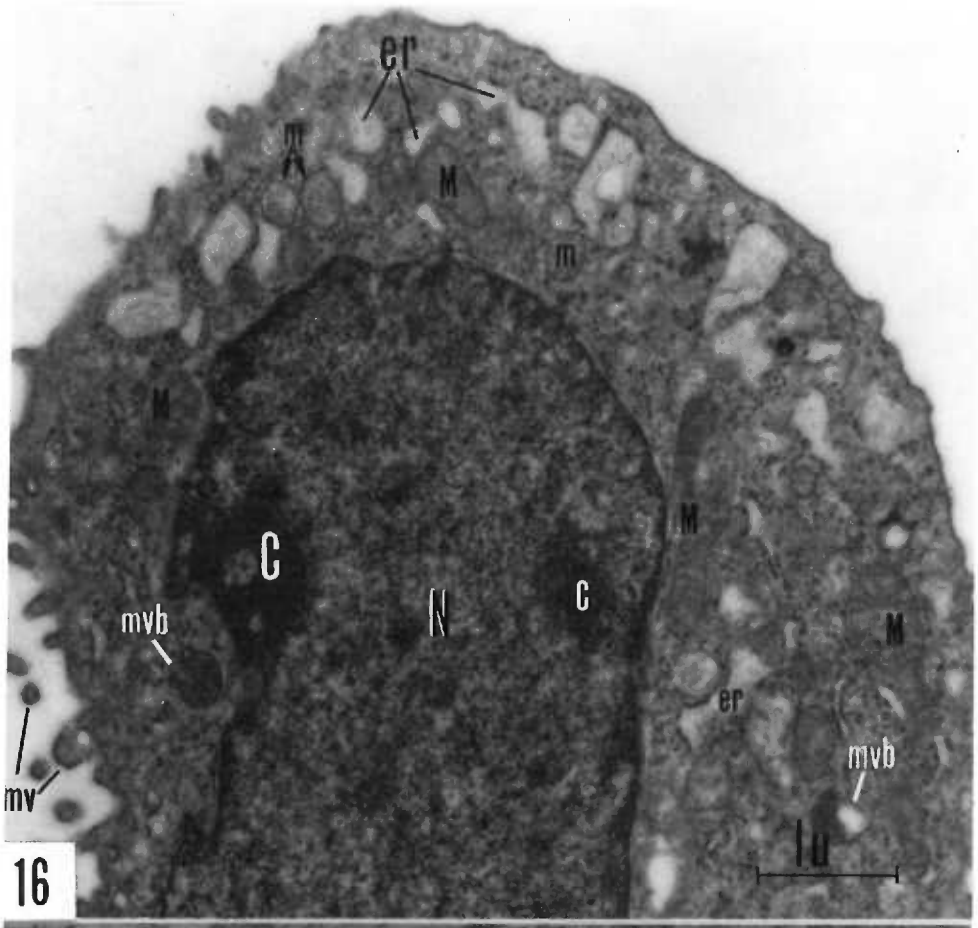
Surface Cell. In this cell the microvilli (mv) are localized to the lateral border of the cell with only occasional small ridges near the apex. The endoplasmic reticulum (er) is markedly distended by an amorphous material of uniform, low density. Near the cell apex three, 0.3 μ to 0.5 μ, oval smooth membrane-bound vesicles (m) contain moderately dense, finely granular material. These differ from the mitochondria (M) by virtue of the absence of internal cristae. Present in the cytoplasm, lateral to the nucleus (N), are circular, dark bodies (mvb), approximately 0.45 μ in diameter, containing numerous small membrane-bound vesicles. One of these multivesicular bodies (mvb) contains a large empty area interpreted as artefact.

X16,400.

Figure 17 (6 hr experimental - type A fixation):

Basal Cell. As compared with the controls, the cell is moderately elongate with its long axis perpendicular to the basement membrane (BM ↓↑). The adjacent cell membranes have zones of villiform interdigitation, but lack prominent intercellular spaces. The large, central nucleus (N) has areas of granular chromatin aggregation (c) near the nuclear membrane with the remaining chromatin granules spread more diffusely throughout the karyoplasm. The cytoplasm contains a few mitochondria (M), and sparse ribosomal aggregates (Rb). Rough-surfaced endoplasmic reticulum (er) is indistinct. The basement membrane (BM ↓↑) is 600-700 Å in width with occasional hemidesmosomes (h).

X16,400.



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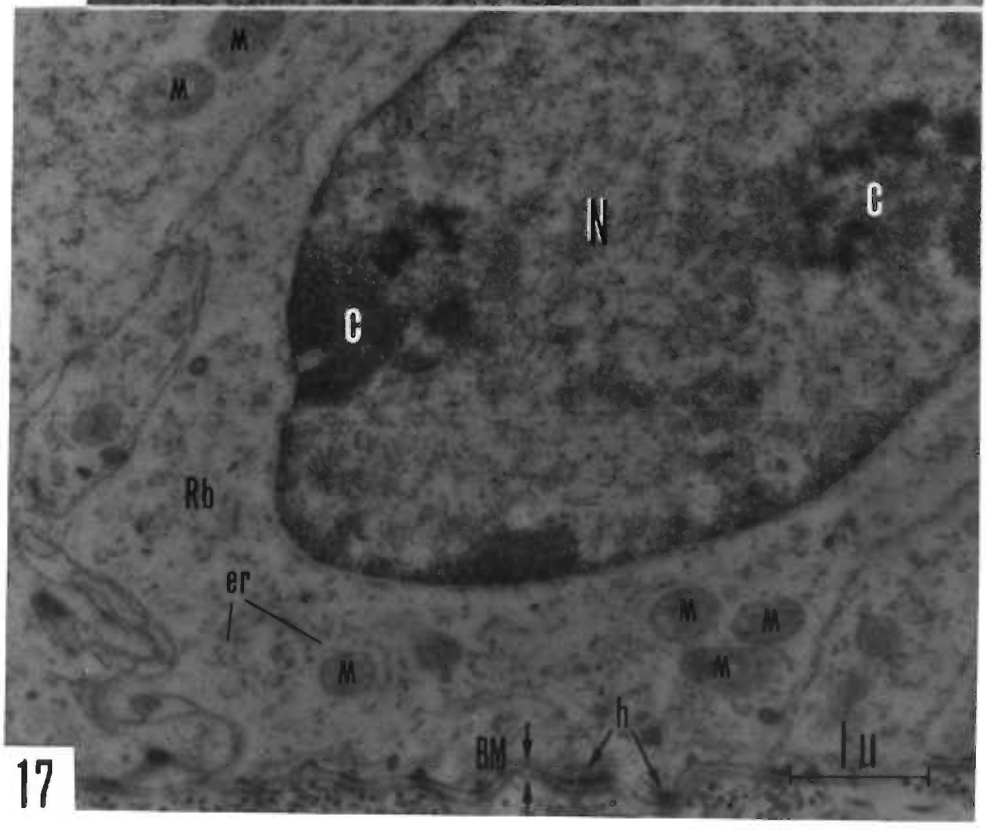


17

Figure 18 (6 hr experimental - type A fixation):

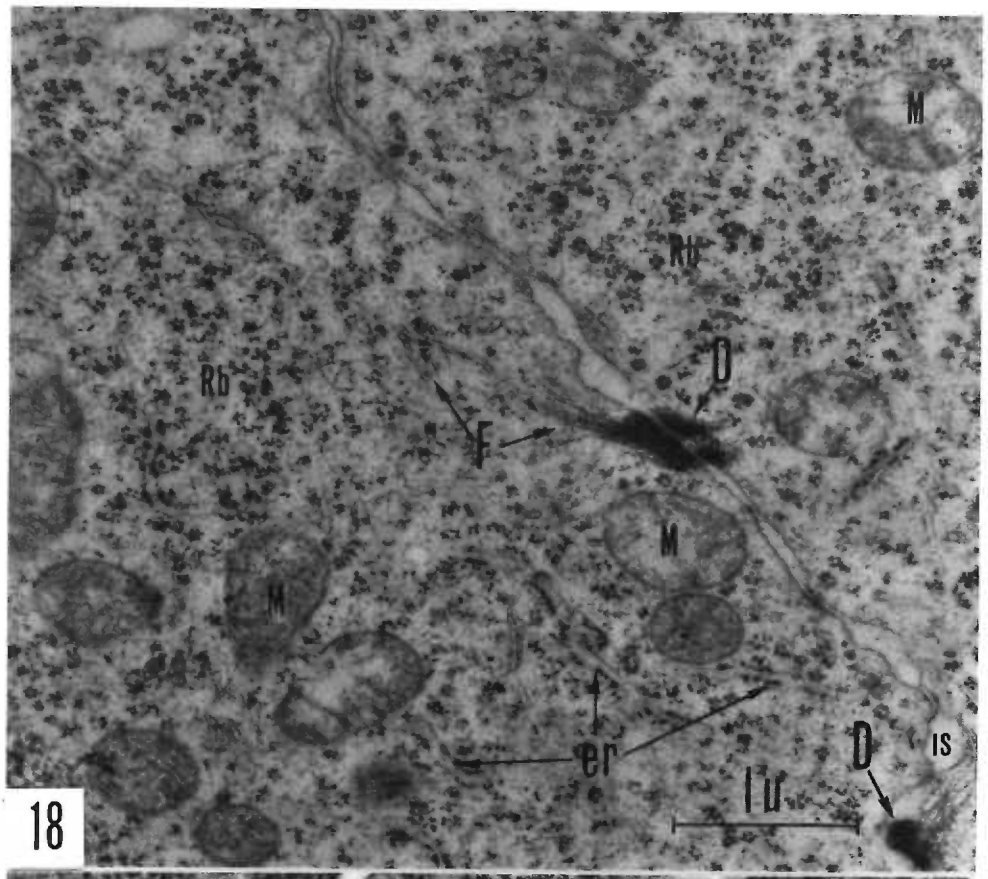
Intermediate Cells. Ribosomal aggregates (Rb) are distributed regularly throughout the cytoplasm. Two desmosomes (D), with their attached fibril bundles (F) extending well into the cytoplasm, are present. Neither the intercellular spaces (IS) nor the rough-surfaced endoplasmic reticulum (er) are dilated.

X22,800.

Figure 19 (6 hr experimental - type B fixation):

Intermediate Cell. In this micrograph, intercellular spaces (IS) are irregularly dilated. A few, fine fibrils (filament groups) (f) are scattered diffusely in the cytoplasm. One small osmophilic body (L) is interpreted to be lipid. The Golgi apparatus (Go) is prominent, measures 2 microns in longest dimension, and has numerous closely approximated vacuoles (v). Additional vacuoles (v), which appear to be empty, are present on the contralateral side of the nucleus (N).

X22,800.



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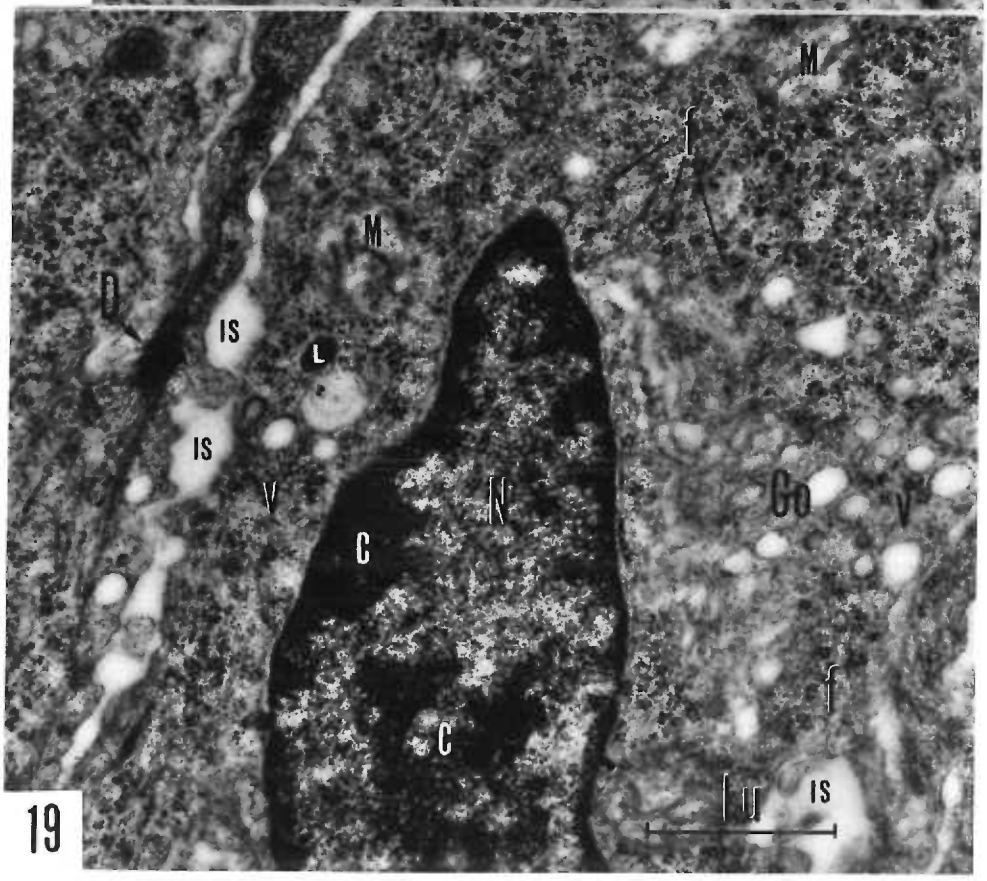


19

Figure 20 (12 hr experimental - type A fixation):

Intermediate Cells. An enlarged Golgi apparatus (Go), approximately 1 micron in greatest dimension with closely approximated dilated rough-surfaced endoplasmic reticulum (er), is seen in many upper level intermediate cells during this time interval. Desmosomes (D) and random filament groups (f) also occur in intermediate cells at 12 hrs.

X22,800.

Figure 21 (12 hr experimental - type B fixation):

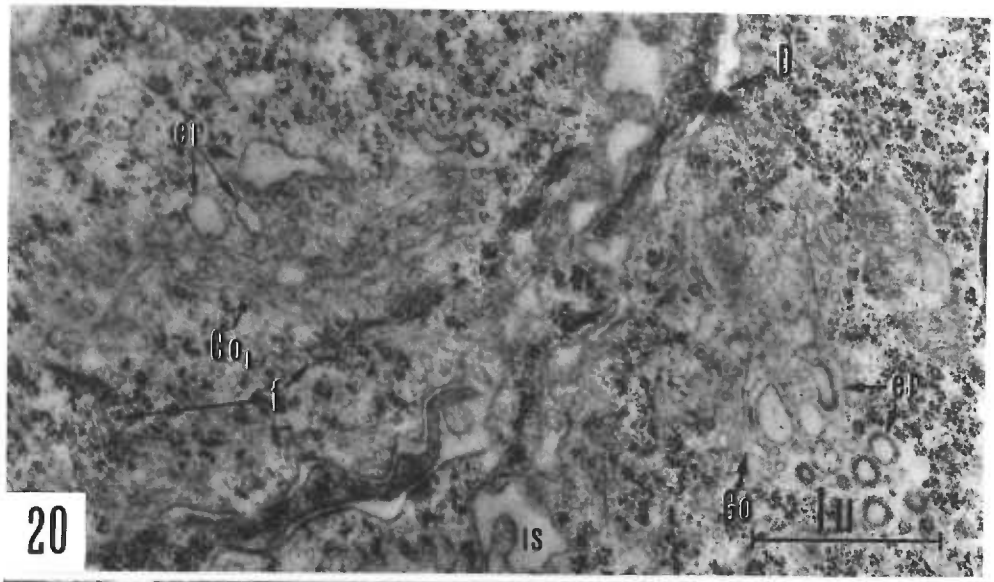
Intermediate Cell. When compared to the cell in Figure 20, this cell is characterized by longer, thicker fibrils (f), desmosomes (D) at closer intervals along the cell borders, and an increase in ribosomes (Rb). These changes suggest a type of differentiation for this intermediate cell quite different from that of the cell in Figure 20.

X22,800.

Figure 22 (12 hr experimental - type B fixation):

Basal Cells. The intercellular spaces (IS) are modified by complex interdigitations of cell membranes. The numbers of hemidesmosomes (h) are increased. Mitochondria (m) may be increased in number; however, their size range is constant at 0.5 to 1.0 microns in diameter. A single, irregular, dense cytoplasmic particle, approximately 0.5 micron in greatest dimension, is interpreted as an artefact (a).

X11,600.



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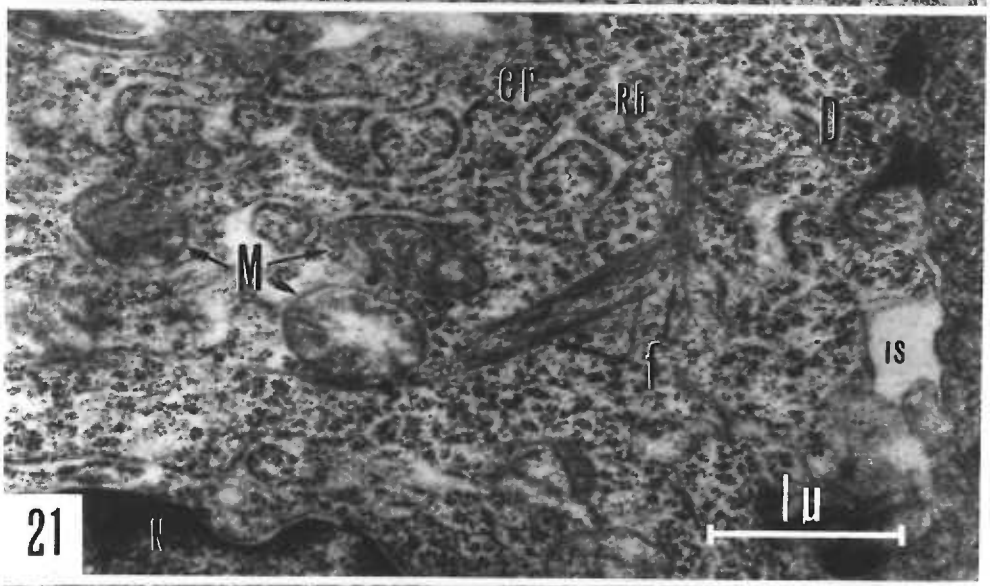

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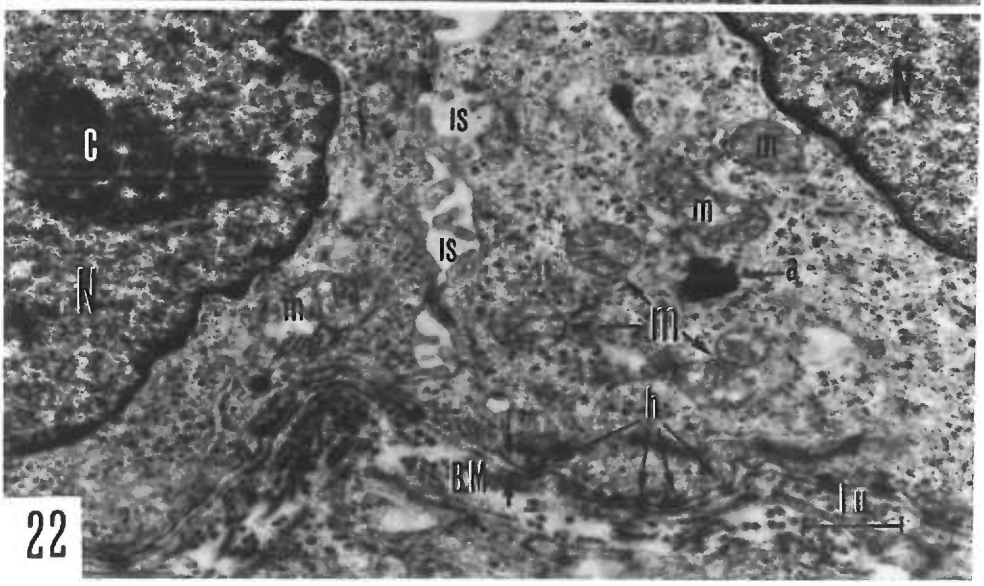


22

Figure 23 (16 hr experimental - type C fixation):

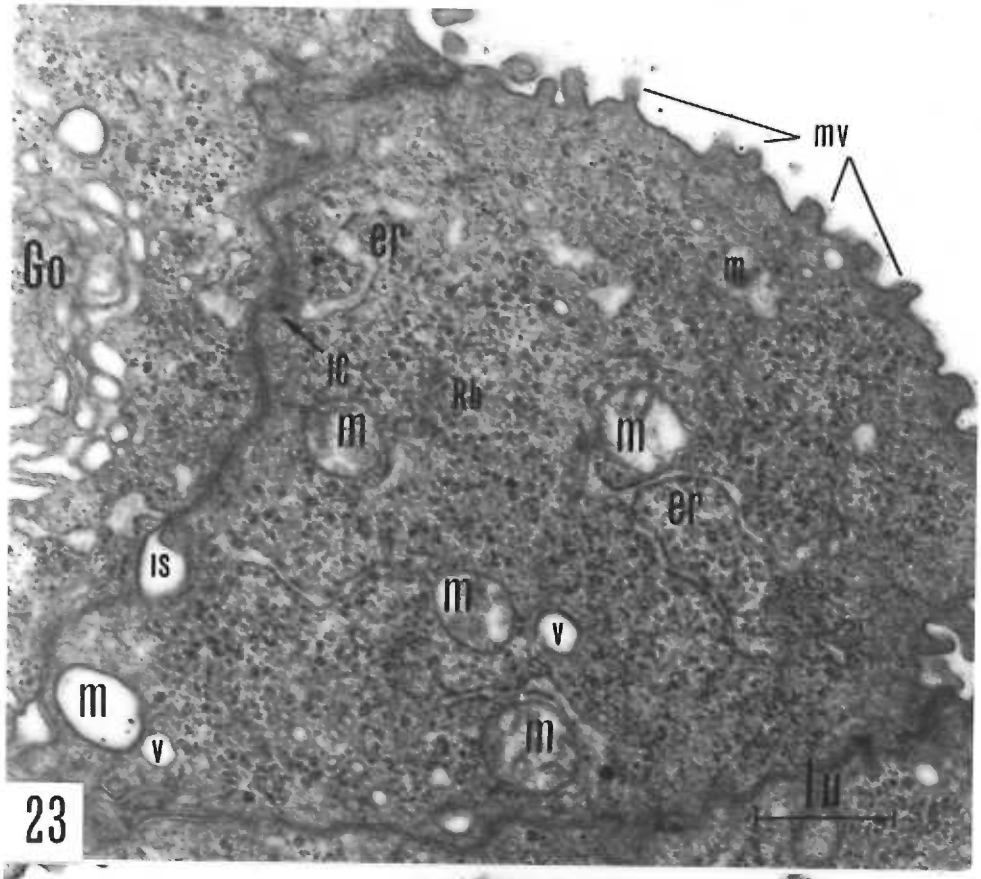
Surface Cells. The large cell is approaching the shape of an inverted cone. The microvilli (mv) with their individual, reticular, apical material are variable in size and shape along the luminal border. The cytoplasm is densely packed with ribosomes (Rb). The rough-surfaced endoplasmic reticulum (er) contains granular material of medium density. At one point along the lateral cell membrane, segments of two cells are in immediate juxtaposition to the cell membrane (IC). There are moderate numbers of cytoplasmic vacuoles (v). In one cell, a Golgi apparatus (Go), greater than 2 microns in longest dimension, is present.

X16,400.

Figure 24 (16 hr experimental - type C fixation):

Surface Cell. In addition to the microvilli (mv), Golgi apparatus (Go), and rough-surfaced endoplasmic reticulum (er) noted in Figure 23, there is an apparent increase in mitochondria (M) in this cell. The size of the largest mitochondrion is less than 0.8 micron, indicating that there is no appreciable increase in mitochondrial size.

X22,800.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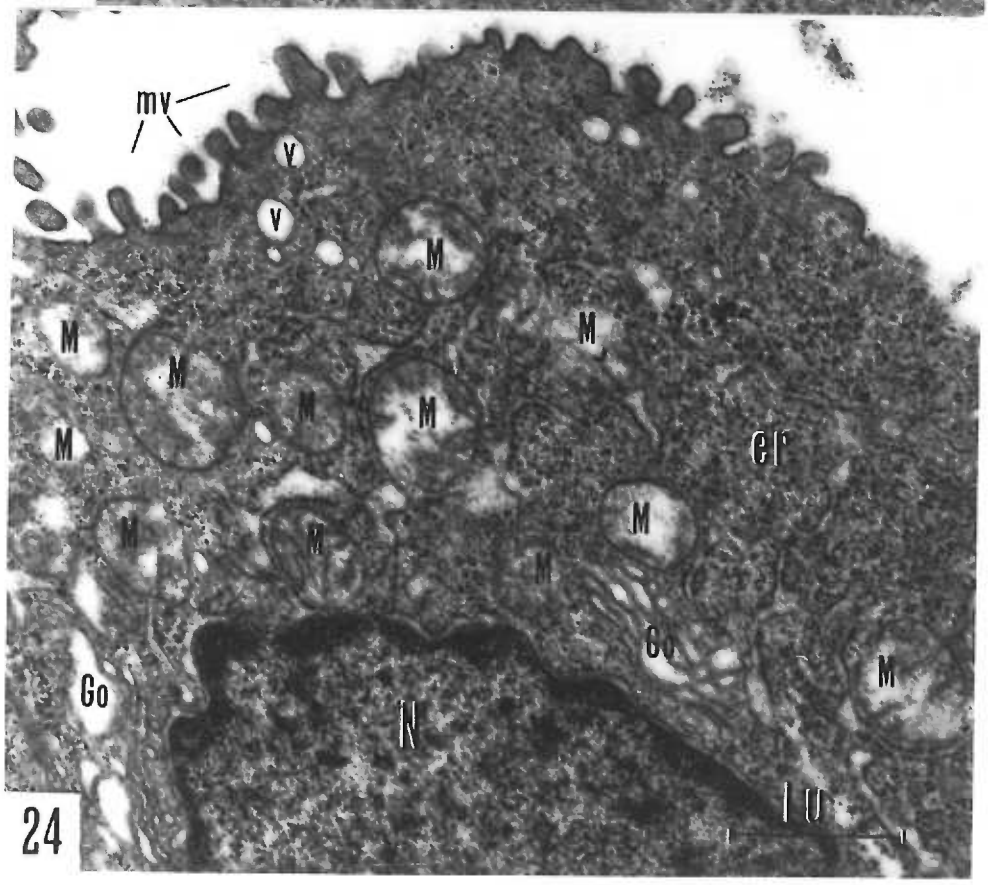
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Figure 25 (18 hr experimental - type C fixation):

Superficial Cells. The cells have assumed a conical shape with the cytoplasm shifted toward the apex of the cell. Dilated endoplasmic reticulum (er) is easily identified along with numerous small vacuoles mainly in the apical cytoplasm. The Golgi apparatus (Go) have increased to greater than 3 microns in longest dimension and material of moderate electron density (mu) can be seen to partially fill vacuoles at the mature face of the Golgi apparatus.

X8,400.

Figure 26 (18 hr experimental - type C fixation):

Superficial Cell. Membrane-bound vacuoles (mu), approximately 1 micron in diameter, contain osmophilic granules in a reticular pattern. Rough-surfaced endoplasmic reticulum (er) also contains material of similar appearance, but less concentrated. The lumen of the endoplasmic reticulum appears to communicate with the lumen of one vacuole at point (c). Mitochondria (m).

X22,800.

Figure 27 (18 hr experimental - type B fixation):

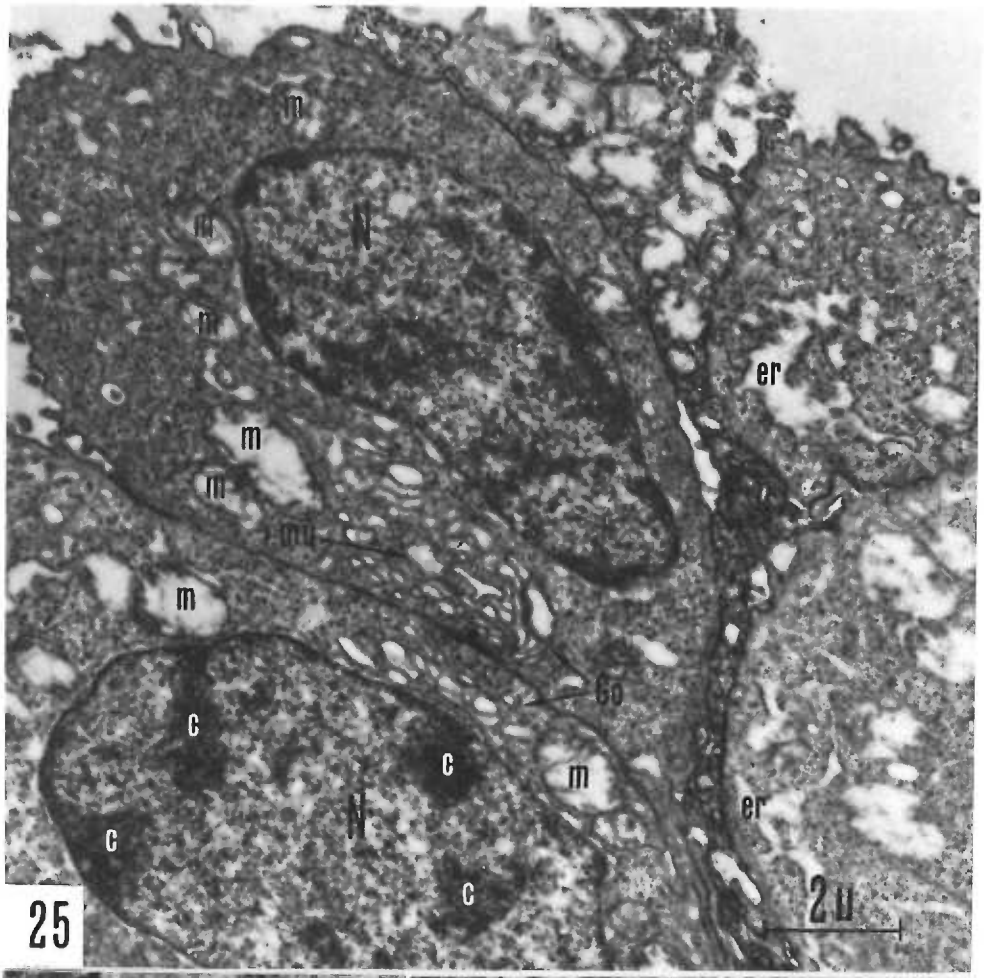
Intermediate Cell. Darkly stained, large granular aggregates of glycogen (Gly) are present. A "myelin body"-like artefact (my) is thought to be due to mitochondrial condensation from overexposure to glutaraldehyde fixation.

X22,800.

Figure 28 (18 hr experimental - type C fixation):

Superficial Cell. A well-defined Golgi apparatus (Go), approximately 4 microns in greatest dimension, illustrates the following characteristics. The Golgi apparatus is cup-shaped with a convex, forming face (FF) and a concave, mature face (MF). The forming face is composed of numerous, empty vacuoles of various sizes. Intermediate between the two faces is one or more elongate cisternae with moderately osmophilic, amorphous material in their lumina. At the mature face numerous very small vacuoles (cv) containing similar amorphous material seem to coalesce into larger vacuoles. The larger vacuoles at the mature face in this micrograph appear empty.

X16,400.



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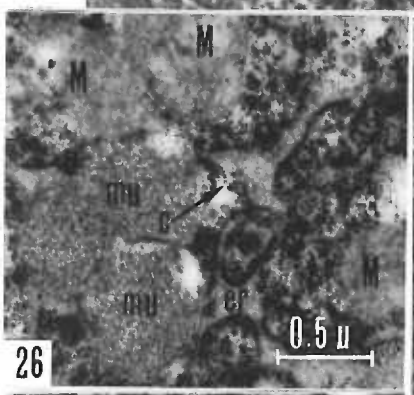

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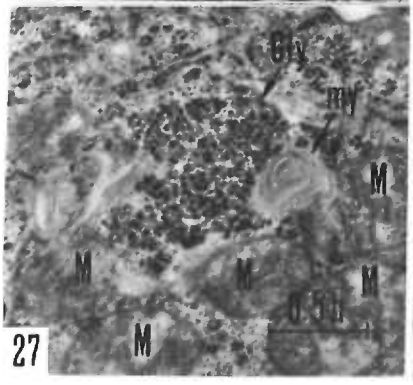

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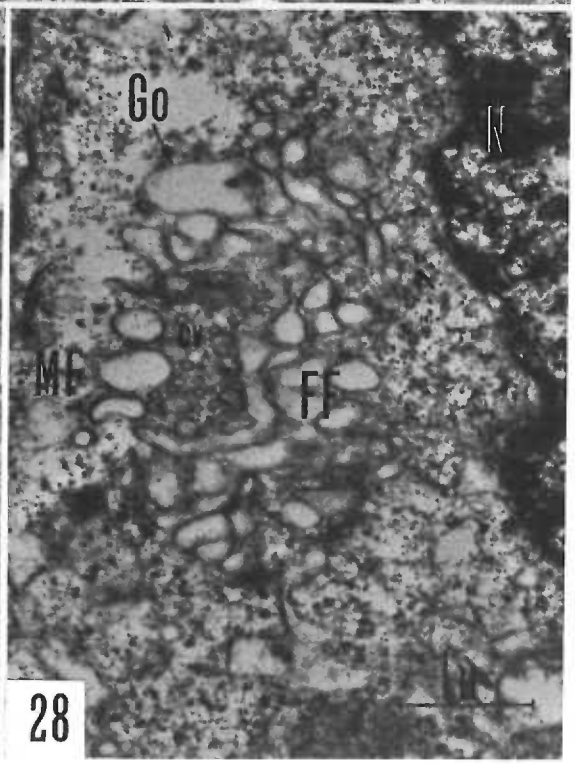


28

Figure 29 (21 hr experimental - type C fixation):

Surface Cell. Microvilli (mv) with their individual investments of moderately dense material are abundant at the surface. Mucus (mu) droplets become progressively more electron dense and smaller in size as they approach the luminal border. They range from > 1 micron near the Golgi apparatus (Go) to < 0.5 micron at the cell border.

X11,600.

Figure 30 (20 hr experimental - type B fixation):

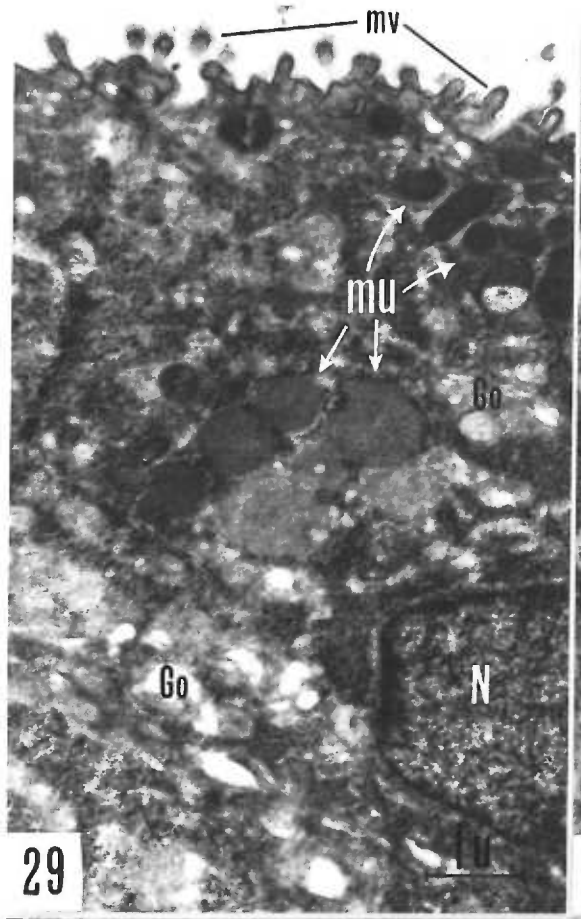
Surface Cell. At a higher magnification, the microvilli (mv) are seen to have linear thread-like projections from the distal one-third of their surface. Dilated rough-surfaced endoplasmic reticulum (er) with small amounts of lightly stained, amorphous material appear in association with the mucus droplets (mu).

X22,800.

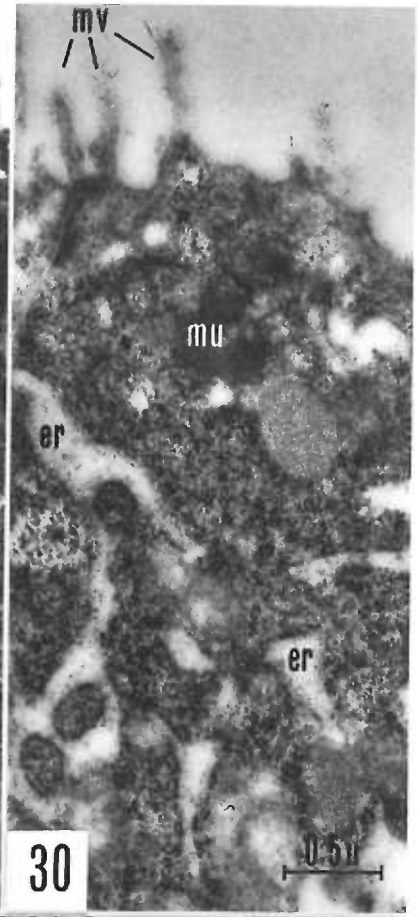
Figure 31 (21 hr experimental - type A fixation):

Intermediate Cell. At this time, these cells have definite squamous characteristics--prominent cytoplasmic fibrils (f), and numerous desmosomes (D) along the cell membranes, which are oriented parallel to the luminal surface. The secretory characteristics of the surface cells (Figs. 29 and 30) are absent.

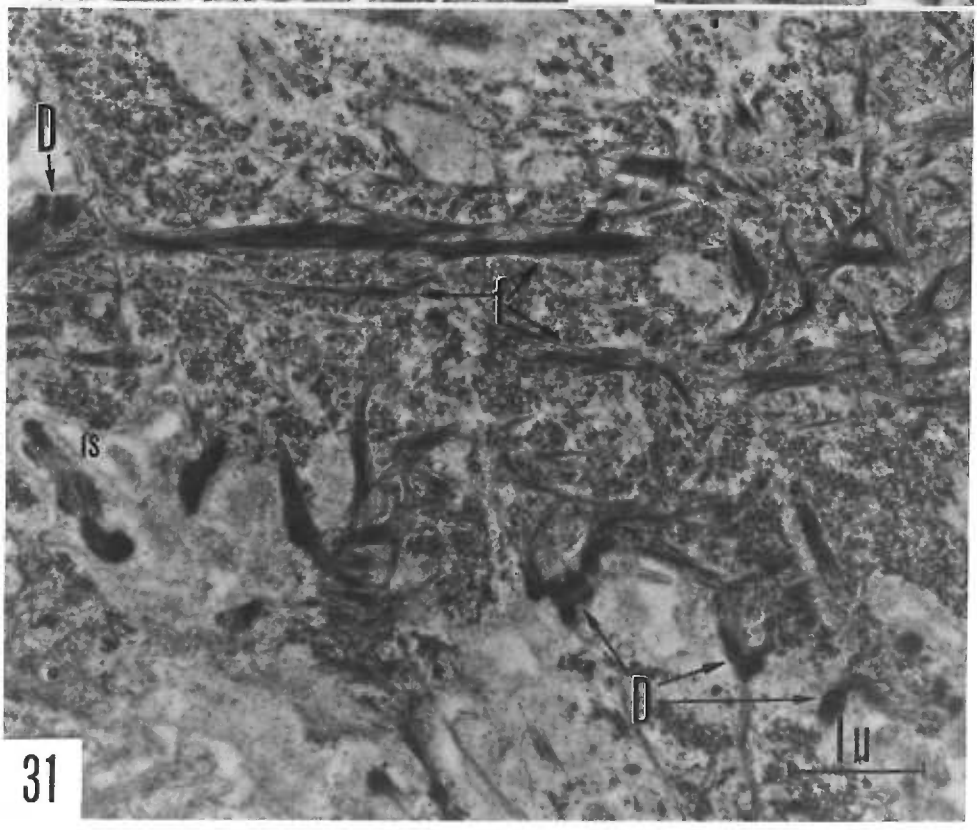
X16,400.



29



30



31

Figure 32 (24 hr experimental - type C fixation):

Surface Cell. The microvillous border (mv) is invested with numerous, individual bristles (gc), approximately 500 Å in height, which are oriented perpendicular to the surface of the microvilli.

X22,800.

Figure 33 (24 hr experimental - type C fixation):

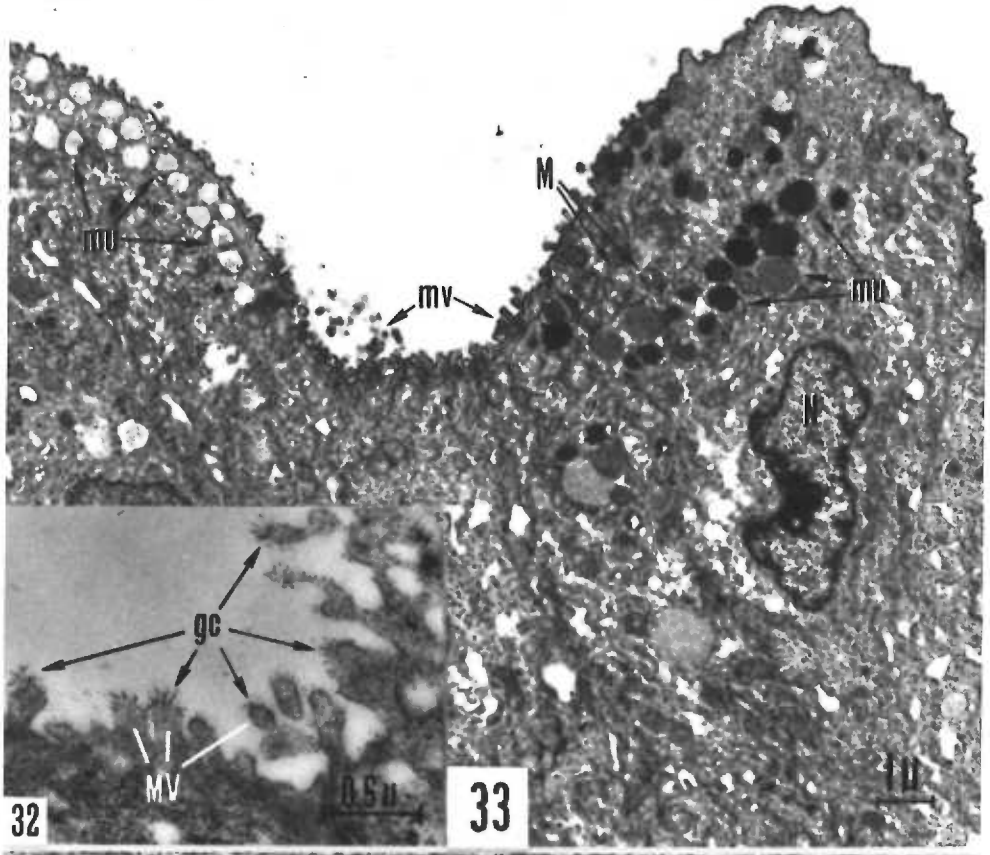
Superficial Cells. In contrast to the densely stained mucous droplets (mu) on the right, the amorphous material on the left is more lightly stained. Therefore, despite their label (mu), the vacuoles on the left may be dilated rough-surfaced endoplasmic reticulum. Other cell features include the well-defined microvilli (mv) and a nucleus (N) with a prominent nucleolus. Mitochondria (M) are generally smaller than the mucous droplets and are less well defined.

X5,800.

Figure 34 (24 hr experimental - type C fixation):

Intermediate and Basal Cells. The intermediate cell (IM), oriented in the horizontal plane, contains a small Golgi apparatus (Go), approximately 1 micron in longest dimension, and has numerous desmosomes (D) along its border. Intercellular spaces (IS) are prominent among the complex interdigitations of basal and intermediate cell borders. Vertically oriented basal cells have prominent desmosomes (D) along their lateral and superficial borders and fibrils (f), up to 2 microns in length, in their cytoplasm. An undeveloped Golgi apparatus (Go) is noted in the lower left basal cell. Mitochondria, although difficult to distinguish due to low contrast, are numerous in all cells.

X8,400.


mu
mv
M
mu
N
gc
MV
0,5 u
32
33
1 u

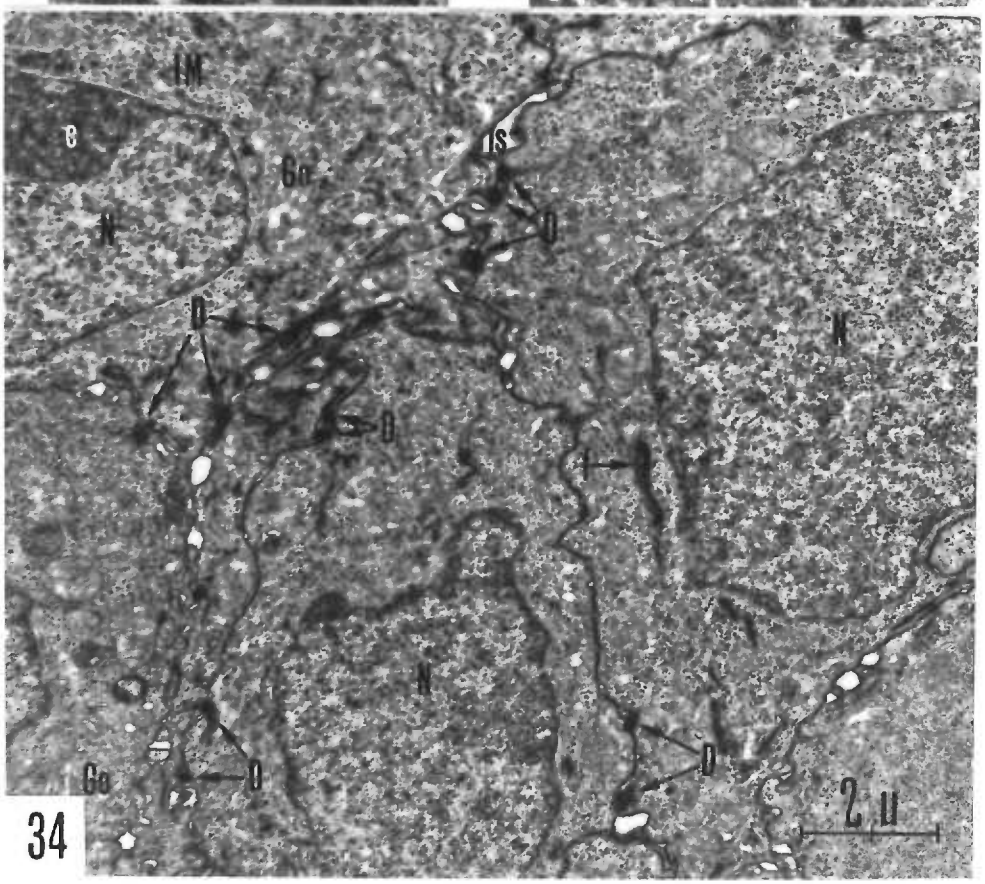
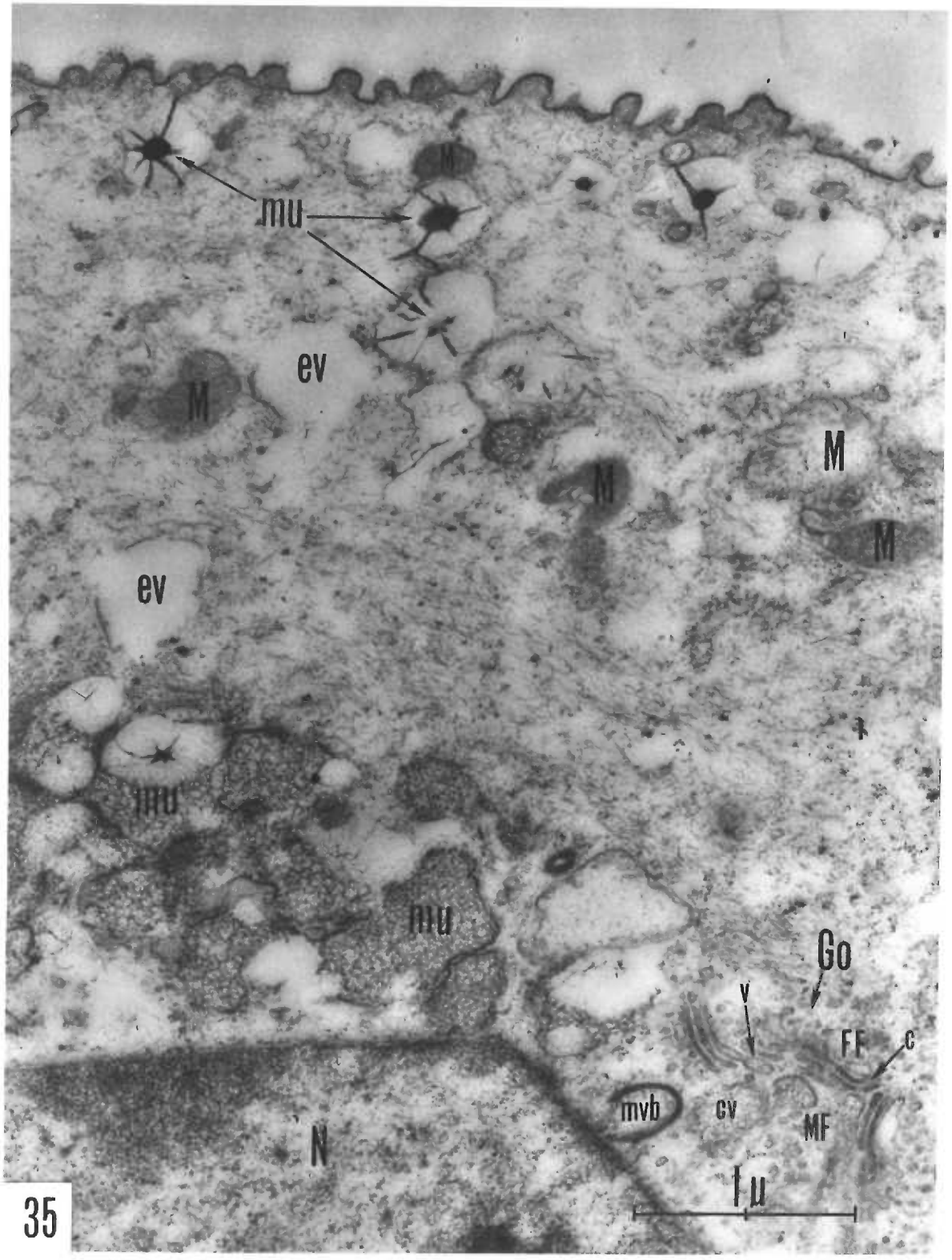

M
c
Go
N
ds
D
D
D
N
N
Go
D
D
2 u
34

Figure 35 (24 hr experimental - type A fixation):

Surface Cell. The presumed sequence of events during mucus formation in the vaginal mucous cell are well illustrated in this micrograph. The Golgi apparatus (Go) with numerous, small vesicles at its convex forming face (FF) is composed of multilayered, flattened cisternae (c). The inner cisternae, containing moderately dense, reticular material in their lumina, pinch off mucus-laden vacuoles (v) into the concave mature face (MF) of the cup-shaped Golgi apparatus. The small vacuoles coalesce into larger vacuoles (cv) until they have formed a mucous vacuole from 0.5 micron to 1 micron in diameter.

The mucous vacuoles (mu) are then displaced from the perinuclear area toward the luminal border of the cell. Secondary to osmium fixation, the mucus in some vacuoles has condensed into central, spherical masses with thick, linear projections to the vacuole membrane. This alteration may explain the appearance of "mucous droplets surrounded by clear, vacuolar areas" in the light photomicrographs. Also noted are empty vacuoles (ev) from which, it is assumed, the mucus has been lost during processing.

X31,600.



35