ULTRASTRUCTURE OF SPONTANEOUS VAGINAL KERATINIZATION IN ORGAN CULTURE (Balb/cCrgl Mice)

by

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INTRODUCTION

The importance of mouse vaginal epithelium as a model for the study of steroid hormone-end organ interaction dates from the original work of Allen in 1922 (1). Early experiments were principally concerned with definition of the cytomorphological characteristics of the estrous cycle in the intact animal and with establishment of the importance of estrogen in the induction of estrus (1, 5, 66). Of the naturally occurring estrogens, estradiol-17-β has been recently shown to be principally responsible for the transition from diestrus to estrus (67).

Under the influence of estrogen *in vivo*, the two to three cell layer cuboidal vaginal epithelium of both immature and castrate mice undergoes a striking transformation (5, 11). This is characterized not only by cell proliferation with active uptake of DNA precursors in the basal cell layer (33) but also by differentiation with early development of a secretory surface epithelium which is subsequently replaced by a stratified and fully keratinized squamous epithelium (5, 11). The product of the secretory cells has been shown to be mucin (5, 9), and the mechanism of its formation (11, 13) closely resembles that occurring in goblet cells in the intestine (23, 24) and involves changes in Golgi apparatus similar to those in rat uterine epithelium (17). The fully keratinized (estrous) vaginal epithelium of the mouse is very similar, at both the light and electron microscopic levels (11), to a variety of continuously keratinizing epithelia (skin, oral mucous membrane, esophagus) (7, 37, 60, 64) and has been shown to give similar reactions with histochemical stains for sulfhydryl and disulfide groups (3).

As a consequence of the development by Fell and Robison (20) of the technique of organ culture, wherein normal cellular relationships and tissue integrity are maintained and outgrowth is inhibited (22), attempts to reproduce *in vitro*, the vaginal changes occurring under the influence of estrogen *in vivo*, have been undertaken. While the early experiments of Rosenthal in 1934 (63) and of Emmems and Ludford in 1940 (18) failed to demonstrate an effect of estrogen on rat vaginal epithelium *in vitro*, Dux in 1941 (16) showed an effect on vaginal explants of the serum of animals previously injected with estrogen; and Coujard in 1943 (14) was able to report a direct effect of estrone on explants of guinea pig and rat vaginal epithelium. It was not, however, until 1953 that Hardy, Biggers, and Claringbold (30) definitively described distinct differences between control and estrogen treated vaginal organ cultures of 6 day old albino mice using hanging-drops and natural media (fowl plasma:chick embryo extract). At 72 to 96 hours, organ cultures grown in medium containing estradiol (either 0.006 or 0.06 µg/culture) were thickened, stratified, and fully keratinized; whereas, control cultures (non-estrogen treated) were cuboidal and non-keratinized with only occasional epithelial thickening. These results were extended to other estrogens, including estrone and estriol, in a subsequent report by Biggers, Claringbold, and Hardy in 1956 (6). From these experiments it was concluded, in contrast to earlier views (16), that estrogens act directly upon the vaginal epithelium and do not require intermediary metabolism in other organs.

Kahn in 1954 (35), using watch glass organ cultures of 3 to 4 week old rat vagina, confirmed the production of precocious keratinization at

1 to 2 days when estrone (1.3 µg/ml) was added to the standard (control) medium. Unlike Biggers *et al.* (6), however, he found that the control cultures underwent *spontaneous* keratinization in the absence of estrogen with development of a keratinized surface on the fourth to fifth day. He suggested that spontaneous keratinization might be the result of low vitamin A concentrations in the culture medium and indicated that vitamin A added to control cultures suppresses keratinization, but that similar concentrations of vitamin A in the presence of estrone only delay cornification. In 1959 (36) Kahn extended his observations to chemically defined medium (CMRL 1066) with and without added estradiol (0.08 µgm/watch glass) and vitamin A (0.3 µgm/watch glass) in a CO_2 atmosphere sufficient to maintain pH 7.2 to 7.4. Spontaneous keratinization was again observed with the control medium, and the rate and degree of keratin formation was enhanced by the addition of estradiol. Vitamin A inhibited spontaneous keratinization with development of "goblet cell" differentiation of the surface at 24 hours; however, by 72 hours there was squamification and keratinization.

In the same year, Martin (44) attempted to reproduce the results of Biggers *et al.* (6) using the watch glass technique and natural medium for the culture of 7 day old albino mouse vaginal rings. He failed to confirm their results and reported 100 percent keratinization of controls at 72 hours as well as a negative dose-response relationship with added estrone in concentrations from 5×10^{-2} to 5×10^{-7} µg. In addition, he studied cultures in chemically defined medium 150 (Parker) with added estrone with similar results. Numerous mitoses were present in the cultures at 24 hours. Addition of vitamin A (1.3 to 40 i.u.) to

the chemically defined medium did not prevent stratification and keratinization at 72 hours. The lack of inhibition of keratinization by vitamin A was possibly attributable to significant depletion of vitamin at 6 hours with none detectable at 18 hours.

In 1961 Koziorowska (39) reported increased alkaline phosphatase in estradiol treated nylon net-roller flask cultures of mouse vaginal fragments in chemically defined medium at 24 hours, and Samso (65) successfully cultured vaginal biopsies from post-pubertal (9 month to 8 year old) dogs. Lasnitzki in 1961 (41) cultured vaginal explants from 2 week old mice using the lens paper watch-glass method with chemically defined medium (Connaught 858) with or without estrone (2 µg/ml) following a 4 hour exposure in culture to vitamin A (100 i.u./ml). Her results confirmed those of Kahn (35, 36) and Martin (44) with respect to the occurrence of spontaneous keratinization of control cultures, and the results of Kahn (35, 36) indicating stimulation of precocious keratinization upon the addition of estrogen. Exposure of the cultures to vitamin A for 4 hours inhibited keratinization at 72 hours; however, keratohyaline granules were present in transitional cells. In cultures pre-treated with vitamin A, which were subsequently placed in medium containing estrone, the epithelium at 72 hours was non-keratinized and lacked keratohyaline granules, but did demonstrate marked clear cell (epidermoid) modification of the transitional cell layer. It was concluded from these experiments that vitamin A inhibits spontaneous keratinization in chemically defined medium and that it antagonizes the keratin stimulating effects of estrone *in vitro*.

In 1963 Bertoli (4) cultured vaginal fragments from prepubertal (18 day old) rats in petri dishes using chemically defined medium 199 with or without added estrogen (0.2 µg/ml) or progesterone (2.0 µg/ml) in a humidified 5 percent CO_2 atmosphere. His results parallel those reported previously and indicate, additionally, antagonism of estrogen action *in vitro* by progesterone with formation of a surface layer of goblet cells.

Most recently, Pullar in 1964 (58) has cultured vaginae from immature white mice (3 to 7 day old) and estrous adult, diestrous adult, and castrate F_1 hybrids (C 57 leaden/jax and A. Heston/jax) by the lens paper-sponge-watch glass technique in chemically defined medium 199 in a 95% O_2:5% CO_2 atmosphere. With immature and oophorectomized vaginal cultures a thin keratin layer developed at 96 hours with full keratinization at 192 hours; while with diestrous adult vaginal cultures, keratinization was well established at 96 hours and a granular cell layer (stratum granulosum) was present. Stains for sulfhydryl and disulfide groups (2) were positive in the keratinized epithelium with greatest intensity of staining in the transitional zone immediately subjacent to the stratum corneum. It was postulated that the final step in the formation of keratin takes place by the oxidation of sulfhydryl groups to disulfide bonds and that the consolidation of the lower keratin layer is less complete *in vitro* than *in vivo*.

Despite numerous differences in culture technique, culture medium, gaseous atmosphere, and age, species, and physiological state of the animals used in the above experiments, several general conclusions seem justified: 1) rodent vaginal epithelium in organ culture undergoes

spontaneous keratinization in the absence of estrogen, 2) keratinization in organ culture is enhanced by estrogen, in appropriate concentrations, in both a quantitative and a temporal sense, 3) vitamin A, under appropriate experimental conditions, antagonizes both spontaneous and estrogen stimulated keratinization *in vitro*, and 4) by light microscopy and histochemistry, keratinization in organ culture, except for the lack of keratohyaline granules in most cases, closely resembles normal cyclic or estrogen induced keratinization *in vivo*.

Although by light microscopy the above conclusions appear valid and although there exist a number of electron microscopic studies of human vaginal epithelium (29, 55, 56) and of rodent vaginal epithelium *in vivo* (8, 48, 49, 62), including two recent studies of mouse vagina *in vivo* (11, 57), there are no ultrastructural studies of vaginal keratinization in organ culture. Thus, a definitive comparison of *in vivo* and *in vitro* vaginal keratinization cannot be made, and a detailed analysis of the morphological similarity or dissimilarity of the two processes at high resolution is not possible. For this reason, and because the histochemical studies of vaginal keratinization by Pullar (58) suggest incomplete oxidation of sulfhydryl to disulfide groups *in vitro*, parallel studies of the light and electron microscopy and histochemistry of spontaneous vaginal keratinization in hanging-drop organ cultures were undertaken.

Hanging-drop organ cultures (12), in chemically defined medium 199 without added serum or crystalline proteins, were used so that analogies could be drawn with previous light microscopic studies using chemically defined media (36, 39, 41, 44, 58) and in order that a baseline be

established against which future studies utilizing hormone additives might be compared. By virtue of the isolation of individual tissue fragments in single wells in this organ culture system (12), establishment of the reproducibility of the culture results and of the ultrastructural changes under control conditions should allow for future studies of hormone-end organ interaction not feasible in *in vitro* organ culture systems which entail the culture of multiple fragments in a common medium.

MATERIALS AND METHODS

Animals:

Adult male and female mice of the inbred strain Balb/cCrgl were obtained from the Cancer Research Genetics Laboratory, University of California, Berkeley, California, through the courtesy of Kenneth B. DeOme, Ph.D. They were segregated into males and females in transparent plastic cages using a mixture of San-I-Cel^{R} and aromatic red cedar shavings as bedding. The animals were fed standard laboratory chow (Ralston-Purina Company) and tap water *ad lib*.

Females were placed in male cages, 3 females/male, and left for 72 to 96 hours. At approximately 14 days gestation, pregnant females were placed in individual cages, and the birth date of each litter was recorded.

Preparation of Tissues for Culture:

Forty-nine day old females with an average weight of 20 gm were ovariectomized by means of the dorsal technique. The adequacy of oophorectomy was confirmed by study of the excised ovaries using a dissecting microscope. On the tenth post-oophorectomy day vaginal smears were taken and evaluated for the presence or absence of estrogen effect. Those animals having a castrate vaginal cytology (66) were sacrificed by cervical fracture. The abdomen was opened under sterile conditions, and the uterus and vagina were carefully separated from other structures. The vaginae were excised at the introitus, and the lower (introital) one-half of the vagina was discarded. After removal of the cervix and urethra, the cranial one-half of the vagina was

divided under the dissecting microscope into 19 to 20 fragments which averaged 1.5 mm in maximum dimension. At all times the fragments were immersed in cold (4° C) chemically defined medium 199 containing 100 μ/ml of penicillin and streptomycin. From each animal approximately 5 fragments were immediately fixed (*vide infra*) as controls, and the remainder were rapidly transferred to the organ culture dish.

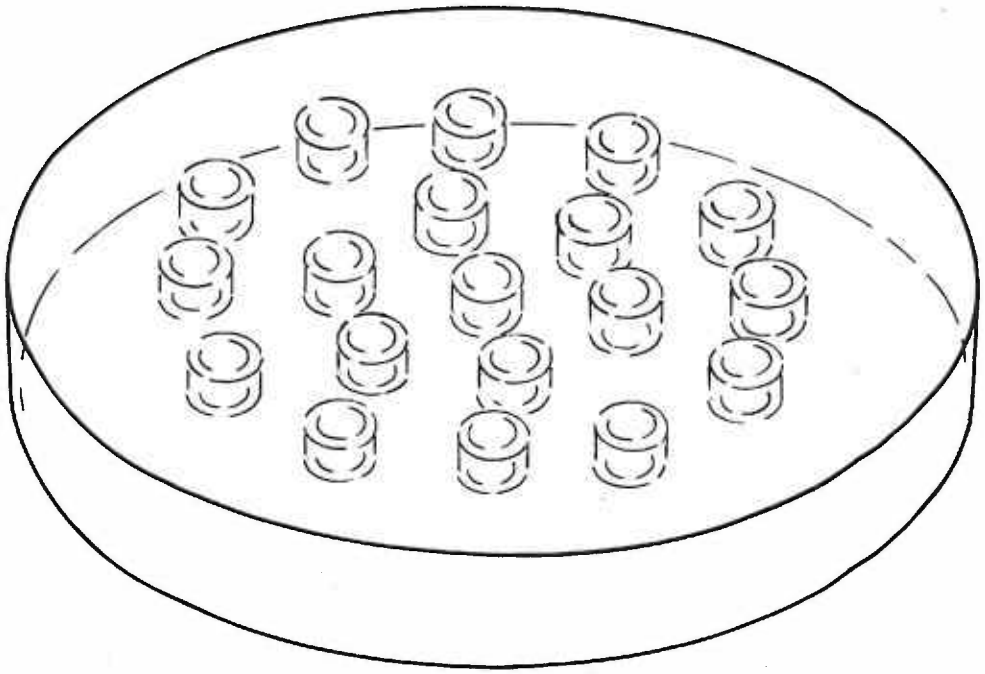
Culture:

Each fragment was placed individually in a single well in a hanging-drop organ culture dish which has been described elsewhere by Cooper, Jentoft, and Wellings (text figure) (12). The wells were filled with an average volume of 0.180 cc of chemically defined medium 199 (53), containing 100 μ/ml of penicillin and streptomycin, giving a fluid to tissue ratio of approximately 60:1. The culture vessel was inverted, the tissue fragments falling to the apices of the menisci of the drops. The culture dish was placed in a plexiglass CO_2 incubator chamber (Labtool Specialties, Ypsilanti, Michigan) which had been pre-humidified by warm sterile distilled water in a large cotton containing petri dish placed at the bottom of the chamber. The chamber was gased with a mixture of 95% O_2:5% CO_2 for 1.5 minutes at a flow rate of 5 liters/minute.

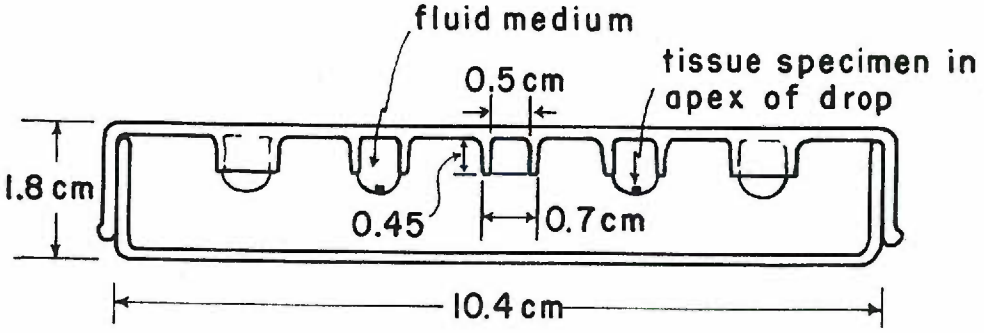
The organ culture chamber containing the hanging-drop cultures was placed in a standard incubator at 36° C. The medium was changed at 48 hours at which time the atmosphere was renewed.

Light and Electron Microscopy:

Individual specimens were removed from the culture vessel at 24 hour intervals over a 4 day period--a total of 14 control, 21 day 1,



HANGING DROP ORGAN CULTURE DISH



14 day 2, 18 day 3, and 23 day 4 specimens were studied by light and electron microscopy. The tissues were immediately fixed for 2 hours in 1% OsO_4 in veronal acetate (pH 7.4) with 0.045 gm/cc of sucrose added (10). They were subsequently rapidly dehydrated in graded cold ethyl alcohol and embedded in Swiss araldite (43). Thin sections were cut on an LKB ultrotome equipped with a Pyrex^R glass knife, mounted on formvar coated copper specimen grids, and triply stained with lead citrate (8 minutes), uranyl acetate (1 minute), and lead citrate (5 minutes) (59, 70). Sections were examined in an RCA EMU-3G electron microscope.

Thick sections were cut from the same blocks and stained by Richardson's method for light microscopy (61).

Histochemistry:

Thick sections of osmium fixed, araldite embedded controls and cultures (*vide supra*) were stained with periodic acid-Schiff reagent (PAS) following treatment with a saturated solution of sodium hydroxide in 100% alcohol (40). Modifications of the method included increases in time of the periodic acid to 15 minutes, of the Schiff reagent to 30 to 45 minutes, and of the hematoxylin to 15 minutes.

Periodic acid-Schiff reactions on sections pre-treated with salivary amylase were performed on control and experimental tissues cultured under conditions identical to those previously described (*vide supra*). These tissues were fixed in glutaraldehyde, embedded in paraffin, and sectioned at 5 microns.

Stains for sulfhydryl and disulfide groups and for sulfhydryl groups alone were performed on the same glutaraldehyde fixed, paraffin

embedded controls and cultures by the method of Barrnett and Seligman (2, 32).

RESULTS

GENERAL CONSIDERATIONS

In all cultures the medium, which contained phenol red as an indicator (53) developed, within 24 hours, a pale yellow color tinged with pink indicative of a pH of approximately 6.0. This coloration was promptly re-established and maintained following the medium change at 48 hours. In none of the cultures was there gross evidence of necrosis or loss of tissue integrity. Drop stability was excellent throughout the culture period, so that righting and inversion of the culture dish was easily accomplished during medium changes and specimen sampling.

By light microscopy, there was minimal to absent necrosis in both epithelium and stroma throughout the 96 hour period in culture. The findings on a day to day basis were reproducible, the major variation being the degree and extent of goblet cell transformation of the superficial secretory cell layer on day 2 (*vide infra* and figs. 3 and 4). By 96 hours, epithelial continuity was established over the entire external surface of each culture fragment (fig. 6). Mitoses were rarely observed in the basal layer on day 1 in culture and were not identified on subsequent days.

LIGHT MICROSCOPY

Control (Day 0):

The epithelium consisted of 2 to 4 layers of cuboidal epithelial cells resting upon a loose fibrovascular stroma. The basal and

intermediate cell layers were formed by cells having oval to round nuclei with irregular chromatin, and slightly granular dense cytoplasm. The superficial cell layer differed by virtue of the presence of scattered single cells having a more abundant cytoplasm with focal cytoplasmic vacuolation and dense intracytoplasmic droplets (fig. 1). The nuclei of these cells were more vesicular with occasional prominent nucleoli.

The stroma in many sections contained scattered mast cells (tissue basophils) with metachromatic granules.

Day 1 Experimental:

After 24 hours in culture the epithelium was 5 to 6 cell layers thick and was well demarcated from the stroma. There was a single layer of basal cells, 2 to 3 layers of intermediate cells, and 1 to 2 layers of superficial cells (fig. 2). The basal cells were enlarged as compared to the controls and polygonal in outline. Their nuclei were larger with generally more prominent nucleoli. The intermediate cells were similar to the basal cells, but were larger, more plate-like, and had more prominent intercellular spaces and intercellular bridges. The superficial cell layer consisted of columnar cells having clear, vacuolated cytoplasm and vesicular nuclei with more homogeneous chromatin. The cytoplasmic vacuoles frequently contained small dense masses interpreted as mucous droplets (fig. 2). The stroma was similar to that of the controls.

Day 2 Experimental:

There was a definite squamoid zone, approximately 6 cell layers

thick, overlain by a highly secretory surface layer of mucinous cells (fig. 3), which in some explants had the characteristics of goblet cells (fig. 4). The basal cells (stratum germinativum) and intermediate cells (stratum spinosum) were similar to the previous day, but there had developed between the intermediate and superficial cells several layers of flattened (precornified) cells whose long axes lay parallel to the surface. These cells (figs. 4 and 10) had denser cytoplasm and more prominent cell membranes than those in the other layers. Their cytoplasm characteristically lacked keratohyaline granules. In addition, their nuclei were generally inapparent. The stroma maintained a density similar to control and day 1 cultures and contained occasional mast cells with intact granules.

Day 3 Experimental:

Very definite and rather abrupt changes were observed at 72 hours in culture. The basal cell-stromal cell junction was irregularly serrated, and the basal cells themselves had further developed features more typical of a stratum spinosum (polygonal outlines, abundant dense cytoplasm, and prominent intercellular bridges) (fig. 5). The cells of the stratum spinosum demonstrated an increasing tendency toward polar orientation parallel to the surface despite preservation of nuclei and prominent intercellular spaces and intercellular bridges. The flattened anuclear layer, first seen on day 2, had developed into a complete stratum corneum characterized by marked density of cytoplasm and indistinct intercellular spaces. The immediate luminal surface consisted partially of degenerating and exfoliating mucinous cells.

Day 4 Experimental:

The epithelium completely invested the supporting stroma and was fully keratinized, the mucinous surface layer having been lost (fig. 6). Serration of the basal cells (stratum germinativum) persisted as did their prominent stratum spinosum-like features. Although the cells of the stratum spinosum proper had undergone further flattening and compression and consisted of approximately 5 cell layers, these flattened cells, unlike the stratum corneum, maintained identifiable nuclei and distinct intercellular spaces and bridges (fig. 7). Keratohyalin was absent in cells beneath the stratum corneum, although occasional cells contained scattered dense masses suggestive of karyorrhetic nuclear debris (fig. 7).

HISTOCHEMISTRY

Periodic Acid-Schiff Reaction:

The superficial cell (luminal) layer of the controls demonstrated occasional single PAS positive cells in an epithelium which was otherwise composed of non-reactive cuboidal cells (fig. 8). In contrast, the majority of the superficial cells of the day 1 and 2 experimental cultures were secretory and contained within their cytoplasm large numbers of PAS positive droplets (fig. 9). In approximately 15 percent of cultures on day 2, the secretory surface cells had pronounced goblet cell-like configurations (fig. 10). On the third experimental day, staining of the superficial cells was less intense and was associated with degeneration and exfoliation of this layer (fig. 12). On day 4,

the epithelium did not demonstrate localized or granular staining. In both the controls and the day 1 to 3 experimental cultures (fig. 11), the PAS positivity of the superficial cells was resistant to digestion with salivary amylase, indicating the mucopolysaccharide nature of the material stained. In neither the controls nor the experimentals was amylase sensitive, PAS positive material (glycogen) identified. PAS staining enhanced the definition of a stratified (precornified) layer of cells which had developed beneath the mucinified surface on day 2 (fig. 10). These cells had more intensely stained, non-granular cytoplasm, and more prominent cell membranes. This latter feature apparently correlated with the development of a peripheral dense cytoplasmic shell in the stratum corneum as seen by electron microscopy (*vide infra*).

Sulfhydryl and Disulfide Histochemistry:

Both the control and the day 1 experimental epithelia were negative for sulfhydryl and disulfide groups (figs. 13 and 14). On the second experimental day, the previously described flattened layer of cells which had developed between the stratum spinosum and the mucinified surface was moderately positive (fig. 15). This staining for both sulfhydryl and disulfide groups increased on day 3 (fig. 16) and reached a maximum in the stratum corneum on day 4 (fig. 17). On the second, third, and fourth experimental days, stains for sulfhydryl groups alone (fig. 18) were comparable in the intensity of reaction product formed to those stained for both sulfhydryl and disulfide groups.

ELECTRON MICROSCOPY

The terminology used for the fibrillary cytoplasmic components involved in the process of keratinization is based on that suggested by Mercer, Munger, Rogers, and Roth (47). Using a transparent grid overlay and randomly selected coordinates for counting, semi-quantitative analysis of the numbers of desmosomes in the control and experimental groups resulted in the following general conclusions: 1) there was no demonstrable difference in the numbers of desmosomes in the basal cell layer in the control and day 1 experimental groups and only a questionable increase on the second experimental day; 2) there was a 3 to 4-fold increase in the numbers of desmosomes in the intermediate cell as compared to the basal cell layer on the first and second experimental days as well as a similar increase in desmosomes in the intermediate cells of day 1 experimental cultures as compared to the controls; and 3) desmosomes were less numerous in the superficial cell layer than in the intermediate cell layer in day 1 and 2 experimental cultures and were more prominent on cell membranes at the intermediate cell-superficial cell junction than on the apposed membranes of the superficial cells themselves.

Control (Day 0):

Basal and intermediate cell layers--The cells in these layers were similar (fig. 19) and were characterized by oval nuclei with irregular chromatin aggregates (fig. 21) and moderately dense cytoplasm containing mitochondria and small numbers of free ribosomes. Endoplasmic reticulum was inapparent. Desmosomes were present in moderate numbers in both

layers and measured up to approximately 2400 Å in the basal cell and 3600 Å in the intermediate cell layers. The basal cells rested upon a distinct, approximately 350 Å, basement membrane from which they were separated by an approximately 700 Å clear zone. There were small to moderate numbers of 70 Å cytoplasmic filaments grouped in loosely aggregated fibrils in both the basal and intermediate cells (fig. 21).

Superficial cell layer--Both non-secretory cuboidal and secretory cells were present (figs. 20 and 22). With the exception of surface microvilli, the former resembled the basal and intermediate cells, while the latter contained within their cytoplasm membrane-limited, reticular aggregates of medium density as well as moderately prominent Golgi apparatus (fig. 20). The immediately subluminal portions of adjacent superficial cell membranes demonstrated occluding zones (*Zonulae occludentes*) beneath which intercellular spaces were dilated and prominent. Desmosomes were more numerous at the intermediate cell-superficial cell junction than between the superficial cells themselves (fig. 22), although those desmosomes present in the superficial cell layer had standard morphological features and were associated with moderately prominent intracytoplasmic filaments (fig. 23). The luminal surfaces of the superficial cells showed occasional zones of inverted cell membrane thickening suggestive of residua of prior desmosomal attachments.

Day 1 Experimental:

Basal cell layer--Cell membrane-basement membrane relationships were similar to the controls as were the numbers of half-desmosomes and

desmosomes (*vide supra* and fig. 24). Maximum desmosomal length (approximately 2500 Å) was also comparable; however, cytoplasmic fibrils, composed of 70 Å filaments, and free ribosomes were more prominent. Rough-surfaced endoplasmic reticulum and Golgi apparatus were poorly developed.

Intermediate cell layer--Large numbers of desmosomes were present measuring up to approximately 4000 Å (figs. 26 and 27). These were associated with marked increases in cytoplasmic fibrils and with prominent free ribosomes.

Superficial cell layer--Mucinified cells were more numerous than in the controls; however, groups of non-secretory cells were still present. The mucinified cells demonstrated not only mucin droplet formation within smooth-surfaced membranes and microvillous surface differentiation, but also concomitant formation of cytoplasmic fibril bundles similar to those present in the intermediate cell layer (fig. 25).

Day 2 Experimental:

Basal cell layer (stratum germinativum)--Cell membrane-basement membrane relationships and definition were still maintained. Cytoplasmic fibrils and free ribosomes were increased with suggestions of polyribosomal aggregation (figs. 28 and 33). Mitochondria contained non-membrane limited dense bodies of unknown nature and significance (fig. 33), similar to those described by Nilsson (52) in the uterine surface epithelium of estrous mice. Nuclear chromatin was more evenly dispersed, with fewer chromatin aggregates, and nucleoli were more prominent. Endoplasmic reticulum and Golgi membranes were again poorly developed.

Intermediate cell layer (stratum spinosum)--These cells now had features typical of a stratum spinosum. Extremely large numbers of complexly interanastomosing fibrils lay within a cytoplasm rich in free ribosomes (figs. 29 and 32). The fibrils were associated with numerous desmosomes, which modified the villiform interdigitations of adjacent cell membranes (fig. 29), and were composed of individual 70 Å filaments which frequently had a beaded appearance (fig. 32). Nuclei had finely dispersed chromatin with small marginal condensates and prominent nucleoli. Mitochondria contained dense bodies similar to those in the basal cell layer (fig. 29).

Superficial cell layer--There was more extensive mucinous differentiation; however, individual non-secretory cells persisted. These cells frequently contained small numbers of approximately 250 Å granules consistent with glycogen, 700 mμ membrane-limited dense bodies interpreted as lysosomes, and non-dilated Golgi apparatus (fig. 30). They did not demonstrate filament and fibril formation like that seen in some secretory cells (fig. 25), and fibrils were consistently less prominent than in the underlying spinal cells (fig. 31). In approximately 15 percent of the cultures, secretory change progressed to generalized goblet cell surface differentiation (fig. 34).

Day 3 Experimental:

Basal cell (stratum germinativum) and intermediate cell (stratum spinosum) layers--These layers had features similar to those described for the same layers on day 4 (*vide infra*).

Superficial cell layer--Portions of the surface consisted of degenerating secretory cells showing both mucin droplet and cytoplasmic fibril formation (fig. 35); while in other areas a stratum corneum was present. The uppermost cells of the stratum corneum demonstrated apparent incomplete keratinization characterized by a failure of filament and fibril aggregation (fig. 36).

Day 4 Experimental:

Basal cell layer (stratum germinativum)--The basal cells had features more characteristic of a stratum spinosum, including prominent cytoplasmic fibril complexes and large numbers of desmosomes (fig. 37). Unlike the controls and the day 1 and 2 experimental cultures, the basement membrane was consistently poorly defined as were the basal portions of the cell membrane, half-desmosomes, and the clear zone which normally separates the cell membrane from the basement membrane.

Intermediate cell layer (stratum spinosum)--Nuclei were less apparent than on previous days, and the cytoplasm consisted principally of fibrils and ribosomes in a clear and moderately abundant matrix (fig. 39). Keratohyaline granules were not seen, and the transition from the stratum spinosum to the stratum corneum was abrupt without formation of a transitional cell layer of the type previously described by Brody (7) and by Cooper *et al.* (11).

Stratum corneum--The lower cells of the stratum corneum had developed a peripheral dense, approximately 250 Å, cytoplasmic shell which had resulted in modifications in usual desmosomal structure (38) at both the stratum spinosum-stratum corneum interface (composite desmosomes,

fig. 42) and within the stratum corneum proper (modified desmosomes, fig. 43). There was a progressive increase in fibril density in the lower and intermediate levels of the stratum corneum (fig. 38) which on both the third and fourth days reached a maximum 1 to 3 cell layers below the surface (figs. 36 and 38). Unlike keratinization *in vivo* (7, 11, 64), however, the uniform distribution of filaments typical of the so-called "keratin pattern" was never established. Filament distribution in irregular, incompletely aggregated, fibrils persisted (fig. 38) as did small groups of ribosomes (fig. 41). In addition, there appeared to be dissolution or regression of filament aggregation in the uppermost layers of the stratum corneum (figs. 38 and 40) with reappearance of individual filaments in a more abundant clear matrix (fig. 40). This was associated with apparent dissolution of desmosomal plates (fig. 44) and with separation of desmosomal halves (figs. 38 and 40) similar to that induced in hamster vaginal epithelium with EDTA (ethylenediaminotetraacetic acid) by Roig de Vargas-Linares and Burgos (62). Nuclei and mitochondria were not clearly identified in cells of the stratum corneum.

DISCUSSION

The findings in this study are similar, at the light microscopic level, to those reported previously using chemically defined media (36, 39, 41, 44, 58) and support the observation that keratinization occurs spontaneously in organ culture in the absence of estrogen (15).

In the present experiments, the 2 to 3 cell layer cuboidal vaginal epithelium of castrate 8.5 week old mice, when placed in hanging-drop organ culture for 4 days, increased progressively in thickness and by the last day was fully keratinized. As compared with estrogen induced keratinization *in vivo* (11), the cells of the stratum spinosum *in vitro* were more extensively spindled and stratified, and the basal cells were more irregular in shape and had features more typical of spinal cells.

Periodic acid-Schiff (PAS) stains, both before and after amylase digestion, demonstrated increasing secretory development of surface cells on the first and second experimental days with goblet cell differentiation in about 15 percent of the cultures. The positivity of the digested PAS stain demonstrates that the secretory product is a mucopolysaccharide (5), and the morphologic similarity of the process of its formation *in vitro* and *in vivo* (11, 13) indicates, unlike prior studies (4, 6), that increased mucin formation *in vitro* is also not dependent on the presence of either estrogen or progesterone in the culture medium. Unlike *in vivo* mucin formation, however, there is coincident formation of filaments and fibrils in the secretory cells. It is interesting that secretory differentiation of mouse vagina *in vitro* occurs in the presence of low vitamin A concentrations (53),

while similar changes with chick ectoderm *in vitro* require medium containing high vitamin A levels (21).

Histochemical stains for sulfhydryl and disulfide groups became progressively more positive from days 2 to 4, indicating increasing formation of keratin precursors (2, 3). The lack of significant differences in the amount of reaction produce formed in sections stained for both sulfhydryl and disulfide groups and for sulfhydryl groups alone was similar to findings reported by Pullar (58) and was consistent with incomplete conversion of sulfhydryl to disulfide groups.

Ultrastructural studies of osmium tetroxide fixed sections from the same or comparable cultures demonstrated, on the first and second experimental days, increased numbers of desmosomes in the intermediate cell layer, as well as marked and progressive increases in ribosomes and 70 Å cytoplasmic filaments, grouped into fibrils having complex intracellular distribution, in both the basal cell (stratum germinativum) and intermediate cell (stratum spinosum) layers. On the third day in culture, a stratum corneum was present, overlain in part by a degenerating mucinous layer; while on the fourth day, the surface consisted exclusively of a stratum corneum. Transition from the stratum spinosum to the stratum corneum was abrupt and did not include the development of either keratohyaline granules or a transitional cell layer. Although progressive aggregation of filaments and fibrils occurred in the stratum corneum, it was incomplete, and the uniform approximation and distribution of filaments typical of the so-called "keratin pattern" seen in epidermis (7, 64) and in mouse vagina *in vivo* (11) did not develop. In the most superficial layers of the stratum

corneum, in fact, there was apparent regression of filament aggregation. This finding of incomplete synthesis of a "keratin pattern" was in keeping with the histochemical findings and strengthens the idea that conversion of sulfhydryl groups to disulfide bonds is incomplete *in vitro* and demonstrates that the processes of *in vivo* and *in vitro* keratinization are neither chemically nor morphologically completely comparable.

Despite these differences in the final stages of keratin synthesis, the early stages of estrogen induced *in vivo* keratinization and of spontaneous *in vitro* keratinization are distinctly similar. These similarities include marked early increases in ribosomes and cytoplasmic fibrils in basal and intermediate cells and significant increases in desmosomes in intermediate cells on the first two days *in vitro*. In addition, certain late changes resembling *in vivo* keratinization did occur *in vitro* and included distinct modifications in cell membranes and desmosomes (composite and modified desmosomes) in the stratum corneum on days 3 and 4. These latter changes are similar to those previously described by Rhodin and Reith (60) in a variety of squamous epithelia and by Farquhar and Palade (19) in amphibian epidermis. It is interesting that in neither *in vivo* (11) nor *in vitro* vaginal keratinization are membrane coating granules of the type described by Matoltsy and Parakkal (45) or small granules as described by Rhodin and Reith (60) and by Farquhar and Palade (19) identified.

The regression of filament aggregation which occurs in the upper stratum corneum *in vitro* is associated with dissolution of the intercellular plates of modified desmosomes and with separation of

desmosomal halves. Since similar changes have been produced *in vivo* by the use of the chelating agent EDTA by Roig de Vargas-Linares and Burgos (62), the possibility exists that these changes *in vitro* may be the result of inadequate concentrations of calcium in the culture medium (53). The absence of keratohyaline granule formation during spontaneous keratinization *in vitro* may, however, be even more important. Although the nature, composition, and function of keratohyalin are still the subject of controversy and discussion (68), ultrastructural studies of normal continuously keratinizing epithelia (7, 60, 64) and of mouse vagina *in vivo* (11) invariably show a close association between keratohyaline granules and cytoplasmic filaments and fibrils in the stratum granulosum. These observations suggest that keratohyalin may be important in the development of interfilamentous matrix and in the final aggregation of filaments into keratin. The incomplete filament aggregation which occurs *in vitro* in the absence of keratohyalin lends support to this hypothesis. In view of these observations, the findings of Pullar (58) that keratohyaline granules are formed during spontaneous keratinization only when diestrous vagina is cultured become extremely important; and comparative ultrastructural studies of filament aggregation in diestrous vaginal fragment keratinization *in vitro* may well help to clarify the role of keratohyalin.

The culture methods employed in the present study were not designed to test the importance of or the nature of the effect of vitamin A on the process of keratinization (21, 41), although spontaneous keratinization did occur in the face of a concentration of 0.1 mg of vitamin A per liter in the medium used (53).

While *in vivo* and *in vitro* keratinization have been shown, by the present study, to differ significantly in the degree to which end stage binding of filaments into keratin takes place, the early changes (*i.e.* at 24 hours) are very similar in both the *in vivo* (11) and the *in vitro* systems. These include significant increases in cytoplasmic ribosomes and filaments and changes in nuclei and nucleoli which correlate well both with biochemical studies which demonstrate marked stimulation by estrogens of ribonucleic acid and protein synthesis in cells of the female reproductive tract in rodents (25, 26, 27, 28, 46, 51, 69) and with studies indicating the importance, respectively, of nucleoli and diffuse chromatin in the synthesis of ribosomal (54) and messenger (42) RNA.

Coincident with work concerning genetic regulatory mechanism of protein synthesis and cellular control systems (34, 50), there has been considerable interest in the elucidation of the mechanism whereby estrogens induce alterations in biochemical activity in cells of the female genital tract (31). In 1964, Talwar, Segal, Evans, and Davidson (69) reported that estradiol-17β-6,7-H^3 associated with macromolecular entities present in the supernatant of homogenized rat uteri after centrifugation at 105,000 g. Partial purification through a sephadex^R G-100 column suggested the existence of a repressor of RNA polymerase, in the first peak of the eluate material, which was sensitive to derepression by estradiol both *in vivo* and *in vitro*. It was suggested that the functions of receptor and repressor might be inherent in the same molecule and that in the absence of hormone the repressor would block the transcription of genetic information. Gorski, Noteboom, and

Nicolette (26) have similarly described the first step of estrogen action as consisting of a stereospecific interaction with a receptor which they have characterized as being sensitive to proteinases and extremes of pH, but insensitive to ribonuclease and deoxyribonuclease. The interaction of this receptor with estrogen apparently results in a change in the biological activity of the receptor protein. The fact that cellular responses to estrogen are blocked by inhibitors of both protein (puromycin) and RNA (actinomycin D) synthesis suggests that synthesis of specific enzymes may be involved. Mueller (51) affirms the interaction of estrogen with a specific "acceptor" through which there is an activation of genetic mechanisms for RNA and protein synthesis and concludes that protein synthesis may be more fundamental than RNA synthesis in the "amplification" of the initial hormone effects.

Most recently, Means and Hamilton (46) have described stimulation of nuclear RNA synthesis and uptake of uridine-H^3 by uteri of ovariectomized rats within two minutes of the intraperitoneal injection of estradiol-17β. They indicate that this rapid stimulation of RNA synthesis occurs before reported effects of estrogen on translation and prior to either nuclear or cytoplasmic protein synthesis. On the basis of available evidence they indicate three possible alternatives for the primary action of estrogen: 1) estrogen first directly or indirectly stimulates RNA synthesis in the nucleus, 2) estrogen acts primarily on the outer membrane of the uterine cell with a resultant influx of precursors permitting increased nuclear RNA synthesis, and 3) multiple effects on cellular or nuclear membranes and on transcription of DNA.

The occurrence of *spontaneous keratinization* when vaginal epithelium from oophorectomized animals is placed in organ culture represents an experimental model wherein the requirement for estrogen activation of cellular synthetic mechanisms is bypassed. The early changes occurring in this system bear a striking morphologic resemblance to those induced by estrogen *in vivo* (11), which implies that similar cellular mechanisms of ribonucleic acid and protein synthesis may be activated.

An understanding of the ability of the artificial conditions of organ culture to induce estrogen-like changes in the absence of estrogen must necessarily await better definition of the action of estrogen *in vivo* (28). Viewed in the light of current concepts of estrogen action (26, 46, 51, 69), however, several possibilities exist with respect to the non-estrogen induced transcription of genetic information which occurs *in vitro*:

1. The repressor material described by Talwar *et al.* (69) and Gorski *et al.* (26) may be altered by the culture conditions in a way such that it is depressed, and nuclear RNA polymerase activity is stimulated, and
2. The artificial conditions of the gaseous atmosphere or of the chemically defined medium itself may alter cell membrane and/or nuclear membrane characteristics in a way comparable to that described by Means and Hamilton (46) with resultant uptake of precursors which stimulate RNA synthesis.

Despite the occurrence of spontaneous keratinization *in vitro*, the addition of estrogen to the medium apparently enhances the rate and degree of keratin formation (6, 35, 41). This implies that although the estrogen dependent mechanism can be bypassed *in vitro*, the basic mechanism for response to estrogen is at least partially maintained. Similarly, the differences observed by Pullar (58) between non-estrogen exposed (castrate and prepubertal) and estrogen exposed (diestrous) vaginal fragments in organ culture suggest that exposure of the vaginal epithelium to estrogen just prior to culture may precondition the cells for more exact replication of the keratinization process as it occurs *in vivo* and thus apparently for more exact transcription of basic genetic information.

The ability of vitamin A in appropriate concentrations to suppress spontaneous keratinization of vaginal fragments in organ culture has been demonstrated by Kahn (36) and Lasnitzki (41). The mechanism of this suppression, as well as the suppression of keratinization of other epithelia *in vitro*, is not known (22). Whether with vaginal epithelium it is related to or entirely independent of the normal estrogen dependent mechanism was not tested in the present study and remains to be determined.

Finally, since the present study demonstrates that *in vivo* and spontaneous *in vitro* keratinization differ significantly, at least in the end stages of keratin formation, data on *in vitro* keratinization cannot at the moment be considered comparable to *in vivo* data. The substitution of *in vitro* for *in vivo* methods must thus await the definition of organ culture conditions which more faithfully reproduce

the normal *in vivo* pattern.

SUMMARY AND CONCLUSIONS

The vaginal epithelium of castrate 8.5 week old mice undergoes *spontaneous keratinization* in the absence of estrogen when placed in hanging-drop organ cultures using chemically defined medium 199. The early stages of *in vivo* and *in vitro* keratinization have been shown to be similar and are characterized by marked increases in cytoplasmic ribosomes and filaments which correspond to known biochemical changes induced by estrogen in cells of the female genital tract. Similarly, there are early changes in nuclear chromatin and nucleoli *in vitro* indicative of active messenger-RNA and ribosomal-RNA synthesis.

In the later stages of *in vitro* keratinization, however, there is, by electron microscopy, a failure of keratohyaline granule formation and incomplete filament aggregation. Thus, estrogen induced *in vivo* and spontaneous *in vitro* keratinization are not truly comparable. The changes occurring in the organ culture system used have, however, been shown to be reproducible and, at the light microscopic level, comparable to those previously described in other *in vitro* studies. The culture vessel used in the present study should offer certain advantages over other organ culture systems.

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Light Microscopy:

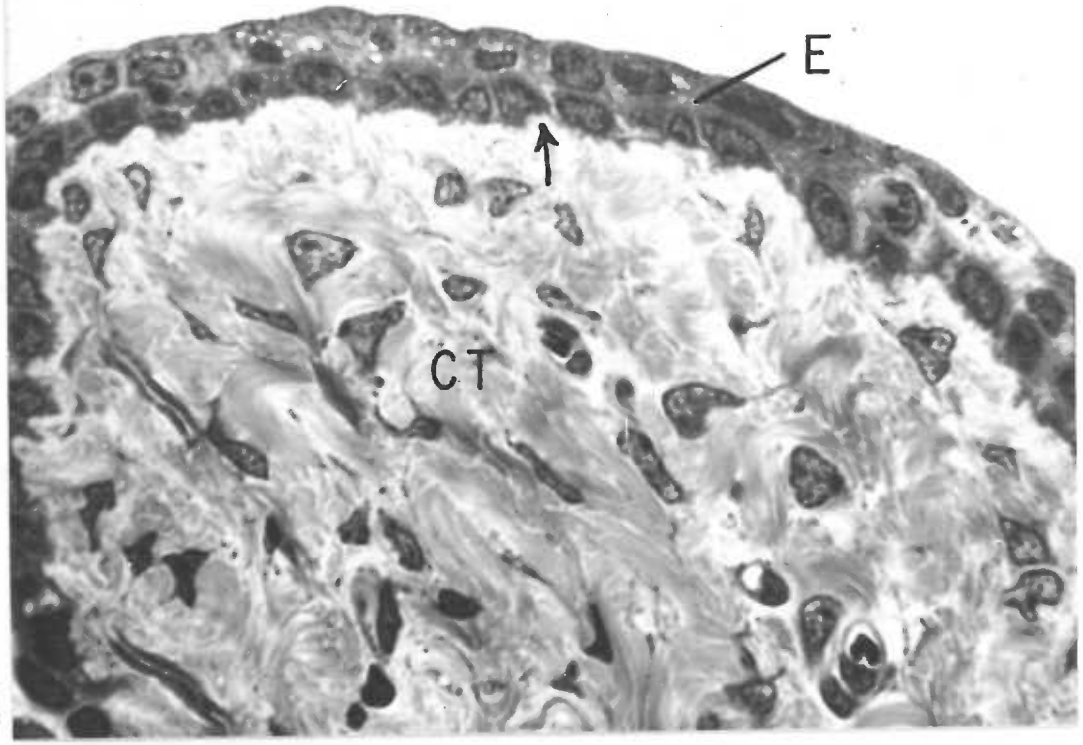
Figures 1 to 7 were prepared from 1 μ thick sections of osmium tetraoxide fixed, araldite embedded tissue stained by Richardson's method (61).

Figure 1: Control (Day 0)

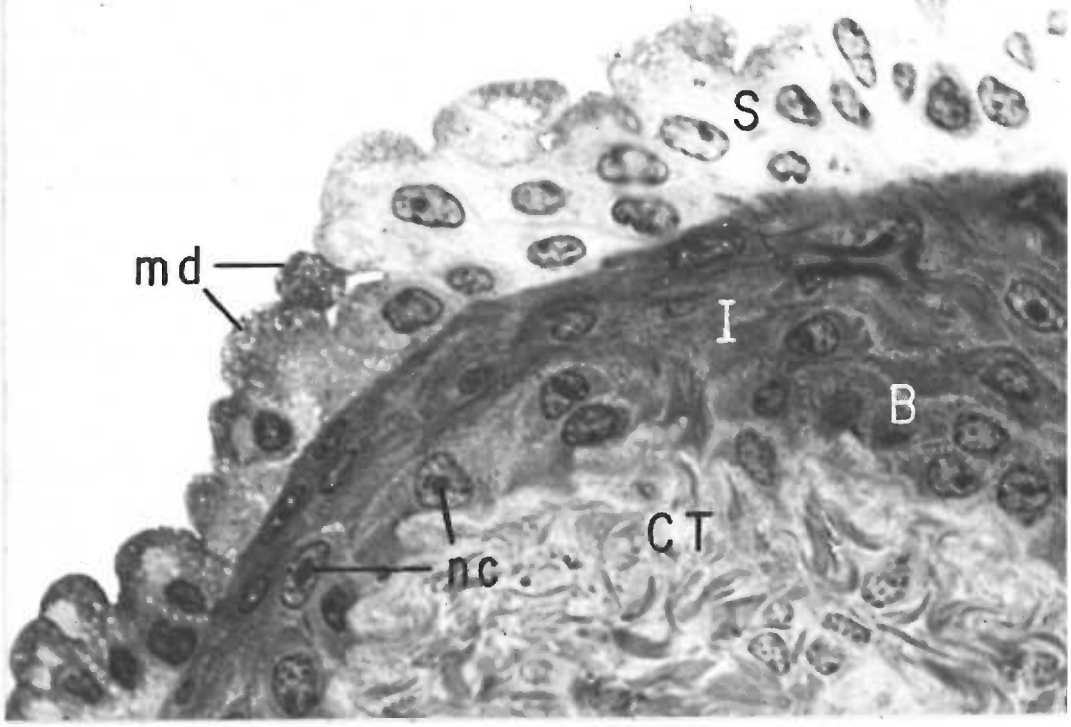
The two to three cell layered cuboidal epithelium (E) overlies a loose fibrovascular stroma (CT) from which it is sharply demarcated (→). Basal cell nuclei have fine, irregularly dispersed chromatin masses and small to inapparent nucleoli. The cytoplasm of the superficial cells is generally more abundant and less dense than that of the basal cells and demonstrates occasional fine vacuolation. Superficial cell nuclei are more vesicular with increased prominence of nucleoli. X900.

Figure 2: Day 1 Experimental

The epithelium is approximately 5 to 6 layers in thickness and consists of a single layer of basal cells (B), 2 to 3 layers of intermediate cells (I), and 1 to 2 layers of superficial cells (S). The superficial cells have pale cytoplasm, frequently containing dense, rounded masses within clear vacuolar spaces (md). Their nuclei are oval and vesicular with occasional prominent nucleoli. The intermediate cells and basal cells are similar and are characterized by polygonal cell outlines, dense cytoplasm, and vesicular nuclei with prominent nucleoli (nc). Connective tissue (CT). X900.



1



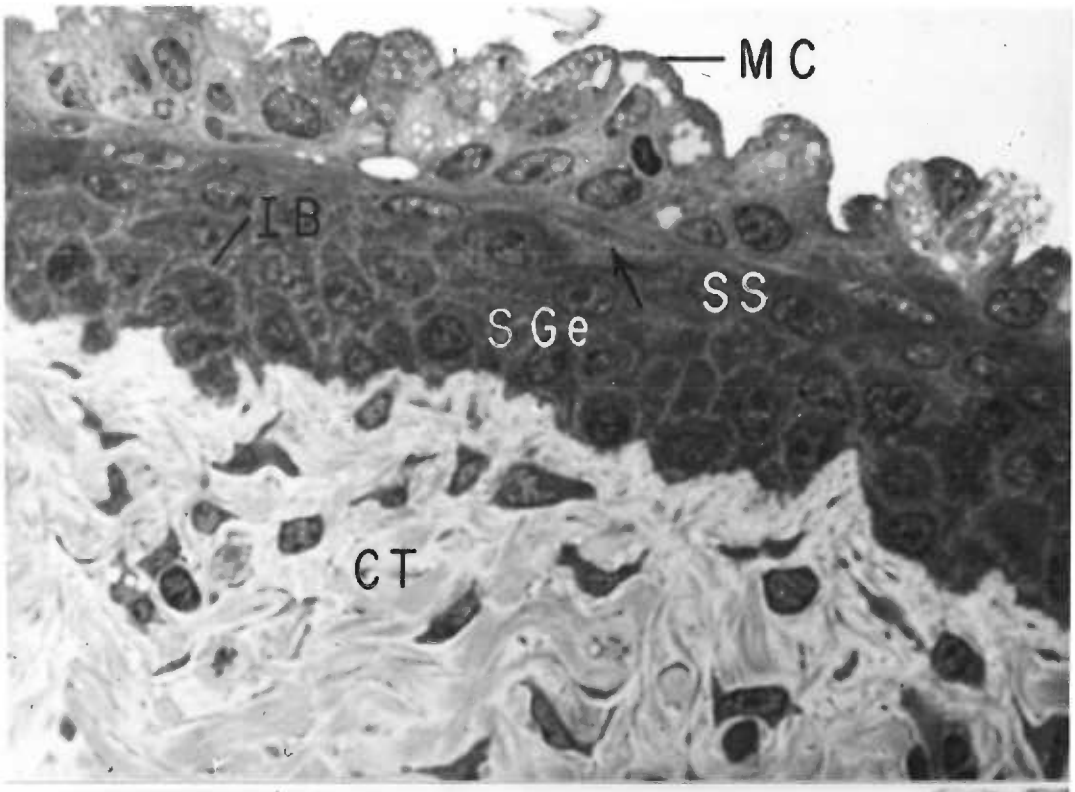
2

Figure 3: Day 2 Experimental

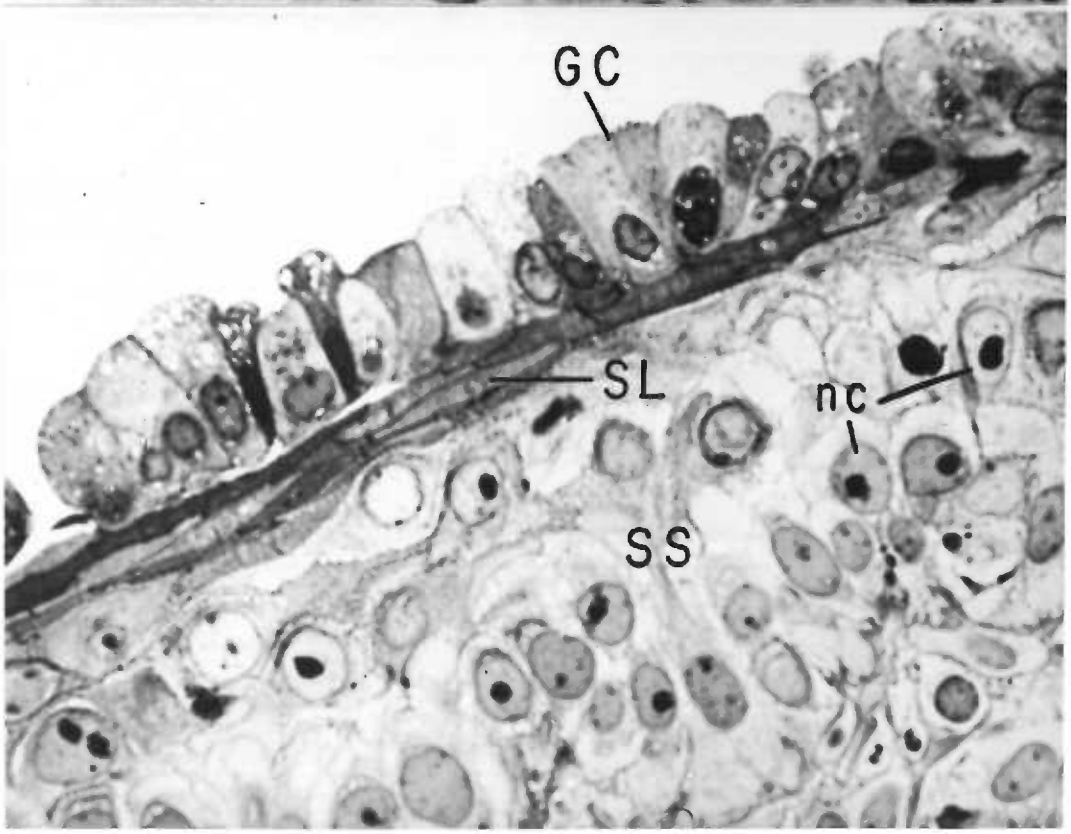
There is a surface layer of mucinous cells (MC) which overlies a now definitely squamoid epithelium. The cells of the basal cell layer (stratum germinativum) (SGe) and the intermediate cell layer (stratum spinosum) (SS) again have similar features. Individual cells are separated by uniform, though prominent, intercellular spaces which are interrupted by numerous intercellular bridges (IB). Between the mucinous surface layer and the stratum spinosum, there are several layers of flattened cells (→) with inapparent nuclei which were not present on day 1 (compare with Figure 2). X900.

Figure 4: Day 2 Experimental

Mucinous surface differentiation has progressed to the formation of distinct goblet cells (GC). These cells are tall columnar with basally placed nuclei and vacuolated, droplet-filled cytoplasm. The layer of stratified cells (SL) between the mucinified surface and the stratum spinosum (SS) is more prominent than in Figure 3. Note the increased staining of the cell membranes in the stratified layer and the lack of nuclei. Prominent intercellular bridges and nucleoli (nc) are present in the stratum spinosum. X1250.



3



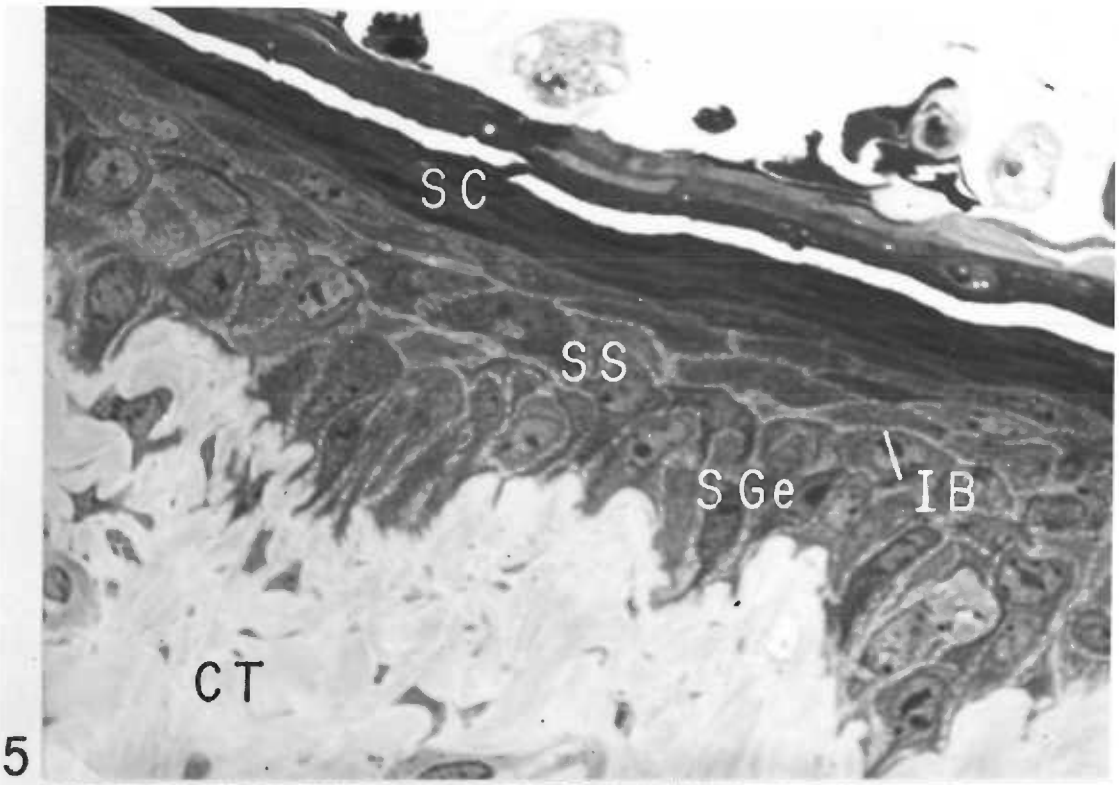
4

Figure 5: Day 3 Experimental

The epithelium consists of approximately 12 to 15 layers of squamoid cells and a degenerate mucinous surface. The stratum corneum (SC) consists of intensely stained, flattened anuclear cells whose long axes lay parallel to the surface. Intercellular bridges (IB), which are prominent in the stratum spinosum (SS), are absent in the stratum corneum. The cells of the stratum germinativum (SGe) have features which are similar to the stratum spinosum. Note that the junction between the stroma (CT) and the epithelium is irregularly serrated. X900.

Figure 6: Day 4 Experimental

Survey view of an entire organ culture specimen demonstrating re-epithelialization of the stroma (CT) and total epithelial keratinization. Stratum corneum (SC); stratum spinosum (SS). X288.



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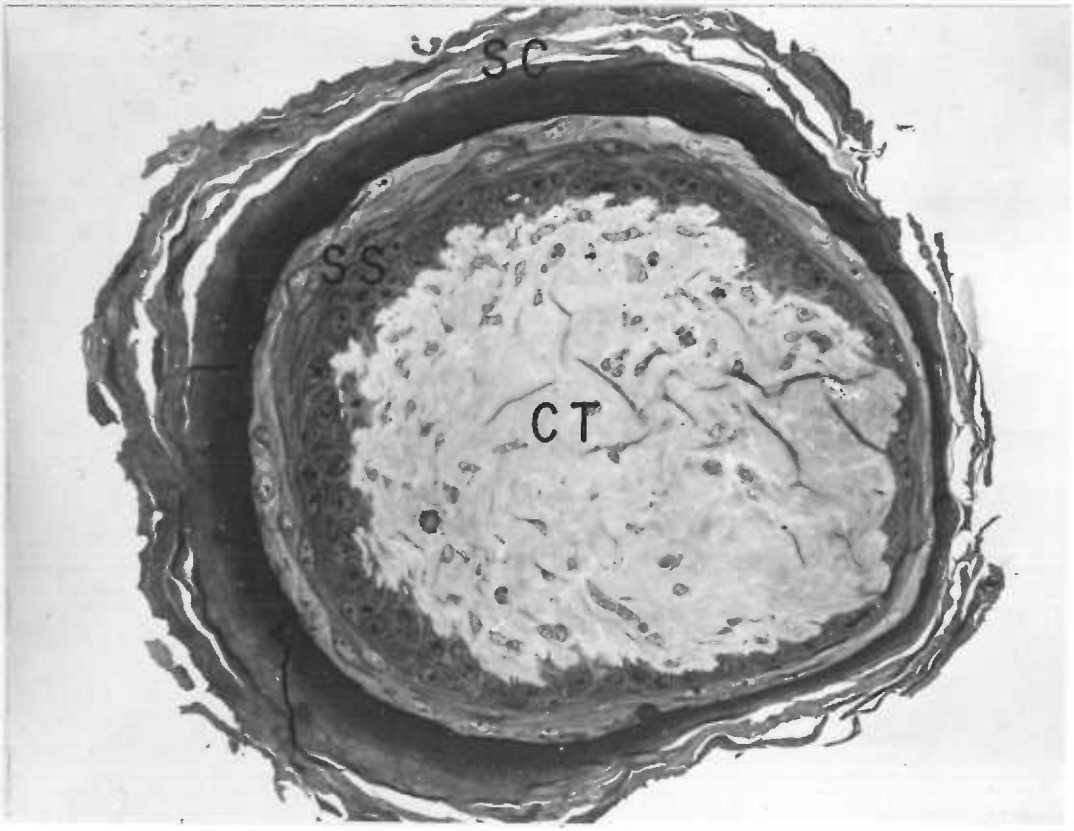

6

Figure 7: Day 4 Experimental

The epithelium has a distinct stratum corneum (SC) with delamination of the upper cell layers. The cells of the stratum spinosum (SS) are flattened, but maintain distinct intercellular spaces interrupted by intercellular bridges. Keratohyalin is absent, although some cells contain karyorrhetic nuclear debris. The lowermost cells (stratum germinativum) (SGe) have irregular outlines, abundant cytoplasm, and vesicular nuclei, features which are more typical of spinal than of basal cells. Similar, though less well developed, findings were present on days 1, 2, and 3 (compare with Figures 2, 3, and 5). X900.

With the exception of Figure 11, which was fixed in glutaraldehyde, embedded in paraffin, and stained with periodic acid-Schiff (PAS) reagent after amylase digestion, Figures 8 to 12 were prepared from 1 μ thick sections of osmium tetroxide fixed, araldite embedded tissue stained with PAS by the method of Lane and Europa (40).

Figure 8: Control (Day 0) (PAS)

There are occasional positively stained mucinified superficial cells (MC) in an otherwise cuboidal epithelium which averages 3 cells in thickness. The demarcation between the epithelium and the stroma is distinct (→). X864.

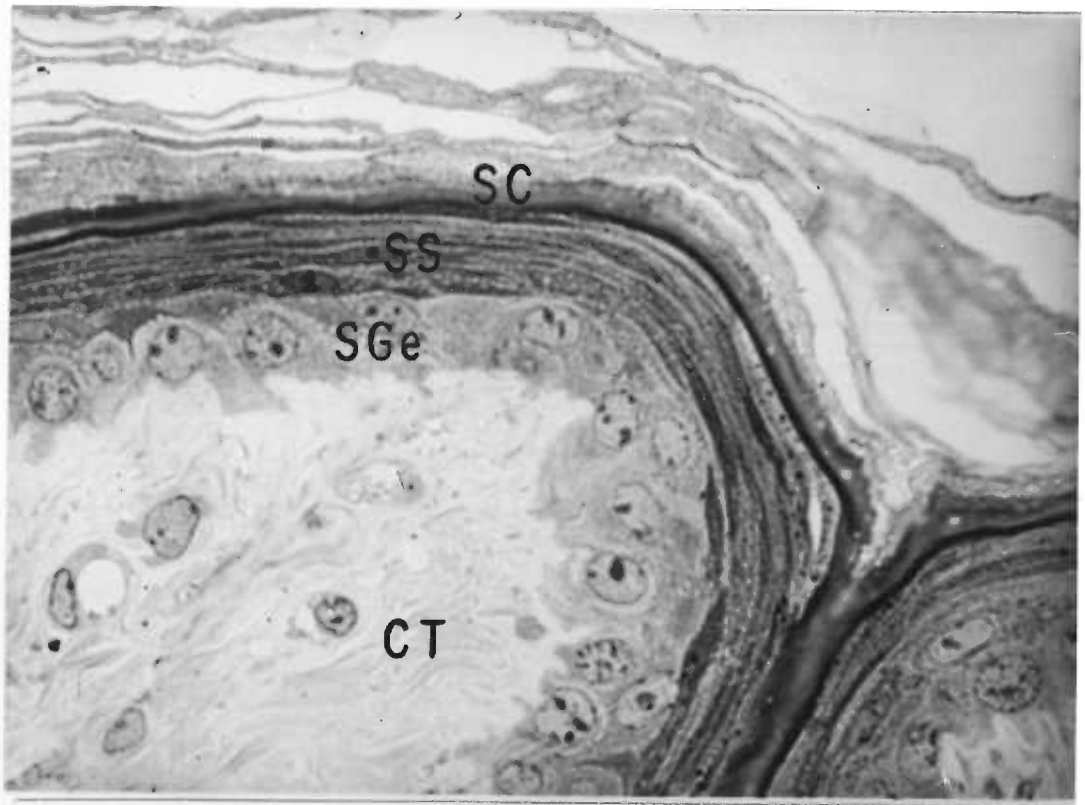

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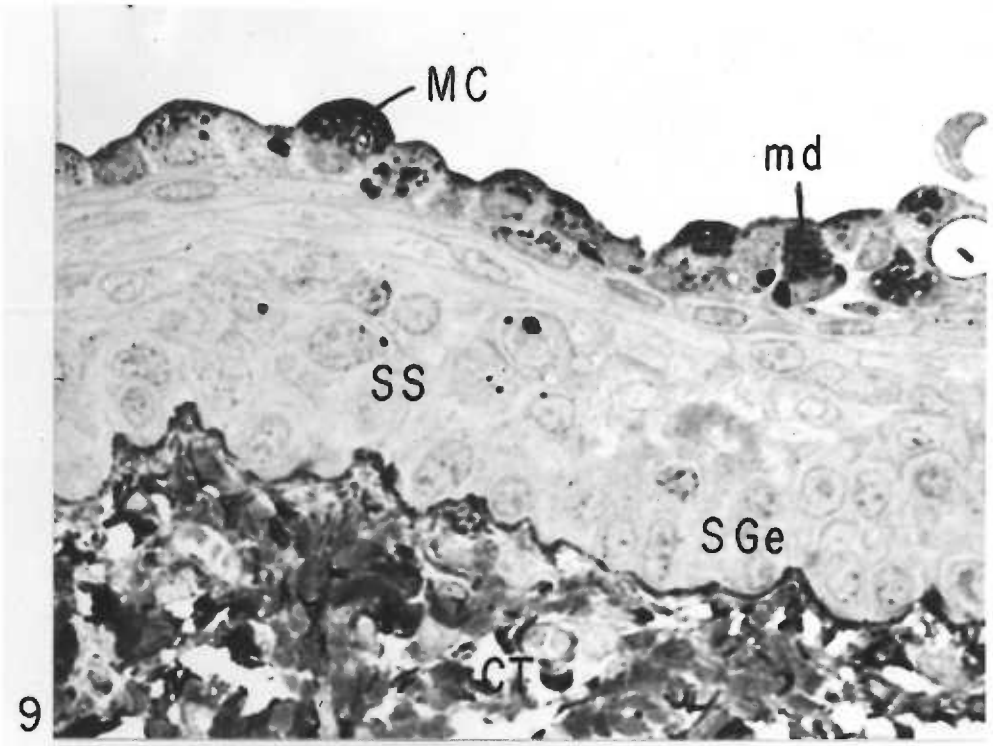
Figure 9: Day 2 Experimental (PAS)

PAS positive granules (md) in the cytoplasm of the superficial cells (MC) are more prominent than in the controls (compare with Figure 8). Note the lack of staining in the stratum spinosum (SS) and the stratum germinativum (SGe). X864.

Figure 10: Day 2 Experimental (PAS)

PAS positive droplets within clear vacuolar spaces are present in the cytoplasm of the goblet cell-like superficial cells (GC). Note the increased staining intensity of the cell membranes in the stratified layer (SL). (Compare with Figure 4.) X864.



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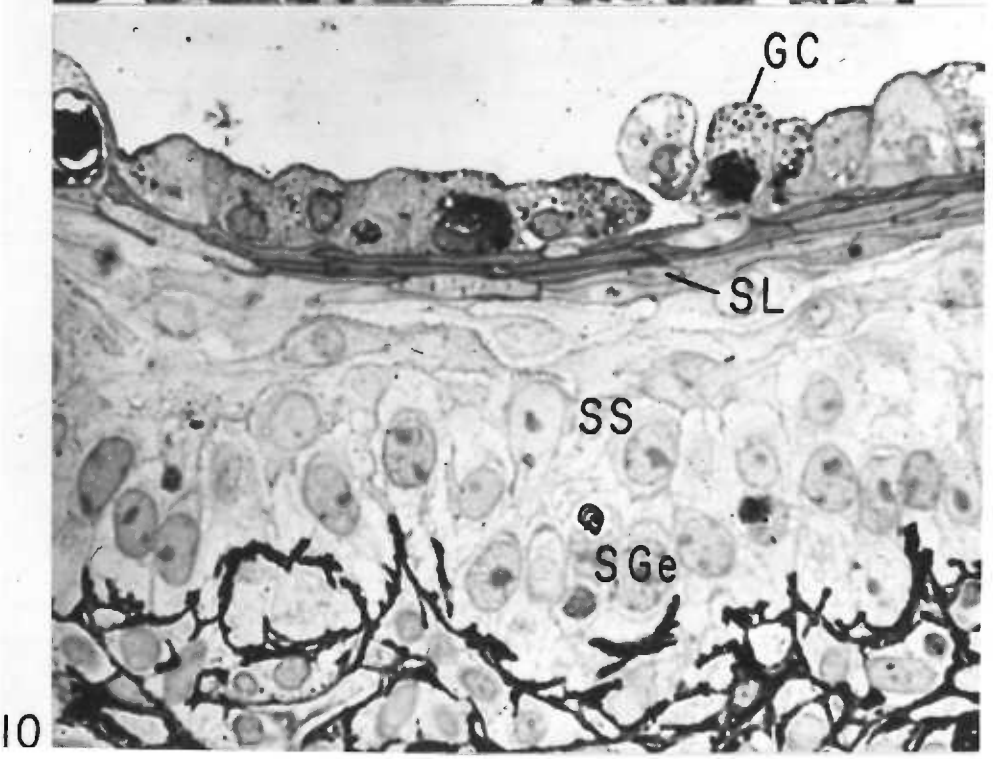

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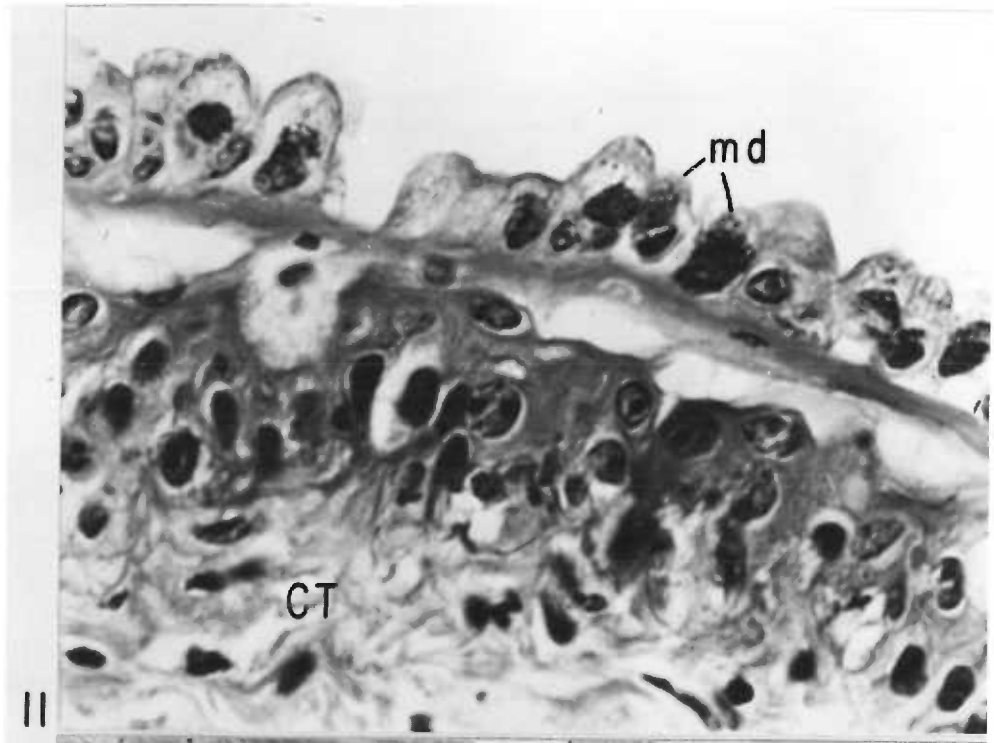
Figure 11: Day 2 Experimental (digested PAS)

The general cytology is comparable to that illustrated in Figures 4 and 10. The intracytoplasmic droplets (md) are resistant to salivary amylase digestion and are thus histochemically consistent with mucin. X865.

Figure 12: Day 3 Experimental (PAS)

The mucinified surface cells (MC) are less intensely stained than on days 1 and 2. The cell membranes of the anuclear cells of the stratum corneum (SC) are prominent, and their cytoplasm is more dense than that of the cells in the stratum spinosum (SS). X864.



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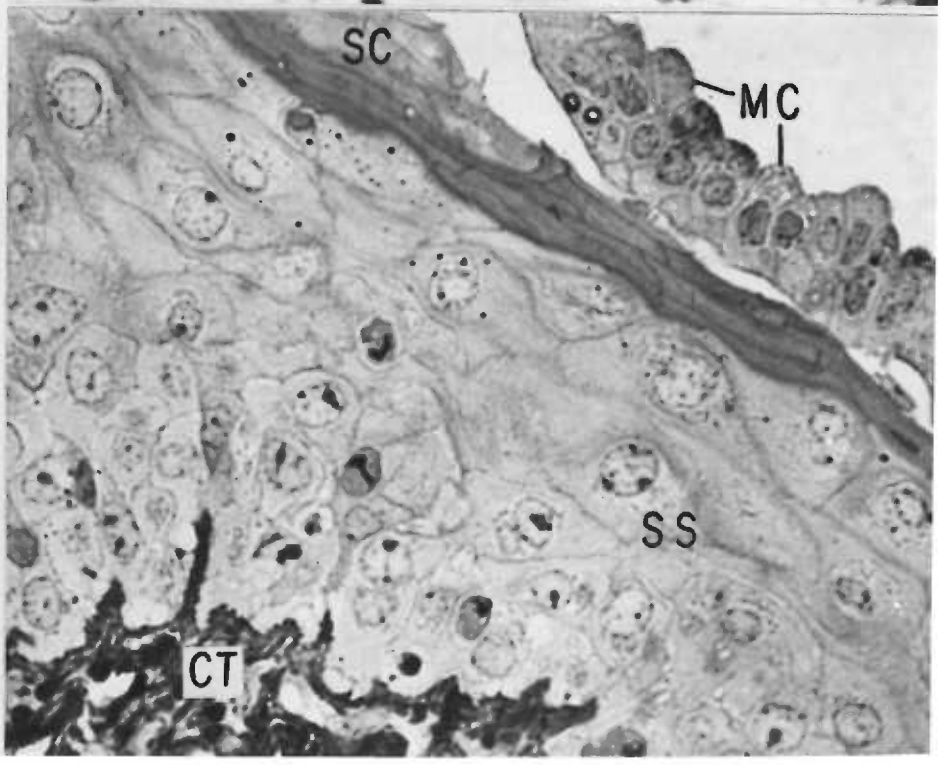

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Figures 13 to 17 were prepared from glutaraldehyde fixed, paraffin embedded 5 micron sections stained for sulfhydryl (SH) and disulfide (S-S) groups by the method of Barrnett and Seligman (2). Figure 18 was stained for sulfhydryl groups alone.

Figure 13: Control (Day 0) (SH and S-S)

The epithelium is thin and is negative for reaction product. X500.

Figure 14: Day 1 Experimental (SH and S-S)

The epithelium is thickened as compared to the control, but lacks definitive staining. X500.

Figure 15: Day 2 Experimental (SH and S-S)

Positive staining has developed in a flattened layer of cells (→) beneath the mucinified surface epithelium. Compare with Figure 10. X500.

Figure 16: Day 3 Experimental (SH and S-S)

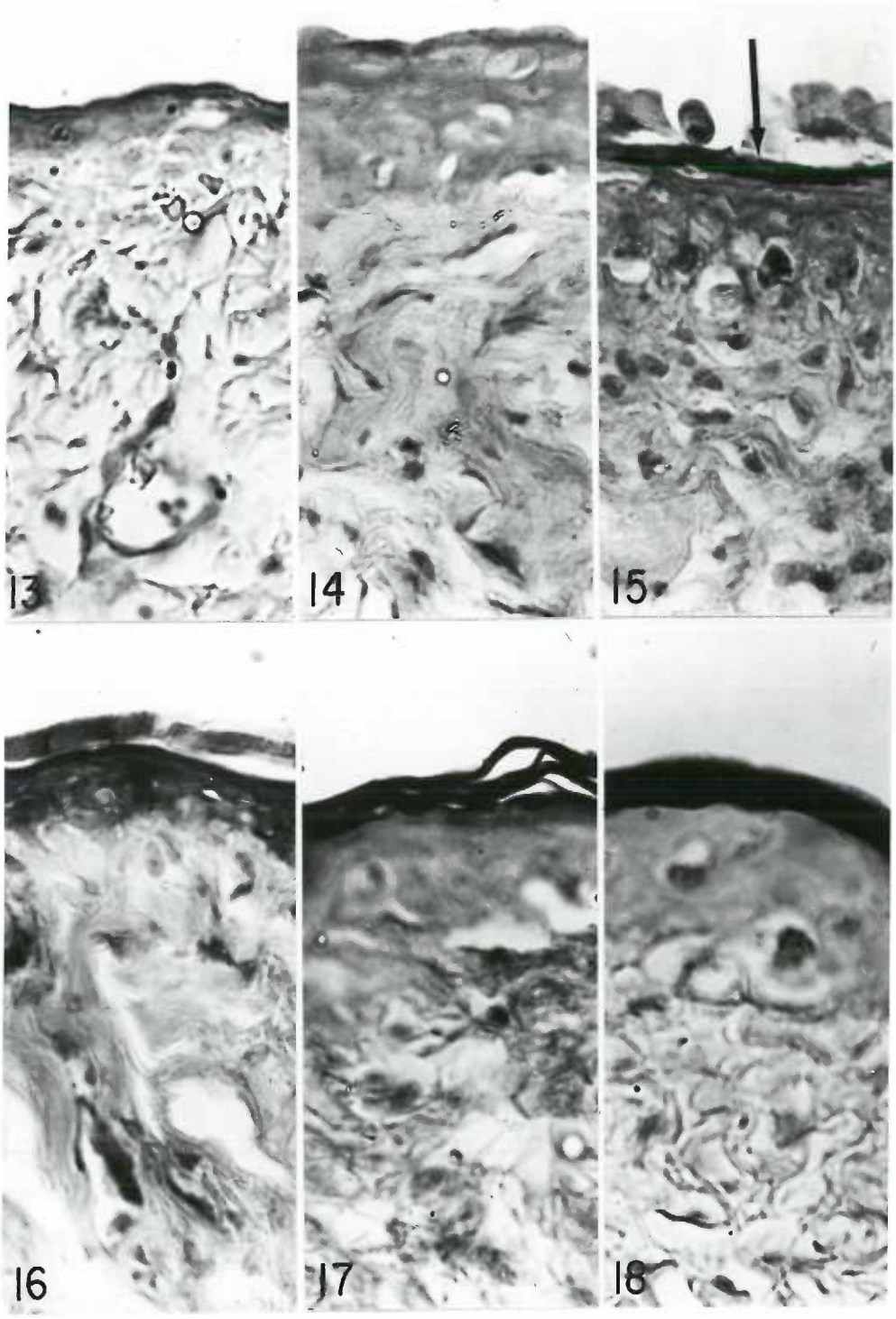
The epithelium is diffusely stained with highest intensity in a linear band beneath an irregular surface layer. X500.

Figure 17: Day 4 Experimental (SH and S-S)

The fully keratinized surface (stratum corneum) is more intensely stained than on previous days. X500.

Figure 18: Day 4 Experimental (SH)

The intensity of staining for sulfhydryl groups alone is comparable to that seen in stains for both sulfhydryl and disulfide groups (compare with Figure 17). This implies, in contrast to normal *in vivo* keratinization (2, 3, 58), incomplete conversion of sulfhydryl to disulfide groups. X500.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Electron Microscopy:

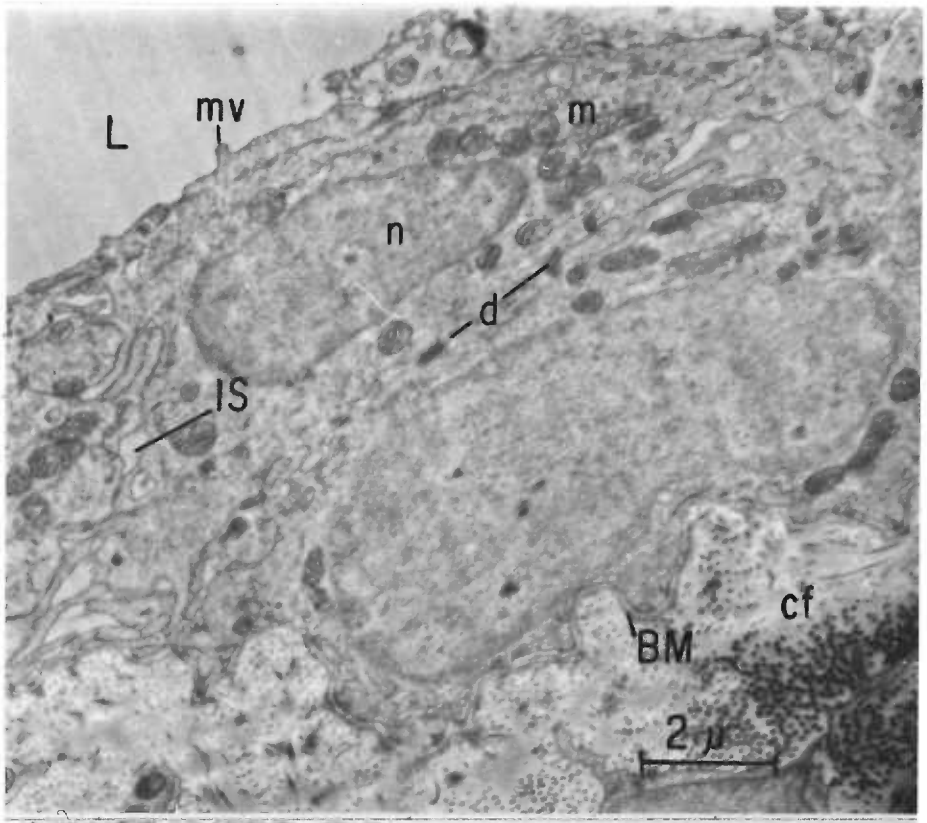
Figures 19 to 44 were prepared from specimens fixed in 1% OsO_4 in veronal acetate (pH 7.4) with 0.045 gm/cc sucrose added and embedded in Swiss araldite. Sections were triply stained with lead citrate and uranyl acetate. The terminology used in the description of keratin related fibrillary cytoplasmic components is taken from Mercer, Munger, Rogers, and Roth (47).

Figure 19: Control (Day 0)

Survey view of entire epithelium. The epithelial cells rest upon a continuous basement membrane (BM) from which they are separated by a clear zone. Desmosomes (d) are present along the closely apposed cell membranes of adjacent cells. In other areas there are distinct intercellular spaces (IS). The luminal surfaces of the most superficial cells have rare microvilli (mv). The cytoplasm contains moderate numbers of mitochondria (m). Collagen fibers (cf) are present in the subepithelial connective tissue. Nucleus (n). X8400.

Figure 20: Control (Day 0)

Secretory surface differentiation is present. Microvilli (mv) are more numerous, and intercellular spaces (IS) are wider and more prominent than in non-secretory areas (compare with Figure 19). The cytoplasm of the secretory cells contains medium density reticular aggregates within smooth-surfaced membranes (md) which are interpreted as mucin droplets. Golgi apparatus are present in the same cells. Desmosome (d); mitochondria (m). X8400.



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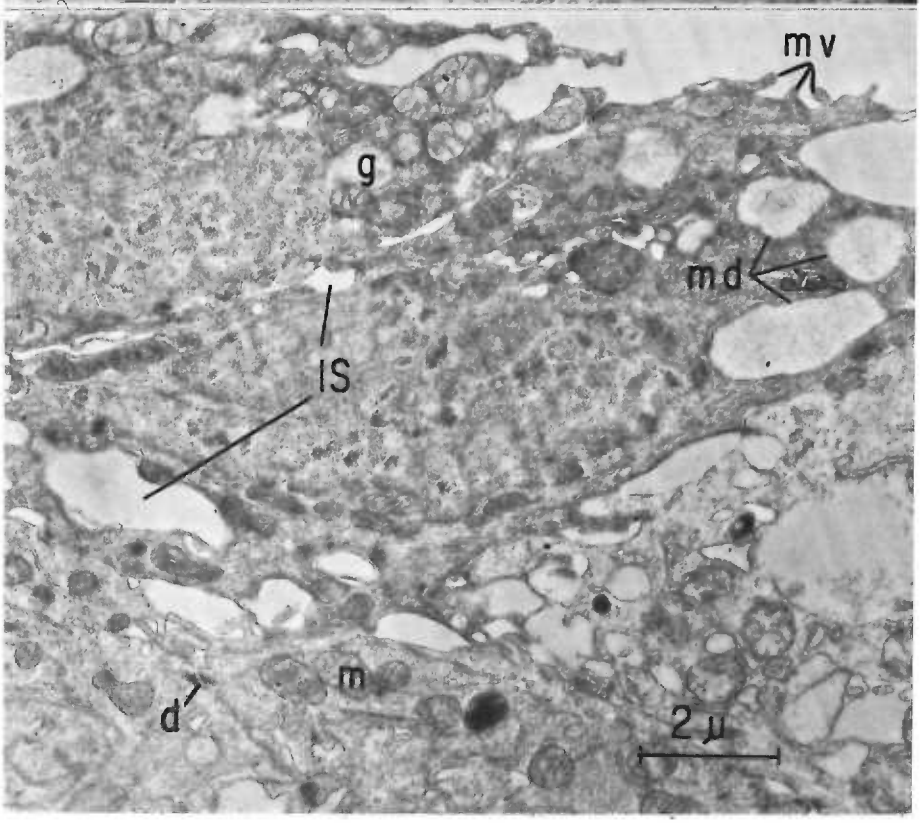

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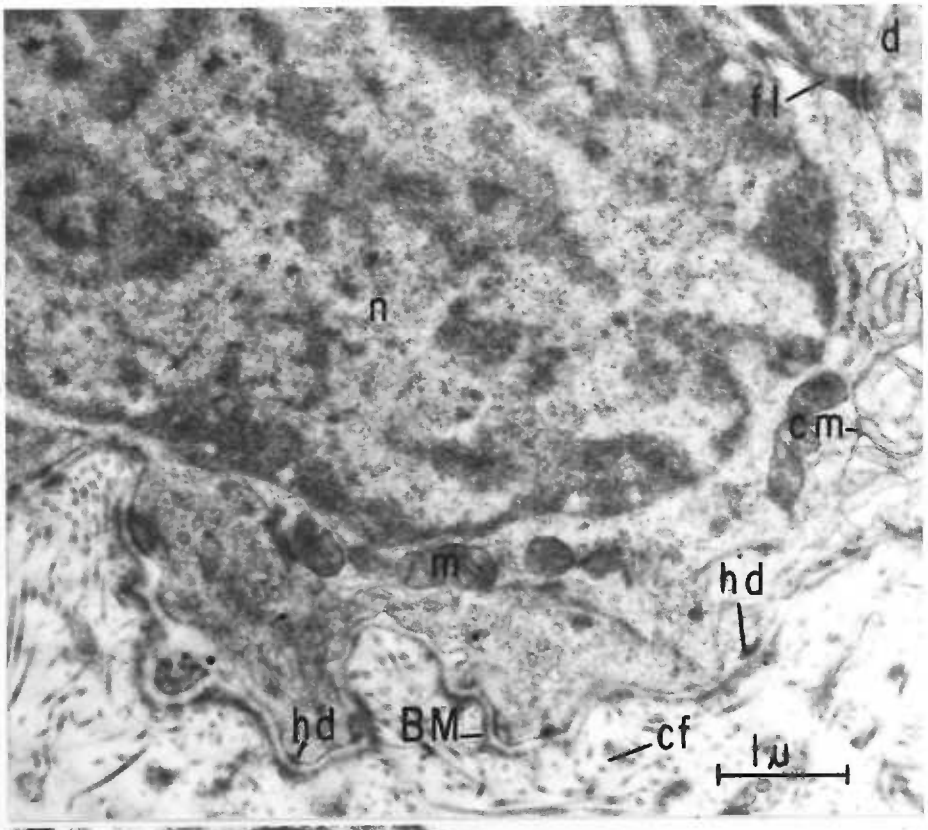
Figure 21: Control (Day 0)

Basal cell layer. The basal portion of the cell membrane shows numerous half-desmosomes (hd) and rests upon an approximately 350 Å basement membrane (BM), from which it is separated by a 600 to 700 Å clear zone. The cell membranes of adjacent basal cells demonstrate desmosomal modifications (d) measuring up to 2400 Å, to which are attached tufts of approximately 70 Å filaments (fl). There are zones of villiform cell membrane interdigitation (cm). Basal cell nuclei (n) have dense, irregularly aggregated chromatin. Mitochondrion (m); collagen fibers (cf). X16,400.

Figure 22: Control (Day 0)

Superficial cells. The surfaces of the cell membranes have numerous microvilli (mv) and in their immediately subsurface aspects are tightly apposed with the formation of an occluding zone (*Zonulae occludentes*) (Zo) (19). Intercellular spaces (IS) are dilated. Multiple desmosomes (d) are present at the junction of the superficial with the deeper cell layers. The cytoplasm of one superficial cell contains numerous mucin droplets (md), while the other superficial cell is non-secretory. The outer and inner leaflets of the nuclear membrane are well demonstrated (nm). Nucleus (n); mitochondria (m). X16,400.



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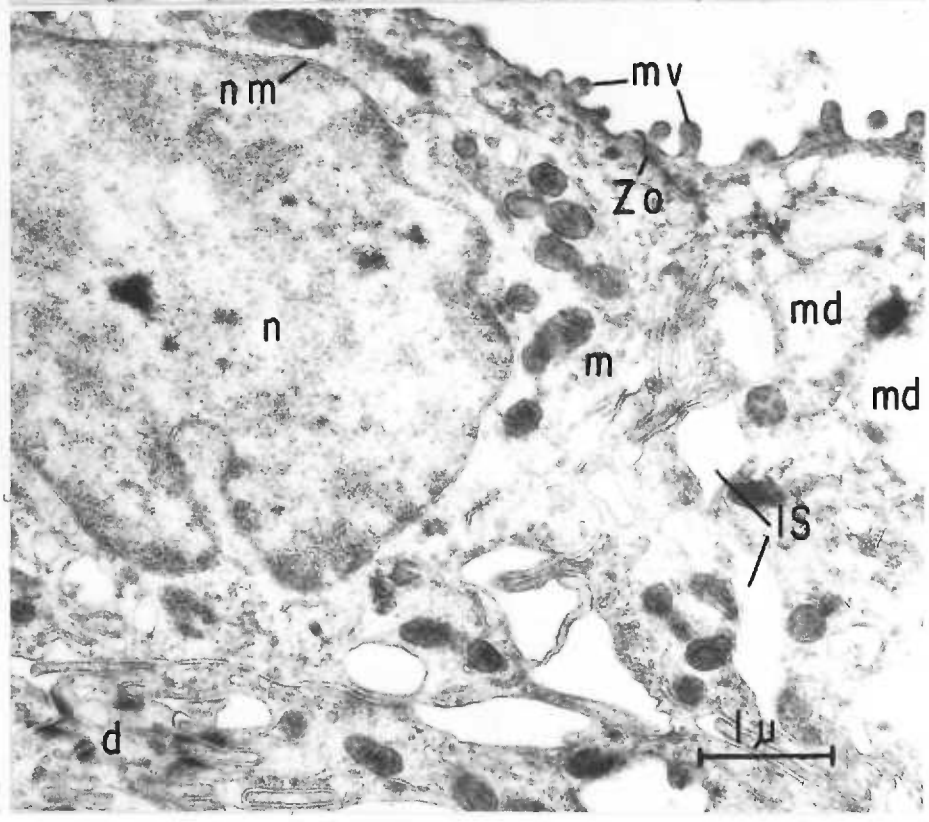

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Figure 23: Control (Day 0)

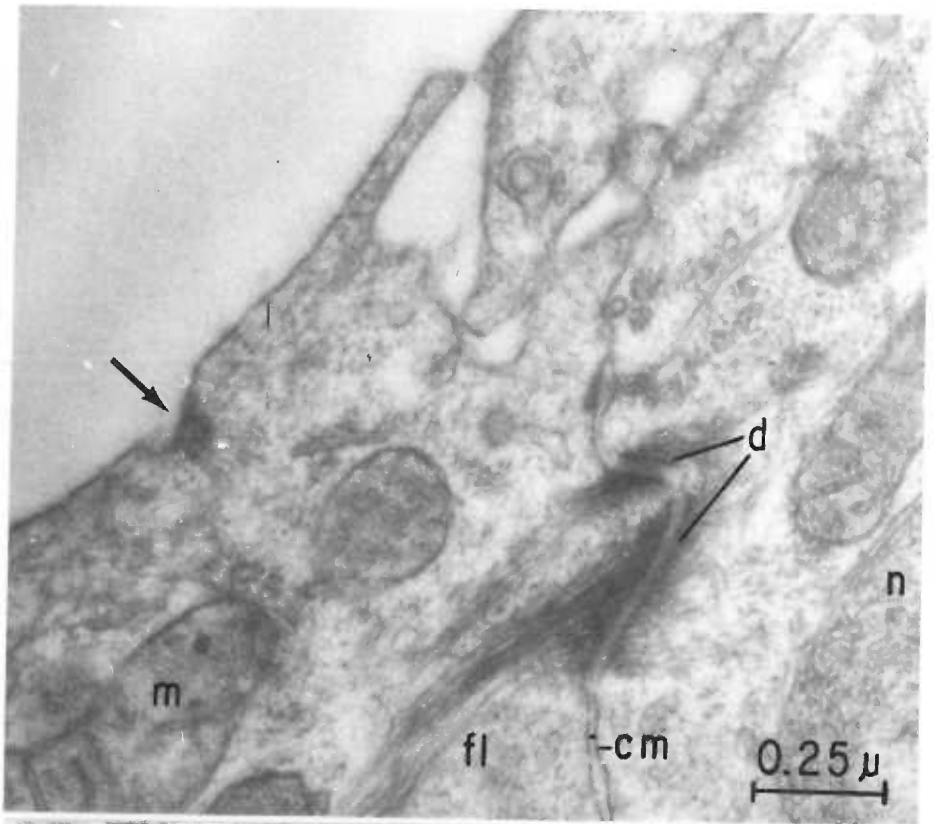
Superficial cells. Desmosomes (d) are present along the apposed cell membranes (cm) just deep to a possible occluding zone. Aggregates of filaments (fl), closely related to the desmosomes, are present in the cytoplasm. The localized inverted area of increased cell membrane density present on the surface (→) is suggestive of a previous point of desmosomal attachment. Mitochondrion (m); nucleus (n). X68,000.

Figure 24: Day 1 Experimental

Basal cell layer. Cell membrane-basement membrane (BM) relationships are the same as in the controls (see Figure 21). Desmosomes (d) are present along the non-basement membrane oriented parts of the cell membranes (cm). Ribosomes (r) are more prominent as are cytoplasmic fibrils (f) composed of 70 Å filaments. Nuclei (n) are similar to controls. Mitochondrion (m).

Inset showing detail of half-desmosome (basal attachment plate). A small tuft of cytoplasmic filaments is attached to a zone of increased prominence of the cell membrane (cm) leaflets. An approximately 700 Å clear zone separates the cell membrane from the basement membrane (BM).

X22,800. Inset X46,800.



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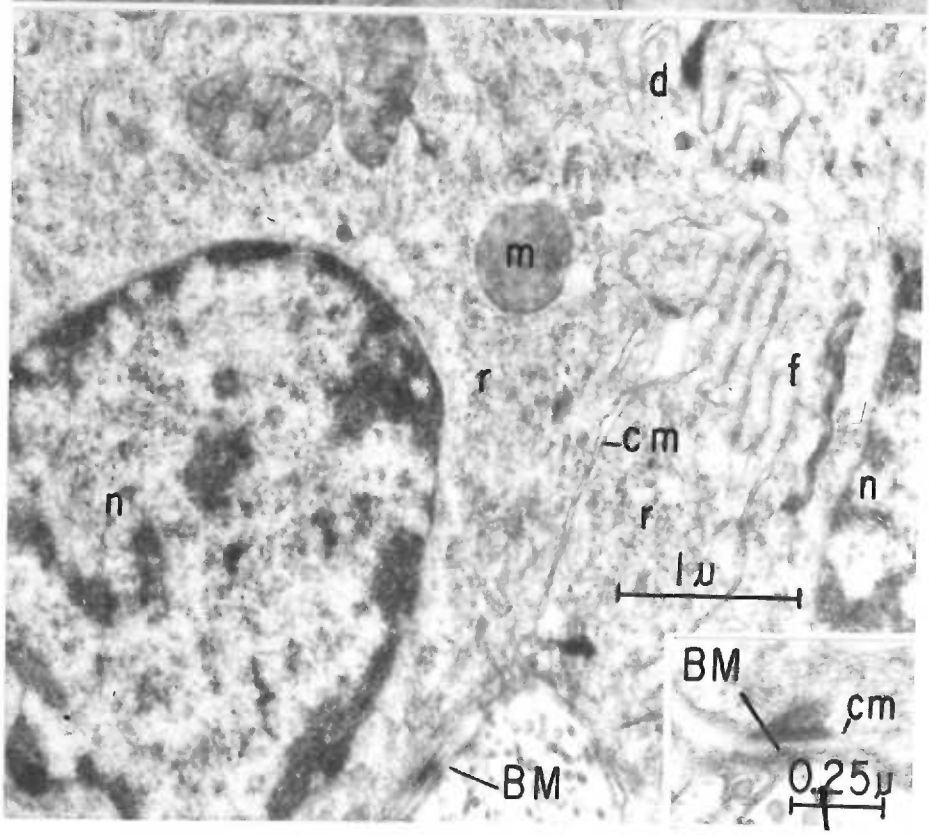

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Figure 25: Day 1 Experimental

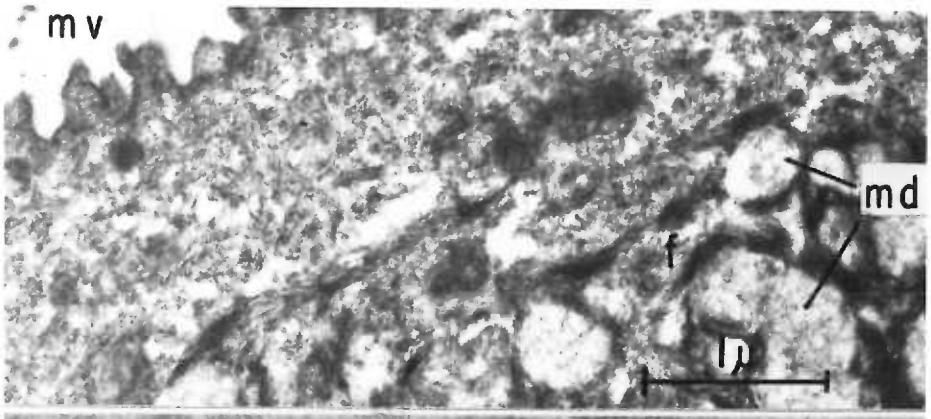
Portion of a superficial cell. The surface has irregular microvilli (mv). The cytoplasm differs from the secretory cells of the controls by development of an extensive interanastomosing system of cytoplasmic fibrils (f) in addition to the mucin droplets (md). X22,800.

Figure 26: Day 1 Experimental

Intermediate cell layer. Multiple standard desmosomes measuring up to 4000 Å modify the apposed cell membranes (cm) of two spinal cells. Cytoplasmic filaments (fl) are related to the desmosomal plaques. X46,800.

Figure 27: Day 1 Experimental

Standard desmosome. The desmosomal plaques (p) end abruptly at the lateral desmosomal limits and lie immediately adjacent to the inner leaflet (il) of the cell membrane (→). The outer cell membrane leaflet (ol) is continuous with the lateral line of the desmosome. The intermediate line (i) is poorly defined. X128,000.



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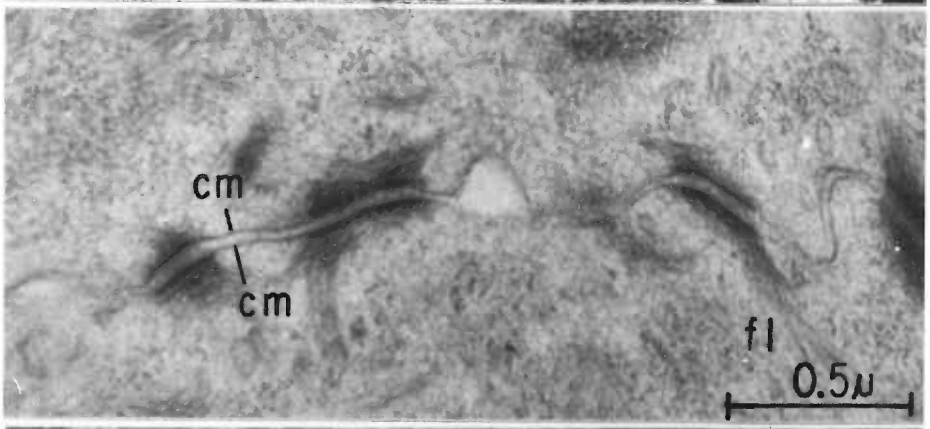

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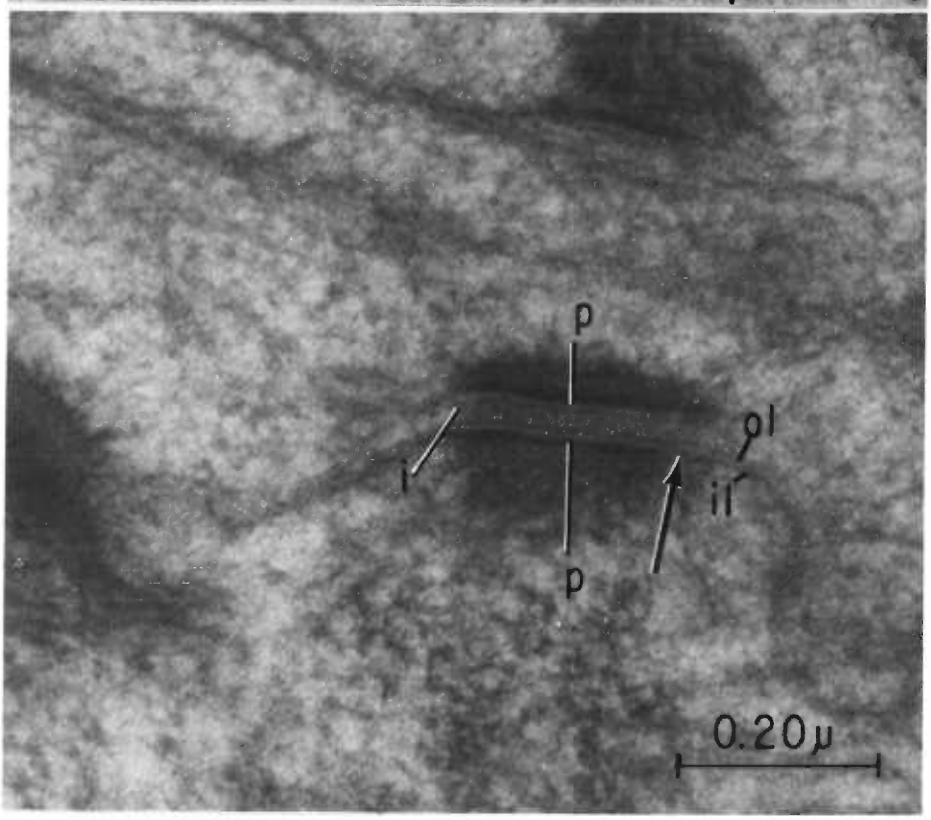

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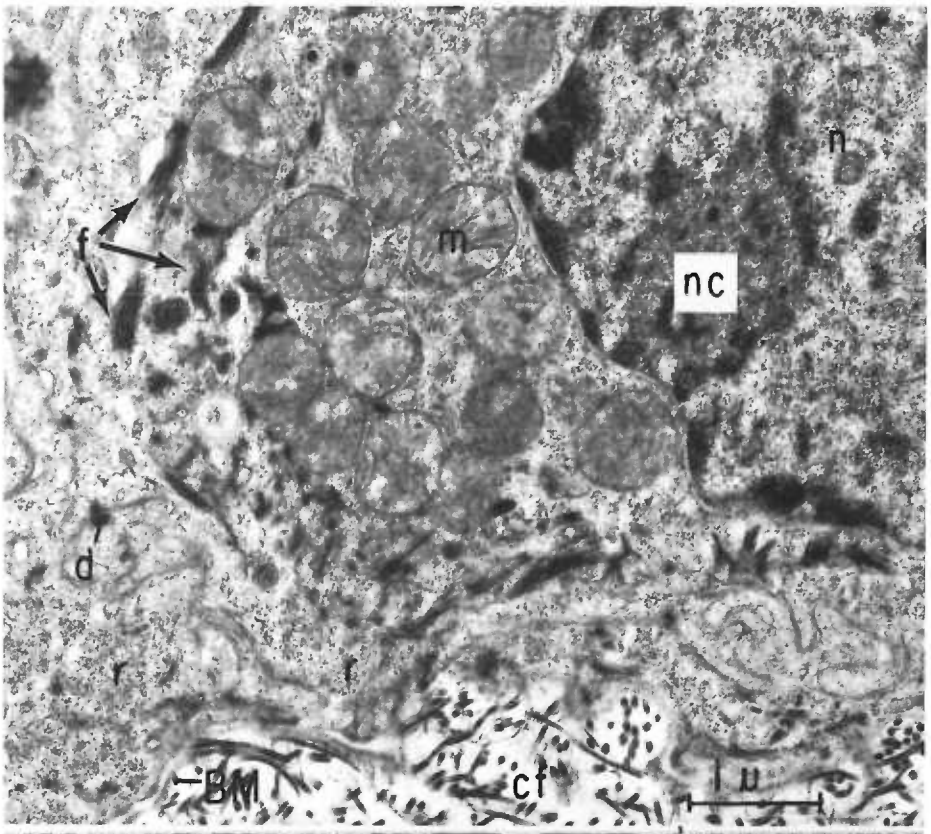
Figure 28: Day 2 Experimental

Basal cell layer (stratum germinativum). Cytoplasmic fibrils (f) are more prominent than on day 1. There are large numbers of free ribosomes (r) with suggestions of polyribosomal aggregates. Nuclear chromatin is more evenly dispersed, and nucleoli (nc) are prominent. Nucleus (n); mitochondria (m); basement membrane (BM); desmosome (d); collagen fibers (cf). X16,400.

Figure 29: Day 2 Experimental

Intermediate cell layer (stratum spinosum). Open intercellular spaces (IS) are present between points of desmosomal attachment (d) along the cell membranes. The cytoplasm of the upper cell consists almost exclusively of large numbers of free ribosomes (r) and of interanastomosing complex fibril bundles (f). Dense osmophilic bodies are present (→) within the mitochondria (m) of one intermediate cell. Similar bodies were seen in basal cell mitochondria (see Figure 33). X16,400.



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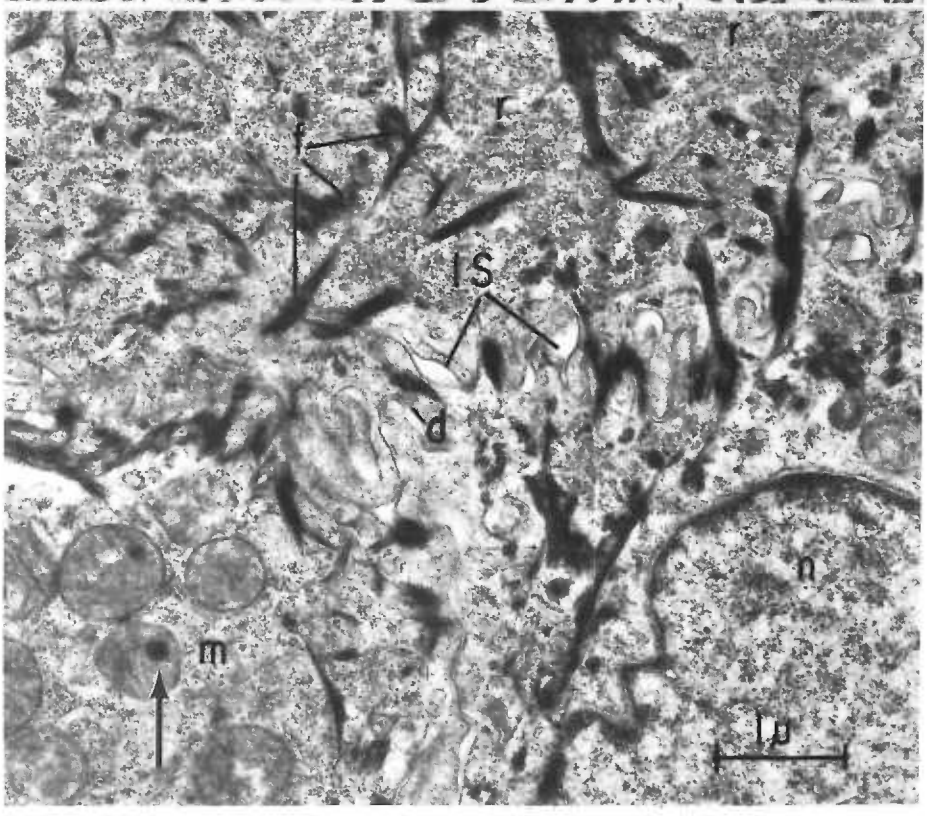

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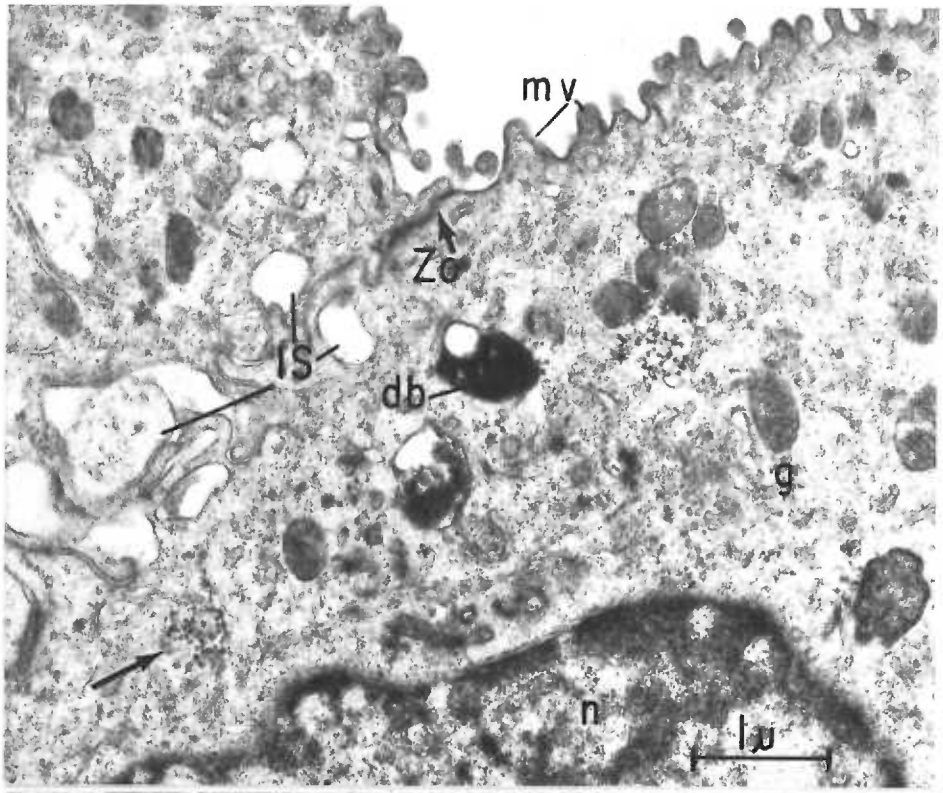
Figure 30: Day 2 Experimental

Non-secretory superficial cells. Intercellular spaces (IS) just beneath an occluding zone (Zo) are dilated, measuring up to 850 mμ, and contain granular material of low to medium density. One cell has small aggregates of dense, approximately 250 Å cytoplasmic granules consistent with glycogen (→) as well as a non-dilated Golgi apparatus (g) and membrane-limited dense bodies (db) interpreted as lysosomes. Microvilli (mv). X16,400.

Figure 31: Day 2 Experimental

Junction between superficial cell and intermediate cell layers. Fibrils (f) composed of individual filament, are more abundant in the intermediate than in the superficial cell. Nuclei (n); nuclear membrane (nm); intercellular space (IS). X46,800.



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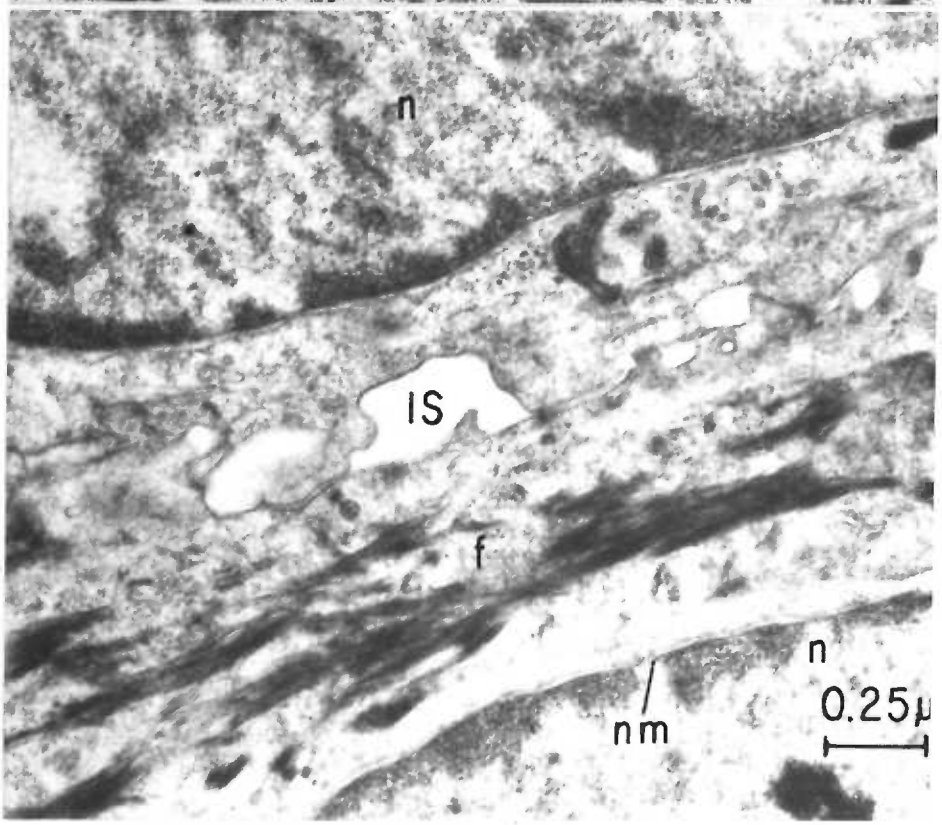

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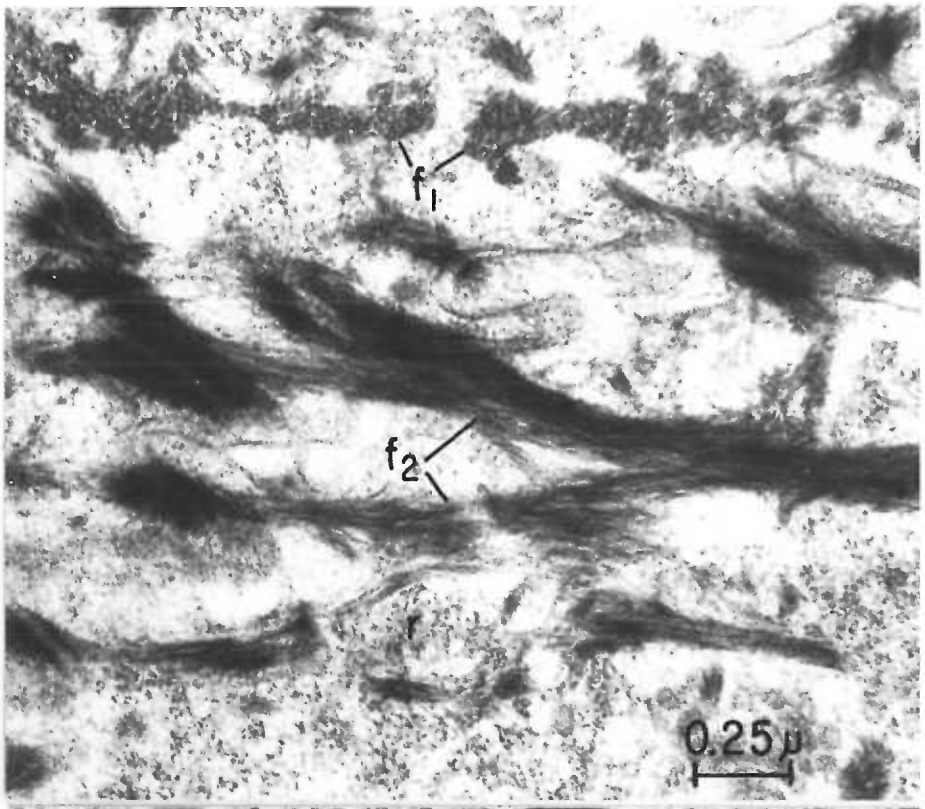
Figure 32: Day 2 Experimental

Intermediate cell layer (stratum spinosum). Fibrils are cut both transversely (f_1) and longitudinally (f_2). The fibrils are composed of closely aggregated, approximately 70 Å filaments, having a somewhat beaded appearance, separated by zones of decreased density. Free ribosomes (r) are present in the cytoplasm between and often intimately associated with the fibrils. X46,800.

Figure 33: Day 2 Experimental

Basal cell layer (stratum germinativum). Cytoplasmic filaments, grouped into interanastomosing fibrils (f) are less numerous than in the intermediate cell layer. Mitochondrial dense bodies (→) are present. Nucleus (n); nucleolus (nc). X22,800.



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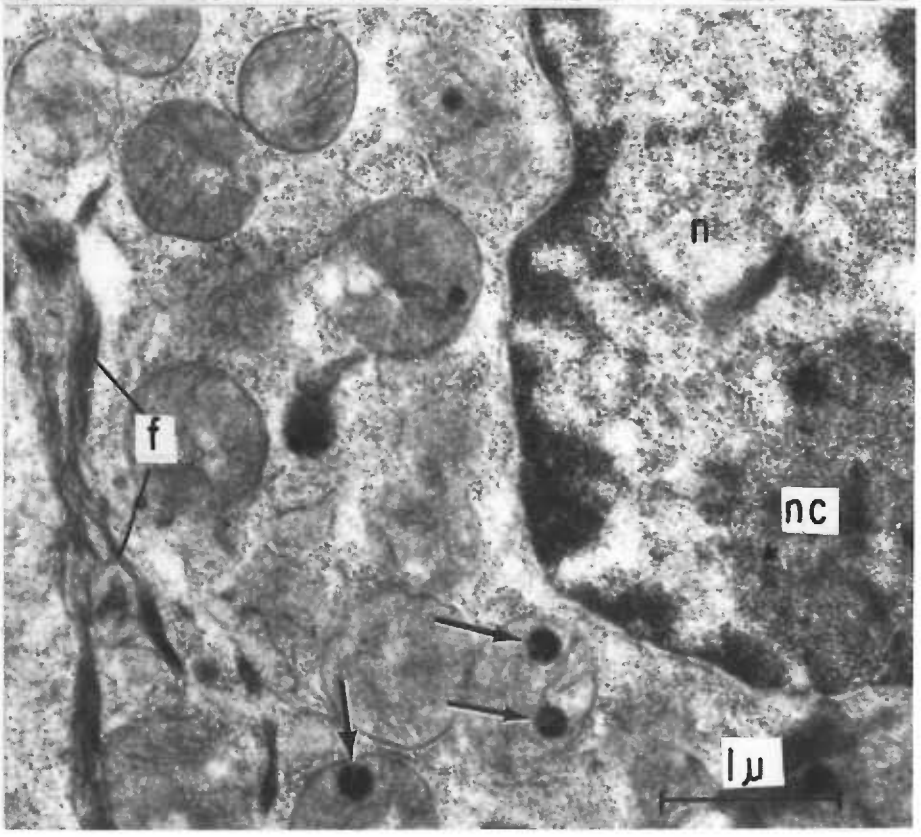

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Figure 34: Day 2 Experimental

Goblet cell (GC) differentiation of superficial cell layer. Note the roughly rectangular cell outlines, basally placed nucleus (n), prominent microvilli (mv), and mucin droplets (md). A stippled, electron dense mass (→) of unknown significance is present in the nucleus. Desmosome (d); segments of rough-surfaced endoplasmic reticulum (er). X8400.

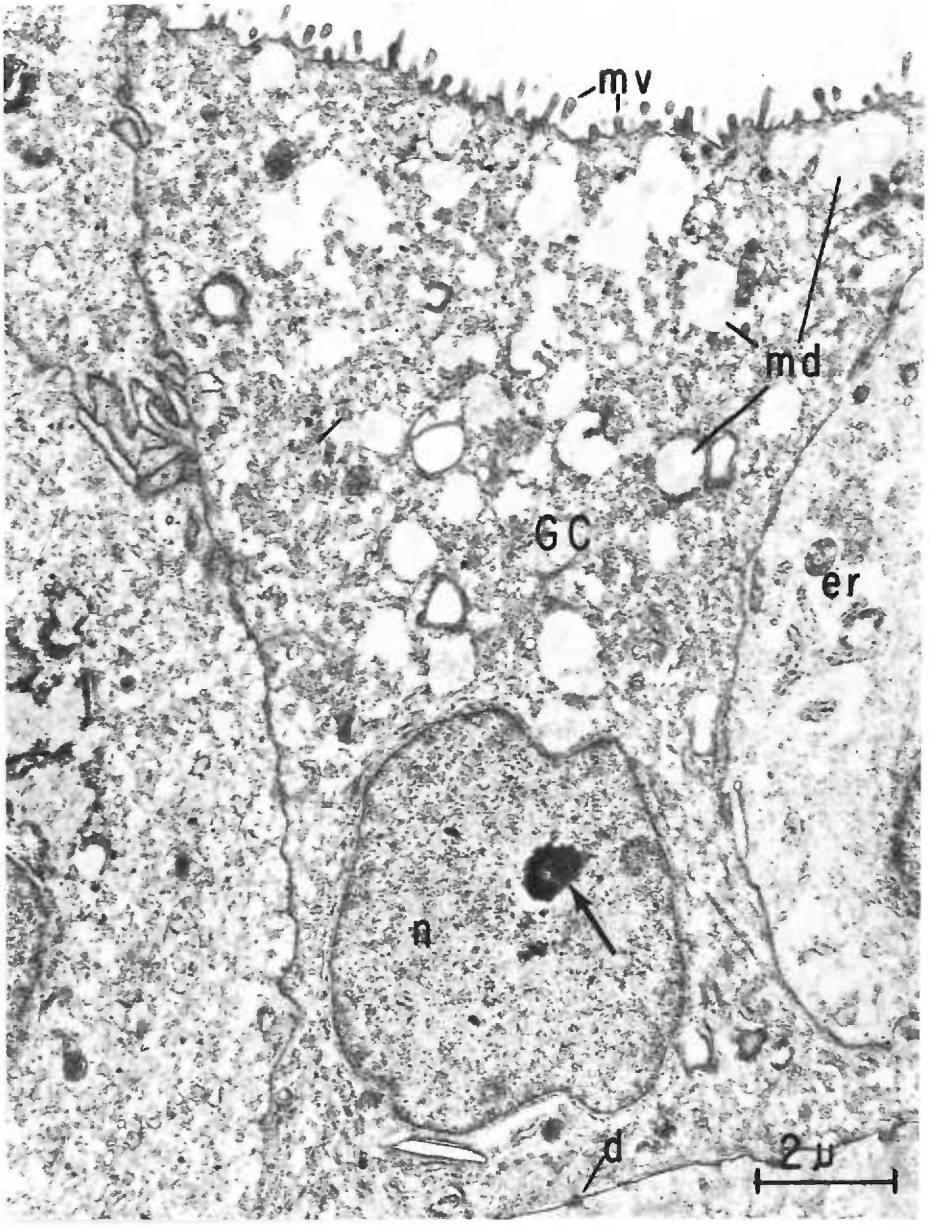

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Figure 35: Day 3 Experimental

Superficial cell layer. A residual mucinified surface cell is present. Note the prominent surface microvilli (mv), and the irregular scalloping of the nucleus (n) with chromatin margination and an irregular nuclear dense body (→). The cytoplasm, which appears moderately degenerate, shows both mucin droplet (md) and fibril (f) formation. The mucinified cell is attached to an underlying more squamoid cell, having prominent fibril bundles (f), by large numbers of desmosomes (d). This latter cell has fewer microvilli. Intercellular space (IS); Golgi vesicles (g). X11,600.

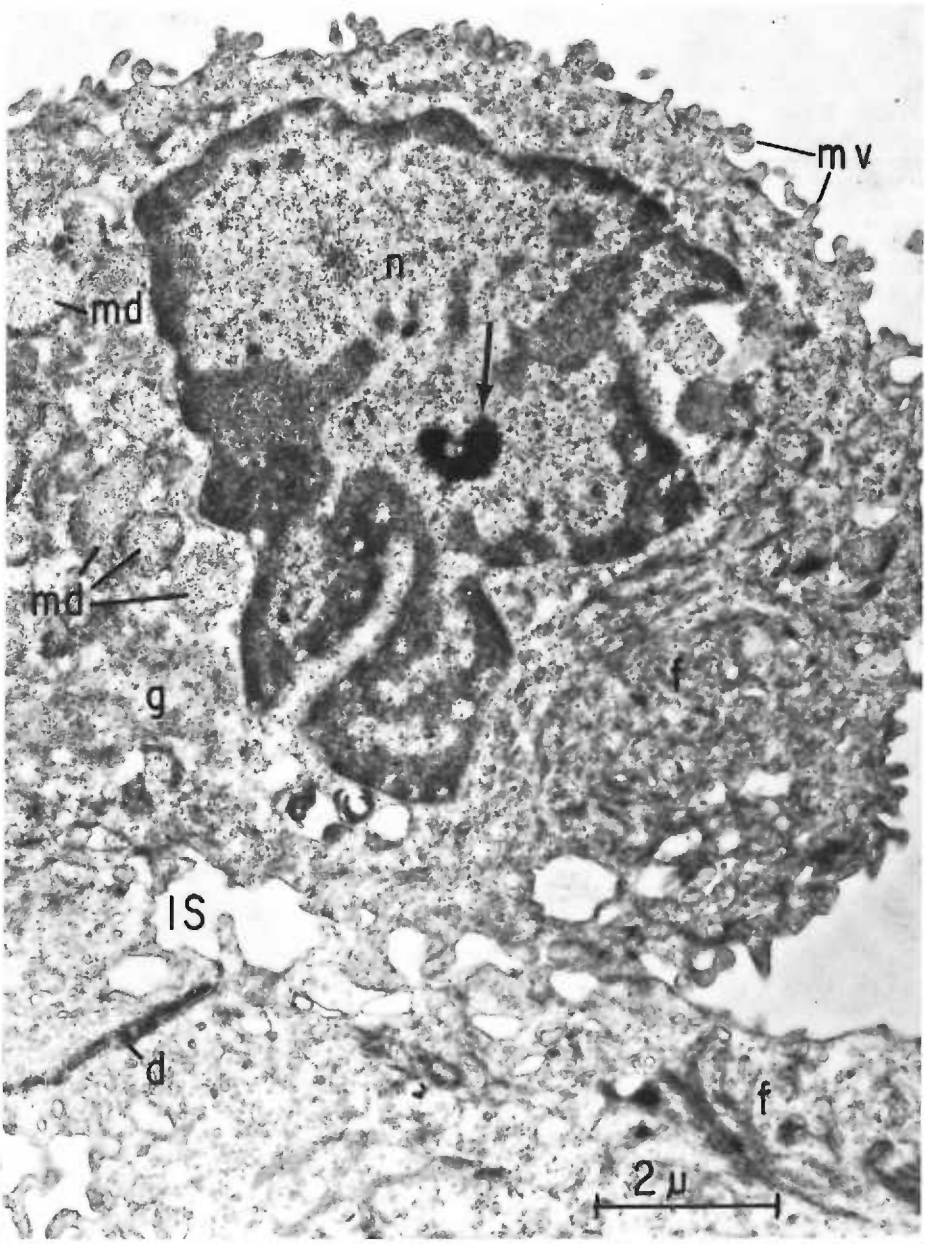

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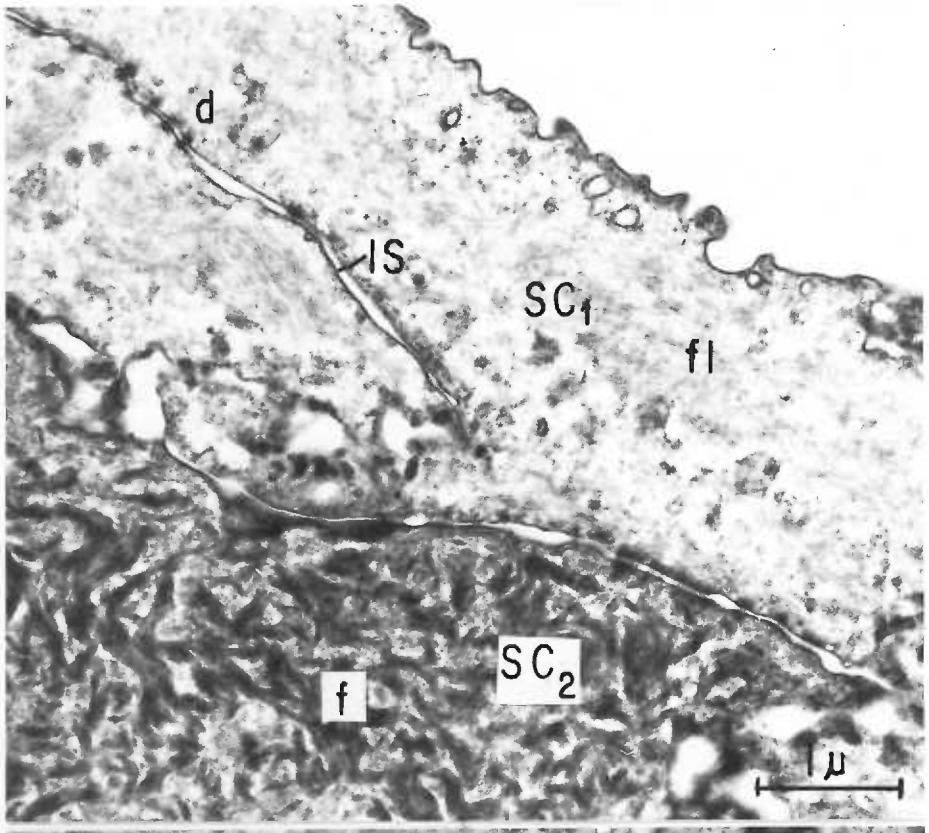
Figure 36: Day 3 Experimental

Superficial cell layer. In this micrograph, squamous cells of the stratum corneum are present at the surface. There are large numbers of desmosomes (d) at the cellular interfaces. In cell SC_2 the cytoplasm is dense and is composed predominantly of irregularly disposed, but distinct fibril bundles (f); while in the uppermost cell (SC_1), individual 70 Å filaments (fl) are loosely distributed in a clear matrix. Intercellular space (IS). X22,800.

Figure 37: Day 4 Experimental

Basal cell layer. Cell membrane-basement membrane (BM) definition and relationships are indistinct, a feature which characterized both the third and fourth experimental days. Desmosomes (d), cytoplasmic fibrils (f), and free ribosomes (r) are present in numbers approaching those found in the stratum spinosum. Collagen fibers of stroma (cf). X22,800.



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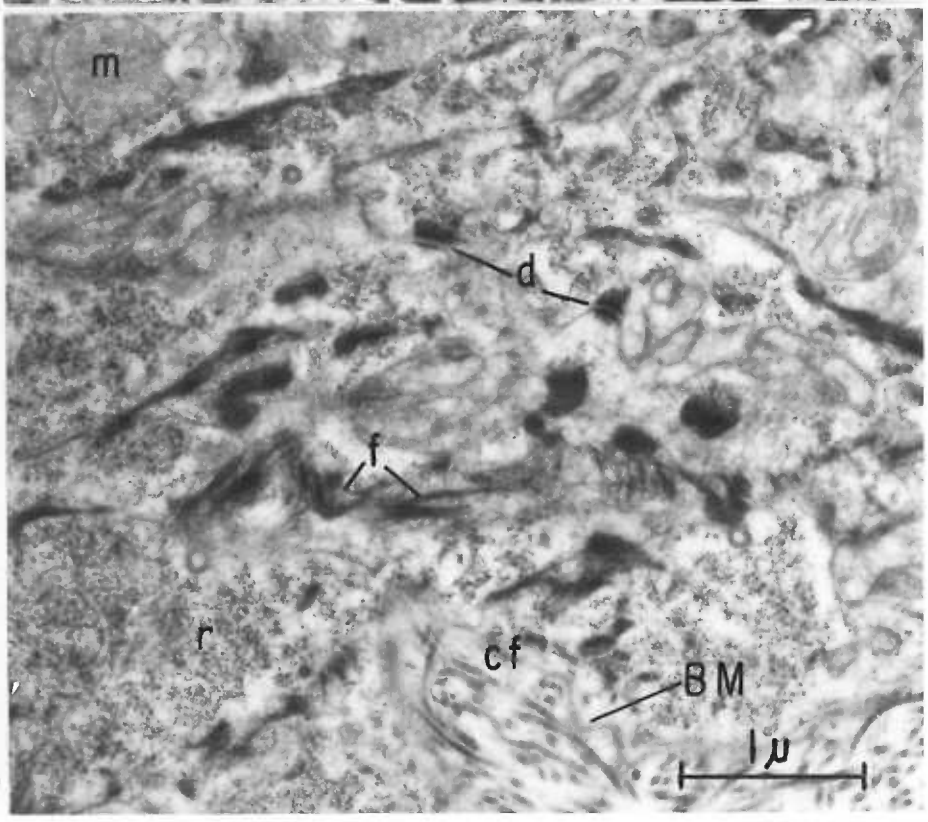

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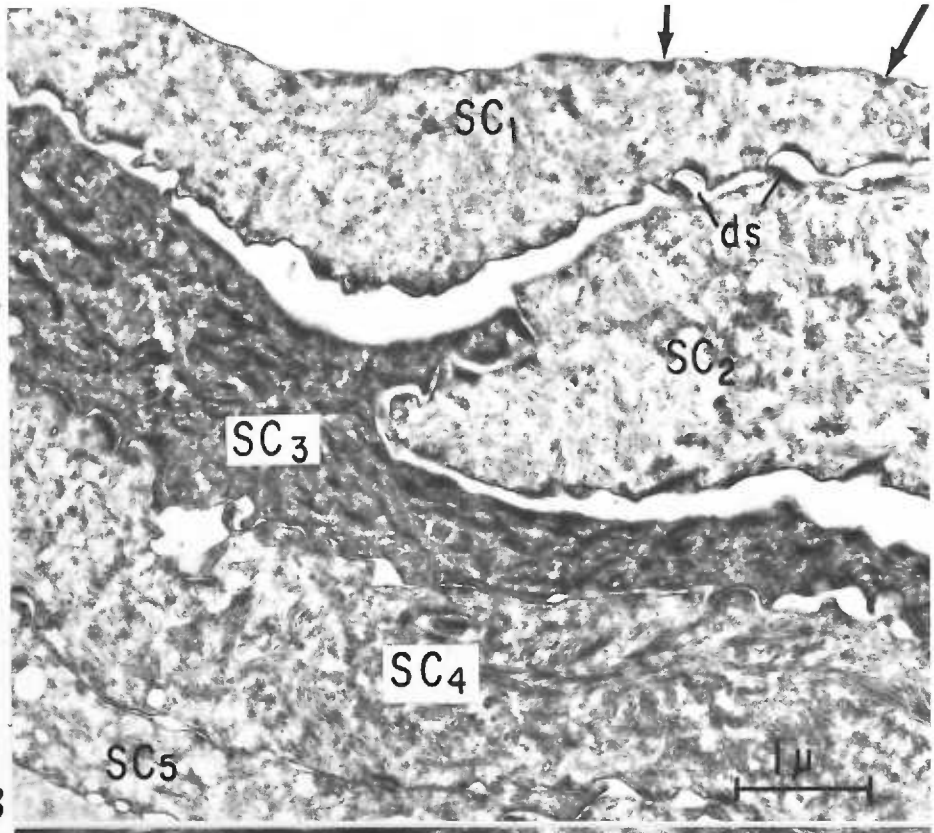
Figure 38: Day 4 Experimental

Stratum corneum. Approximately five layers of cells are present (SC_1-SC_5). Note the decrease in cytoplasmic matrix and increase in fibril density from SC_5 to SC_3 which does not persist in the upper two cell layers (SC_1 and SC_2). Exfoliation of surface cells is associated with separation of points of desmosomal contact (ds). Note the residua of desmosomes (→) on the luminal surface of cell SC_1. X16,400.

Figure 39: Day 4 Experimental

Junction between stratum spinosum (SS) and lower stratum corneum (SC). Note the absence of keratohyaline granules. The lowermost cell of the stratum corneum (SC_2) has developed, in contrast to the spinal cell, an approximately 250 Å peripheral dense cytoplasmic shell (dm) which lines the inner cell membrane leaflet. This shell of dense material has resulted in modification of standard desmosomal structure between the cornified cells (md). Note the increased density of fibrils (f) in the stratum corneum (SC) as compared to the stratum spinosum (SS), but the failure of the complete fibril aggregation as seen in epidermis (7, 64) and in mouse vagina *in vivo* (11). X22,800.



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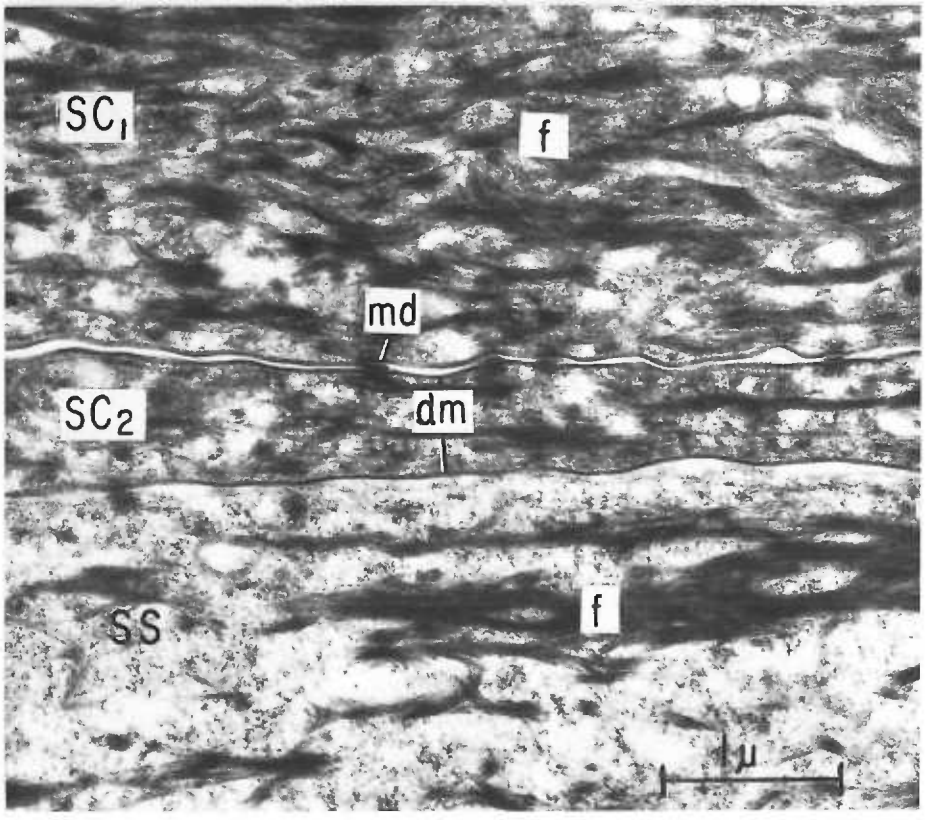

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Figure 40: Day 4 Experimental

Upper stratum corneum. The inner leaflets of the cell membranes are lined by the dense cytoplasmic shell (dm) which is first seen in the lowermost cells of the stratum corneum. Note the lack of complete fibril aggregation (f) and the persistence of identifiable individual filaments (fl). The uppermost cell demonstrates the abrupt increase in clear cytoplasmic matrix which typifies the uppermost cell layers and which is associated with desmosomal separation (ds) and widening of the intercellular space (IS). Modified desmosomes (md). X22,800.

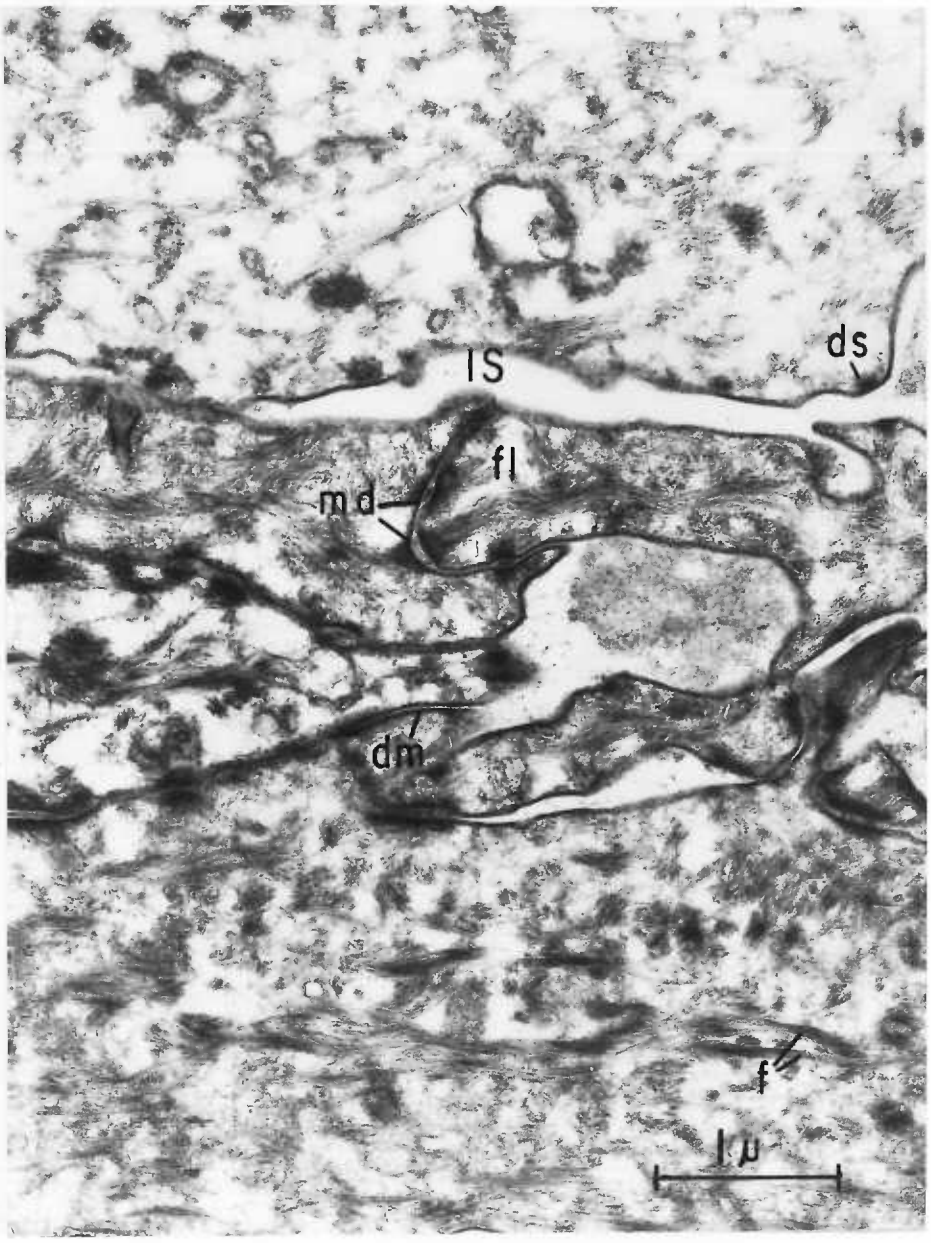

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Figure 41: Day 4 Experimental

Stratum corneum. Note the persistence of individual 70 Å filaments (fl), which have a beaded appearance, and of scattered ribosomal aggregates (r). X46,800.

Figure 42: Day 4 Experimental

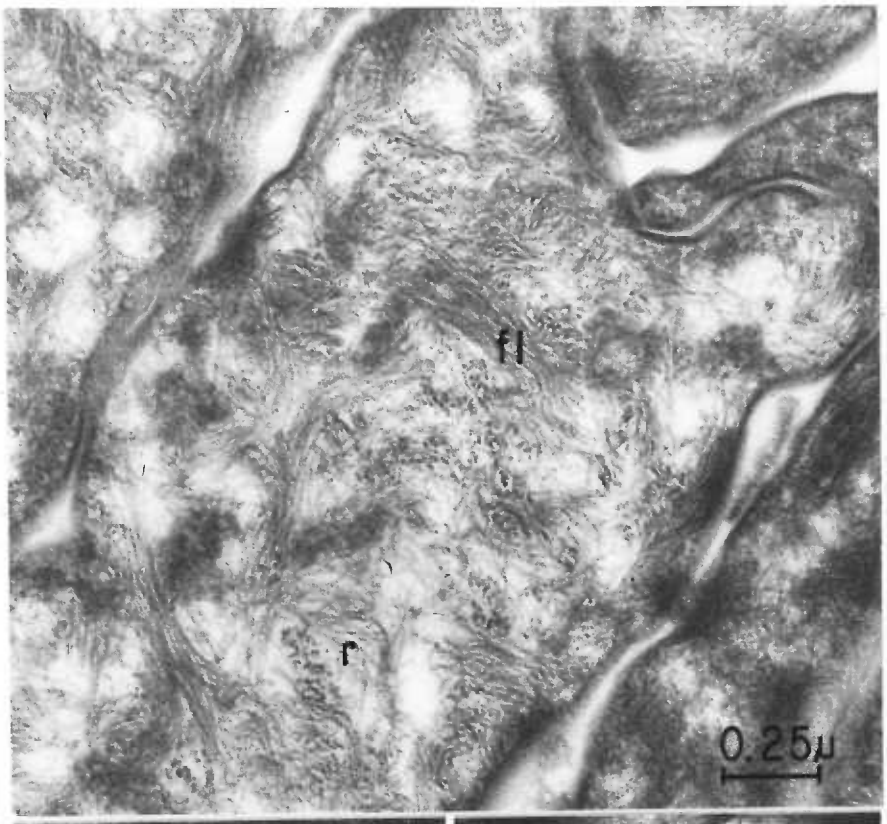
Composite desmosome at junction between stratum spinosum and stratum corneum. In the stratum spinosum (SS), the desmosomal plaque (p) ends at the lateral desmosomal limits (→); while in the cell of the stratum corneum (SC), the plaque (p) is continuous with the shell of dense material (dm) which lines the inner leaflet of the cell membrane. X46,800.

Figure 43: Day 4 Experimental

Modified desmosome. These occur between cells of the stratum corneum. Both desmosomal plaques (p) are continuous with a dense cytoplasmic shell (dm), and the desmosomal plate (→) is more homogeneous than in standard and composite desmosomes (compare with Figures 27 and 42). X46,800

Figure 44: Day 4 Experimental

Junction between three cells (SC_1-SC_3) of the upper stratum corneum. Note the loss of definition of the desmosomal plates (→). These changes resemble desmosomal changes induced in hamster vaginal epithelium with EDTA by Roig de Vargas-Linares and Burgos (62). X46,800.



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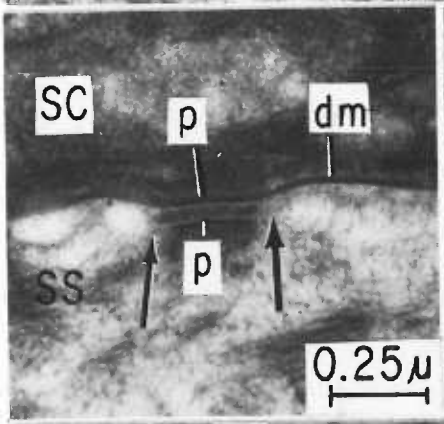

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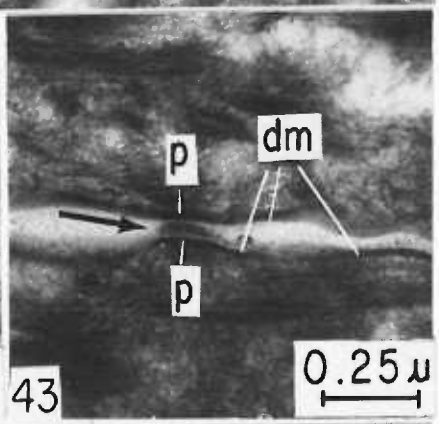

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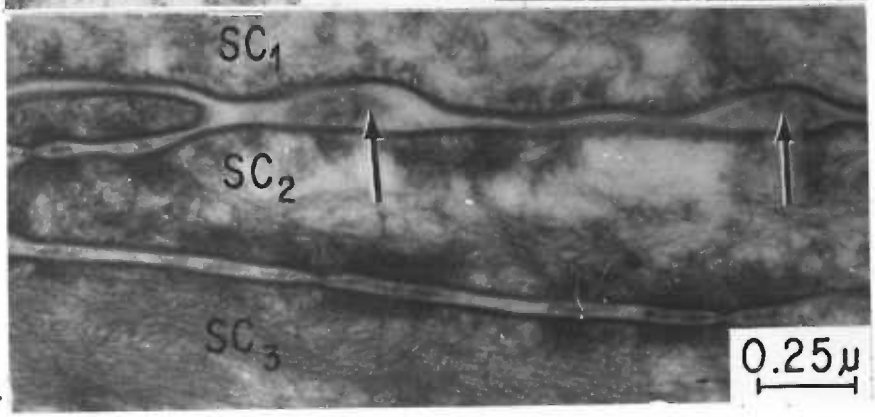

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