SOME ULTRASTRUCTURAL EFFECTS OF INSULIN, HYDROCORTISONE, AND PROLACTIN ON MAMMARY GLAND EXPLANTS

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ABSTRACT

The effects of insulin, hydrocortisone, and prolactin on the morphology of explants from midpregnant mouse mammary glands were studied. Insulin promotes the formation of daughter cells within the alveolar epithelium which are ultrastructurally indistinguishable from the parent cells. The addition of hydrocortisone to the medium containing insulin brings the daughter cells to a new, intermediate level of ultrastructural development by effecting an extensive increase of the rough endoplasmic reticulum (RER) throughout the cytoplasm and an increase in the lateral paranuclear Golgi apparatus. When prolactin is added to the insulin-hydrocortisone medium, the daughter cells complete their ultrastructural differentiation. There is a translocation of the RER, Golgi apparatus, and nucleus and the appearance of secretory protein granules within the cytoplasm. There is excellent correlation between the ultrastructural appearance of the alveoli and their capacity to synthesize casein.

INTRODUCTION

The development and differentiation of the mouse mammary gland, in vitro, depends on the addition of insulin (I), hydrocortisone (F), or some other glucocorticoid, and prolactin (P) to the synthetic culture medium (Elias, 1957, 1959; Rivera and Bern, 1961; Juergens et al., 1965; Stockdale et al., 1966). Some of the effects of insulin and prolactin at various stages of this process have been reported (Lockwood, Voytovich et al., 1967; Voytovich et al., 1969). It was also discovered that during the insulin-induced proliferation of mammary epithelium the daughter cells formed in the presence of hydrocortisone were covertly different from those formed in the absence of hydrocortisone (Lockwood, Stockdale, and Topper, 1967). However, detailed information related to the influence of hydrocortisone on this system has been lacking. The present ultrastructural studies reveal a cytological effect of the glucocorticoid which can be directly correlated with known functional manifestations, and also enhance our insight into the actions of prolactin.

MATERIALS AND METHODS

Animals

11-12-day pregnant C5H/HeN mice, staged according to the schema of Gruneberg (1943), were used in these experiments. The animals were killed by cervical dislocation. The abdominal mammary glands were removed bilaterally under sterile conditions and cut into explants weighing approximately 1 mg. Precise details of the culture methods and hormone solutions used in this laboratory have been
Experimental Design and Preparation of Tissues for Microscopy

The double incubation procedure developed by Lockwood, Stockdale, and Topper (1967) was employed. During the first incubation period, the explants were cultured for 96 hr in either: (1) Medium 199, containing penicillin and insulin (I systems) or (2) in Medium 199, containing penicillin, insulin, and hydrocortisone (IF systems). At the end of the first incubation, the explants from both the I and IF systems were transferred to media containing Medium 199, penicillin, insulin, hydrocortisone, and prolactin (IFP media). Explants from eight animals were fixed at: (1) zero time, the beginning of incubation; (2) 96 hr, the end of the first incubation and (3) 48, 72, and 96 hr after the beginning of the second incubation period during which all explants were cultured in IFP media (see Fig. 1).

Each explant was blotted free of excess medium, cut into four pieces, and immersed in the fixative described by Karnovsky (1965) for 2 hr at room temperature. The material was washed overnight in 0.2 M phosphate buffer, pH 7.6, and subsequently postfixed in 2% osmium tetroxide in phosphate buffer, pH 7.6, dehydrated in a graded series of ethanol and propylene oxide, and infiltrated and embedded in Maraglas (Spurlock et al., 1963). The plastic was allowed to polymerize for 48 hr at 55°C. At least five blocks per time point, from each of eight animals, were sectioned and studied with the electron microscope. Sections with silver-to-gold interference colors were cut on a Porter Blum MT-1 ultramicrotome, mounted on unsupported copper grids, stained with aqueous uranyl acetate and lead citrate (Reynolds, 1963), and viewed in an RCA EMU-3G electron microscope. Pictures were taken at original magnifications of 1800-24,000.

For light microscopy, 1-2-μm-thick plastic sections were cut, mounted on glass slides, and stained with a solution of 1% toluidine blue in 1% boric acid.

Procedure for Casein Assay

The ability of explants from both I and IF systems to incorporate 32P into casein was studied by using the methods developed in this laboratory for casein isolation and identification on starch-urea gels (Turkington et al., 1965). The experiment was repeated on three animals. At 44, 68, and 92 hr after the beginning of the second incubation in nonisotopic IFP media, explants from each system were pulse-labeled for 4 hr with 32Pi (final concentration, 70 μCi/ml) in IFP medium. At the end of the pulse, the explants were weighed and homogenized. A calcium-rennin-precipitable phosphoprotein fraction was isolated from the 100,000 g supernatant and applied to a starch-urea gel for electrophoretic separation. The final gel was radioautographed for 2 wk.

Published elsewhere (Juergens et al. 1965; Stockdale et al. 1966) and will not be repeated here; however, it is important to point out that each experiment was conducted with tissue from a single mouse.
RESULTS

Morphological Observations

Uncultured Tissue (Zero Time Explants from the Midpregnant Mouse Mammary Gland)

At zero time the explants were composed primarily of large, unilocular fat cells, connective tissue cells, and blood vessels. Although moderate numbers of secondary and tertiary ducts were present within the explants, relatively few true alveoli could be found. At the light microscope level, the cross-sections of the alveoli were small, being composed of approximately four to ten cuboidal-to-columnar epithelial cells. The lumina of the alveoli were small and usually were devoid of any recognizable inclusions; however, some lumina contained lipid droplets and dense granules presumed to be secretory protein. Most alveolar cells had large nuclei containing small nucleoli. Their cytoplasm often contained one or more large lipid droplets and occasionally a protein granule in the apical cytoplasm. A few mitotic figures were present, but, in general, the majority of the alveolar epithelial cells exhibited little cytological evidence of secretory or proliferative activity. These findings are in agreement with previous observations made in this laboratory (Stockdale et al., 1966).

When viewed in the electron microscope, the alveolar epithelial cells showed much greater variability in structure than was apparent at the light microscope level. This disparity made it impossible to describe a “typical” or prototype epithelial cell. Instead, it must be kept in mind that the alveolar epithelium at zero time was not uniform in appearance, an indication that this cell population was not synchronized in vivo. In general, the epithelial cells within a given alveolus had similar morphology, but cells in adjacent alveoli often appeared to be in different states of development.

Certain structural characteristics were common to all alveolar cells. These included surface features such as the presence of: (1) numerous, short, apical microvilli (see Fig. 2); (2) occasional lateral plications which interdigitated with similar structures from adjacent alveoli; and (3) a smooth basal plasmalemma overlying a well defined basal lamina. Desmosomes were rarely encountered except near the apex of the cells. The intercellular space was constant (ca. 200 Å) despite the occasional irregularities in the lateral surfaces of the epithelial cells. Frequently, there were small structures which resembled pinocytotic vesicles on both the lateral and basal membranes of the cells. Smooth-surfaced and coated vesicles were found throughout the cytoplasm. Multi-vesicular bodies and structures presumed to be lysosomes (i.e., membrane-bounded structures containing myelin figures or cellular debris) were seen in the cytoplasm of many cells.

The cytoplasm of the alveolar epithelium exhibited pronounced variability in its ultrastructural organization and complement of organelles. The majority of the alveoli were composed of cells which had little structural specialization, a few alveoli contained cells which displayed advanced secretory activity, while still other alveoli had structural features intermediate between these two extremes.

The least specialized cells had a few rod-shaped mitochondria with transverse cristae and no mitochondrial granules, a few profiles of rough endoplasmic reticulum (RER), and numerous free ribosomes scattered throughout their cytoplasm (see Fig. 2). A rudimentary paranuclear Golgi apparatus was present in the lateral cytoplasm. Lipid droplets were found occasionally in cells of this type, but protein granules were never encountered. The nucleus was centrally located, occupied approximately one-third of the cytoplasm, and had a well defined fibrous lamina and a peripheral rim of heterochromatin. The nucleoli varied in size.

In contrast, the small percentage of epithelial cells exhibiting the most pronounced structural specialization at zero time were characterized by a fairly discrete polarity of organelles and the presence of secretory products. These cells possessed moderate-to-large amounts of RER, the bulk of which was located at the base of the cell in the form of nondilated, parallel cisternae, as well as many free ribosomes throughout their cytoplasm. The Golgi apparatus was well developed, located in the apical cytoplasm, and had numerous vacuoles containing protein granules in what appeared to be various states of maturation, i.e., various states of electron opacity (Bargmann and Knoop, 1959; Hollman, 1959, 1966; Sekhri et al., 1967; Verley and Hollman, 1966; Wellings et al., 1960-1966). Vacuoles presumed to be condensing vacuoles were seen
FIGURE 9 This low-power electron micrograph shows portions of several alveolar epithelial cells at zero time. Most of the alveoli in the midpregnant C3H/HeN mouse are composed of cells like the ones shown here. The epithelium shows little structural specialization and is devoid of secretory protein granules. The microvilli (mv) are short and often irregular. There are many free ribosomes (r), a few short profiles of rough endoplasmic reticulum (rer), and several small mitochondria (m) throughout the cytoplasm. The Golgi apparatus (g), usually situated in the lateral cytoplasm close to the nucleus (n), is small and appears empty. The nucleus is centrally located and has little heterochromatin. × 33,000.

in the Golgi region. Sometimes there were three or more granules within a single vacuole. Large protein granules were present in the apical cytoplasm, while lipid droplets could be found throughout the cytoplasm. The mitochondria were more abundant than in the less well developed cells, although in shape and internal organization they were identical to mitochondria of the less specialized cells. The nucleus usually was present in the basal cytoplasm and the nucleoli were very large. Sloughed cells were frequently present in the lumens of these alveoli.

**Cultured Tissue**

**IF CELLS AT THE END OF THE FIRST INCUBATION PERIOD (IF96):** Explants cultured with insulin and hydrocortisone for 96 hr contained more epithelium and less adipose tissue than the zero time samples. There was a striking increase in the number of alveoli within the explants as well as an increase in the average number of cells per alveolar cross-section. By counting the number of cells per alveolar cross-section in 100 zero time alveoli and 100 alveoli of the IF96 samples, it was found that at zero time there was an average of eight cells per alveolar cross-section, whereas at the end of the first incubation period in IF medium there was an average of 16 cells per alveolar cross-section. In addition, there were more mitotic figures in both the alveolar and ductal epithelium than at zero time. The lumina of the alveoli were wider than at zero time and usually contained no secretory material. A very few cells contained granules presumed to be secretory protein; and many cells had one or more lipid droplets in their cytoplasm. The epithelial cells within the alveoli were approximately the same size as at zero time, and frequently they were
Figure 3 In this electron micrograph, the basal portions of several alveolar cells from explants cultured 96 hr in "Medium 199" containing insulin and hydrocortisone are shown. Compared to RER at zero time, the RER (rer) has become highly developed throughout the cytoplasm. Small and medium-sized lipid droplets (L) are present throughout the cytoplasm, but secretory protein granules are absent. Cells with these characteristics do not synthesize detectable amounts of casein. Lateral plications (arrows), presumably surface specializations for cell-to-cell attachment, can be seen clearly. × 28,000.
pseudostratified. The nuclei of these cells were centrally located and comparable in size to those of the zero time cells, although the nucleoli usually were larger. These histological findings are in agreement with those of other investigators (Stockdale et al., 1966; Mayne et al., 1968; Wellings et al., 1966).

At the ultrastructural level, the alveolar epithelium had more RER than at zero time (see Fig. 3 and compare with Fig. 2). The RER filled most of the cytoplasm except for the microvillous border and the narrow rim of cytoplasm surrounding the periphery of the cells beneath the plasmalemmas. In most cells, the RER was not dilated and appeared to be randomly distributed; however, it sometimes formed parallel cisternae and whorls. Cells with dilated RER occasionally contained fine flocculent material within the cisternae of the RER (see Figs. 3 and 4).

The mitochondria were structurally similar to those seen at zero time. Strands of RER often were closely applied to the mitochondria, but these organelles, like the RER, showed no preference for any portion of the cytoplasm (see Fig. 3).

The Golgi apparatus was more extensively developed than at zero time; however, its vesicles, vacuoles, and cisternae usually appeared empty. In some cells, the Golgi apparatus was supranuclear, but in most cases it was lateral to the nucleus.

Small lipid droplets were occasionally encountered (see Fig. 3), but protein granules were rarely seen. Moreover, fewer multivesicular bodies and structures presumed to be lysosomes were seen in these cells than at zero time.

The nucleus was situated in the middle of the cell. It contained little heterochromatin and one or two fairly large nucleoli (Fig. 3).

Although there were a few cells at the end of the first incubation which resembled the least developed cells seen at zero time, a pronounced change in the ultrastructure of most of the alveolar epithelium occurred during the 96 hr in IF medium. At the end of this period, the majority of
the IF epithelial cells showed an extensive development of RER throughout their cytoplasm, and had a larger Golgi apparatus than the least specialized cells at zero time. However, they usually were devoid of secretory granules.

IF CELLS DURING THE SECOND INCUBATION PERIOD: 48, 72, and 96 hr after the beginning of the second incubation period in IFP medium, explants were removed from the cultures, fixed, and prepared for microscopy. The tissue underwent remarkable changes in its histology during this period. The explants contained significantly less adipose tissue and larger alveoli than at the end of the first incubation in IF medium. There was an average of 18 cells per alveolus, compared to 16 cells at the end of the first incubation. The lumina of the alveoli were greatly enlarged and filled with lipid and protein granules. The alveolar epithelium was no longer pseudostratified. Instead, it formed a simple, regular layer around each alveolus. The epithelial cells were larger than at zero time or the end of the first incubation period. Their nuclei and nucleoli were also considerably larger, and usually the nucleus was located in the basal cytoplasm. There were many lipid droplets throughout the cytoplasm, and protein granules of various sizes were present in the apical cytoplasm of most cells.

All of these changes were obvious in the majority of cells and alveoli 48 hr after the initiation of the second incubation. However, examination of the 72- and 96-hr explants revealed that, with longer exposure to IFP medium, progressively more cells and alveoli assumed the above-mentioned characteristics.

The fine structure of the alveolar epithelium also was considerably different from that of the zero time and IF96 samples (see Fig. 5 and compare with Figs. 3 and 2). The cells now closely resembled those seen by others in mammary glands from late pregnant rats and mice (Bargman and Knoop, 1959; Hollman, 1959; Wellings et al., 1960–1966). Although the lateral and basal plasmalemmas were essentially unchanged, the apical microvilli were now longer, more regular, and more abundant than before. The nuclei and nucleoli were also larger. The nucleolar growth was associated with an increase in size of both the pars amorpha and the nucleolonema.

The changes in the cytoplasm were even more dramatic, the most important being the appearance of secretory products and a shift in polarity of the organelles. Now, the bulk of the RER was located in the basal half of the cytoplasm. In some cells, the RER formed nondilated, parallel cisternae, while in other cells it was dilated and filled with fine flocculent material of low electron opacity. The Golgi apparatus was supranuclear and much larger than it was in the earlier samples. Many Golgi vacuoles contained granules of moderate-to-high electron opacity which were presumed to be secretory proteins, while the apical cytoplasm was filled with membrane-bounded protein granules. Lipid droplets of various sizes could be found at almost any level of the cytoplasm, and they were not consistently associated with any organelle. Free ribosomes and short, rod-shaped mitochondria were scattered throughout the cytoplasm. Occasionally, structures presumed to be lysosomes and small multivesicular bodies were found in the apical cytoplasm.

I CELLS AT THE END OF THE FIRST INCUBATION PERIOD (196): When viewed with the light microscope, the I96 explants were comparable in appearance and in the average number of cells per alveolus to the IF96 explants, with the following exceptions: (1) the lumina tended to be smaller, and (2) usually there were no lipid droplets within the cells.

Examination of these explants with the electron microscope revealed that although the cells in all alveoli were remarkably similar in appearance they were significantly different from the IF96 cells (see Fig. 6, compare with Fig. 3). The lateral and basal surfaces of the cells remained essentially the same as at zero time; however, the microvilli were less numerous than at zero time or in the IF96 cells. The I cells exhibited little structural specialization and in most respects were like the least well developed cells seen at zero time (compare Figs. 6 and 2). They had numerous free ribosomes throughout their cytoplasm and only a few isolated profiles of RER. They possessed a few small mitochondria and a rudimentary Golgi apparatus in the lateral cytoplasm. No lipid droplets were encountered. Structures presumed to be lysosomes were frequently found.

In short, the most striking features of the alveolar epithelial cells at the end of the first incubation period in I medium were: (1) their structural similarity; (2) their relatively sparse covering of
Figure 5 Electron micrograph showing a tangential section through an alveolus of an IF explant, 48 hr after the addition of prolactin to the medium. The epithelium now is distinctly polarized and contains visible secretory products. The bulk of the RER is located in the basal half of the cytoplasm and is frequently dilated (z). The Golgi apparatus (g) is large. It has become supranuclear and contains membrane-bounded protein granules (arrows). Protein granules (p) are also present at the apex of the cells beneath the microvilli. Cells with these structural features can synthesize all of the major casein bands. × 19,000.
FIGURE 6 The alveolar epithelial cells shown are from an explant cultured 96 hr in "Medium 199" and insulin. During this time, the epithelium in the I explants underwent as much cell proliferation as that within the IF explants; however, the daughter cells formed in the presence of insulin are indistinguishable from the majority of alveolar epithelial cells present at zero time (cf. with Fig. 2). There are many free ribosomes throughout the cytoplasm, but very little RER (arrow). The Golgi apparatus (g) is usually in the lateral cytoplasm and appears empty. Cells with these ultrastructural characteristics do not synthesize secretory proteins, but are as effective as the IF₉₆ cells in synthesizing a nonmilk, nonsecretory protein fraction. × 26,000.
microvilli; (3) their small, lateral Golgi apparatus; (4) their paucity of RER; (5) the lack of polarization of the RER and nucleus; and (6) the absence of secretory products.

I CELLS DURING THE SECOND INCUBATION PERIOD: The epithelium in the I explants after 48 hr of exposure to IFP varied greatly in appearance. Approximately 1/3 of the alveoli seemed unchanged, whereas the remaining alveoli were noticeably different from those in the I96 explants. The altered alveoli were enlarged and were composed of a simple layer of epithelial cells. A few of these alveoli had lipid and protein granules within their cells and distended lumina. The nuclei and nucleoli were larger than at the end of the first incubation. With longer exposure to IFP medium, progressively more cells and alveoli developed these characteristics, until, at 96 hr, the explants were comparable to the IF explants 48 hr after the beginning of the second incubation.

The fine structural changes which occurred during the second incubation were equally impressive. The alveoli within the 48-hr explants were not uniform in appearance; however, on the basis of their ultrastructural characteristics, they could be divided roughly into three groups. The first group, representing about 1/3 of all alveoli studied, was indistinguishable from the alveoli of the I explants at the end of the first incubation. These alveoli were small and their lumina contained no secretion. The epithelium usually was pseudostratified. The cells had a centrally located nucleus, small nucleoli, many free ribosomes, a few short profiles of RER, and few mitochondria. The microvilli were sparse and often irregular. The Golgi apparatus was small and located in the lateral cytoplasm. There were no signs of secretory activity or polarization of organelles.

Approximately 3/4 of the alveoli studied seemed to belong to the second group. These alveoli were enlarged. Their lumina were wider, and their cells were larger than in the I96 samples. The epithelium was simple, not pseudostratified. Usually there was no secretion in the lumina, but small lipid droplets frequently were present in the cytoplasm of these cells. The microvilli were more abundant and regular than before. The nuclei and nucleoli were larger than at the end of the first incubation. The cytoplasm was filled with RER, and the Golgi apparatus, which was usually lateral to the nucleus and occasionally supranuclear, was more extensively developed than before. The cells in these alveoli were very similar to those in the IF explants at the end of the first incubation period (see Fig. 7 and compare with Fig. 3).

The third type of alveoli was less abundant than the other types. These alveoli also were enlarged. Their lumina were filled with secretory products, and the epithelium surrounding the lumina was simple and regular. The cells were larger than after the first incubation. The nuclei and nucleoli also were enlarged and were situated deeper in the basal cytoplasm. The microvilli were very long and regular. The cytoplasm of these cells was structurally polarized. The bulk of the RER, which was often dilated, was present in the basal half of the cytoplasm. The Golgi apparatus was extensively developed and supranuclear. Its vacuoles often contained electron-opaque products presumed to be secretory protein. The apical cytoplasm frequently contained membrane-bound protein droplets. Large fat droplets in the form of blebs surrounded by a thin rim of cytoplasm were often found at the apical surface of the cells where they were being pinched off into the lumina. The alveoli in this group were indistinguishable from the well-developed alveoli in the IF explants 48 hr after the initiation of the second incubation period.

The alveoli within the 72- and 96-hr explants also could be classified according to the above schema; however, there was a pronounced shift in the frequency of certain types of alveoli (see Figs. 8–10). The 72-hr explants contained few alveoli with the characteristics of group one, and a much larger population of well-developed alveoli of group three. At the end of the second incubation, the majority of the alveoli were well developed. It appeared that after 96 hr of culture in IFP medium the I explants were structurally identical to the IF explants incubated 48 hr in IFP medium.

Casein Synthesis

Since the ultrastructural observations showed that explants from both I and IF systems developed identical morphological characteristics during the second incubation in IFP medium, it was of interest to compare their ability to synthesize casein. Previous work from this laboratory (Lockwood et al., 1967) indicated that: (7) explants, cultured in I media during the first incubation...
Figure 7  Electron micrograph showing I cells after 48 hr of exposure to IFP medium. The RER (arrow points to one profile) is now elaborately developed and extends throughout most of the cytoplasm (cf. with Fig. 6). Most of the alveolar epithelium resembles that seen in explants cultured 96 hr in IF medium (cf with Fig. 3). Cells with these ultrastructural characteristics do not synthesize detectable amounts of casein. X 27,000.
The ultrastructural appearance of the alveolar epithelium within the I explants after 72 hr of culture in IFP medium. The microvilli (mv) are longer, more regular, and more abundant than at earlier times. The Golgi apparatus (g) is greatly enlarged and occupies a horseshoe-shaped band of cytoplasm above the nucleus (n). Protein granules (arrow) are present within Golgi vacuoles and occasionally in the apical cytoplasm (p). A small, but detectable, amount of all major casein bands is synthesized by the explants at this time. Note that there is still a moderate amount of RER in the apical cytoplasm. X 21,000.

period were unable to synthesize casein during a second incubation period in IFP media; whereas (2) explants cultured in IF media during the first incubation were able to make casein during the second period in IFP media. In these early experiments, the second incubation period was only
FIGURE 9  The large cell in this micrograph shows the polarization of organelles which occurs in the I cells during exposure to IFP medium. The Golgi apparatus (g) is supranuclear, while the bulk of the RER (r) and the nucleus are in the basal cytoplasm. The basal plasmalemma and its associated basal lamina (b) can be seen. X 13,000.

24 hr. During the present studies, therefore, the question arose as to whether or not the I systems would synthesize casein once the synthetic apparatus, as viewed in the electron microscope, had developed.

The radioautographs showed that the IF explants, during the second incubation period, were able to synthesize each of the four major casein bands (Turkington et al., 1965). These bands were synthesized at each of the time points studied;
Figure 10  Electron micrograph showing apical portion of an alveolar epithelial cell from an I explant after 96 hr of culture in IFP medium. Note presence of protein granules (p) in apical cytoplasm and within the Golgi apparatus (g). × 20,000.
however, there tended to be more of each band synthesized at 72 and 96 hr than at 48 hr.

The I explants responded differently. No radioactive casein bands 2, 3, and 4 were discernible in the 48-hr samples. However, small-to-moderate amounts of these major casein bands were synthesized at 72 hr. By 96 hr, the synthesis of casein bands 2, 3, and 4 was comparable to that of the IF explants at 48 hr.

RESULTS

DISCUSSION

The fine structural observations showed that explants from midpregnant mouse mammary glands underwent many changes in their histology and ultrastructure during organ culture in chemically defined medium 199 with added hormones. The information derived from the micrographs provides new insight into the morphological effects produced by the individual hormones and the structural requirements for casein synthesis. In addition, the ultrastructural changes observed in response to the hormones are in good agreement with previously reported functional changes.

It has been shown that, at zero time, explants from midpregnant mouse mammary gland are able to synthesize detectable amounts of the major casein bands (Lockwood et al., 1966). The casein formed is referred to as base-line casein, and its synthesis gradually and predictably falls off with time in culture in I or IF medium (Turkington et al., 1967). From the electron micrographs, we have concluded: (1) that the so called base-line casein producers are cells within the few well developed alveoli at zero time (i.e. cells with secretory products and discrete polarization); and (2) that the decline in base-line casein production which occurs with prolonged culture in I or IF medium is related to the fact that the internal structure of these cells is not maintained by either hormone system.

At the end of the first incubation, the alveoli within the I systems were remarkably alike and their ultrastructure was indistinguishable from that of the least specialized alveoli at zero time. Previous workers have shown that I is associated with DNA synthesis in the mammary gland (Stockdale et al., 1966; Stockdale and Topper, 1966; Lockwood et al., 1967). Explants from midpregnant mouse mammary glands cultured in I medium increase their rate of total DNA synthesis compared to zero time. The increased rate of DNA synthesis in I medium is unaffected by the addition of F and/or P, and there is no increase when I is omitted from the medium. In addition, 70% of the alveolar epithelium cultured in I or IFP medium becomes labeled during a 48-hr exposure to thymidine-3H (Stockdale et al., 1966). Although it was assumed that the epithelial cells, which synthesized DNA, divided and formed daughter cells, no data were compiled to directly test this theory. In the present study, it was found that there were approximately twice as many cells per alveolus at the end of the first incubation, in either I or IF medium, as there were at zero time. These results indicate that during the first incubation period in the presence of insulin most of the alveolar epithelial cells not only synthesized DNA but also underwent division to form daughter cells. In the I systems, the daughter cells so formed were structurally indistinguishable from the relatively undifferentiated parent cells.

In contrast, the IF cells at the end of the first incubation were significantly different from their zero time precursors. The IF cells had an elaborate RER and a large Golgi apparatus (Mills and Topper, 1969). The structural differences between the I and IF systems at the end of the first incubation are not reflected, however, in their capacity to synthesize nonmilk, nonsecretory proteins. Lockwood et al. (1966) demonstrated that the synthesis of nonmilk proteins proceeds at a maximal rate in I medium and that the addition of F and/or P to the I medium did not affect the rate of synthesis. Hence, we conclude that the production of nonmilk proteins within the mammary epithelium is not dependent upon an elaborate RER or enlarged Golgi apparatus.

The fact that I96 cells with very few membranes and many free ribosomes are maximally effective in the synthesis of nonsecretory proteins fits well with the idea that membranes are not primarily involved in the synthesis of intracellular (non-secretory) proteins (Slautterback and Fawcett, 1959; Hay, 1963). The structural differences between the I96 and IF96 cells are of particular importance, however, when we consider the problem of secretory protein formation. Previous work from this laboratory (Turkington et al., 1967) showed that both I96 and IF96 explants are incapable of casein synthesis. These findings correlate well with the lack of visible secretory proteins in the I and IF cells at the end of the first incubation. They indicate that the organization and degree of structural differentiation seen at
this time are insufficient for the synthesis of secretory protein. Yet, an important difference exists between IF and IFP explants: explants cultured in IF medium for 96 hr can make casein within 12 hr after the addition of P to the medium (Turkington et al., 1967); whereas, IFP explants cannot synthesize detectable amounts of casein after 12 hr of exposure to IFP medium (Lockwood et al., 1967). The reason for this difference was previously unknown. The electron micrographs, however, have given us new insight into the effect of hydrocortisone on the alveolar epithelium. They indicate that F is in some way related to the formation and maintenance of the RER and Golgi apparatus. The presence of these organelles enables the IFP cells to rapidly respond to prolactin in terms of casein synthesis. In contrast, the absence (or minimal development) of these organelles has no deleterious effect on the capacity of the IFP cells to synthesize nonsecretory proteins.

F and other steroids have been implicated in the biosynthesis of another type of cytomembranes, the agranular endoplasmic reticulum, in several other tissues (Jones and Fawcett, 1966; Conney et al., 1965; Rancourt and Litwack, 1968). This, however, the first time that F has been linked to membrane proliferation in the mammary gland. In the future, this phenomenon should be investigated more fully. In this context, it would seem worthwhile to consider the action of F on the nucleoli, for it is possible that the enlargement of the nucleoli affected by F may be related to the action of this hormone on the membranes.

The morphological changes which occurred in the IF explants during the second incubation focus attention on the action of P. 48 hr after the addition of prolactin to the IFP medium, the majority of the alveoli and their lumina were enlarged. The alveoli were lined by a single, regular layer of epithelial cells. There were an obvious polarization of organelles within the cytoplasm, a shift in position of the nucleus, an enlargement of the nuclei and nucleoli, an increase in size of the cells, and the appearance of secretory products within the cells and lumina. At this time, the explants also were able to synthesize significant amounts of all four casein bands. Thus, it appeared that functional specialization and structural specialization coincide. As was mentioned earlier, it has been shown that IF explants require somewhere between 4 and 12 hr of exposure to IFP in order to make detectable amounts of casein (Turkington et al., 1967; Voytovich et al., 1969). In addition, recent work shows that there is a functional synergism between I and P during the second incubation and that P can exert part of its functional effect in the absence of I (Voytovich et al., 1969). In view of these results, it will be worthwhile to investigate: (1) the ultrastructural changes occurring in the IF explants during the first 12 hr of the second incubation and (2) the effect of I and P on the structural changes during the second incubation; i.e., do I and P act synergistically in respect to both structural changes and casein synthesis, and is it necessary for the ultrastructural changes effected by P?

The changes in the I explants during the second incubation reflect the influences of both F and P. The IFP explants during the second incubation underwent dramatic alterations in their structure. The changes paralleled what took place in the IF explants at earlier times. During the first 48 hr, most of the epithelial cells developed an extensive but randomly distributed RER; and, although the previously rudimentary Golgi apparatus underwent no translocation, it, too, became significantly larger. No detectable casein synthetosis occurred at this time. By 72 hr, the Golgi apparatus was supranuclear and contained a few protein granules. Simultaneously, the bulk of the RER and the nucleus moved into the basal cytoplasm. After 96 hr in IFP medium, when most of the alveolar epithelium was polarized, contained secretory products, and was indistinguishable from the IF epithelium after 48 hr of the culture in IFP, the IFP explants were able to synthesize all major casein bands. These results show that there is good correlation between the ultrastructural appearance of the alveolar epithelial cells and their capacity to make casein. From the IF explants during the first incubation, we learned that F can exert its effect, in terms of ultrastructure (i.e., membrane proliferation), in the absence of P. The sequence of events observed in the IFP explants during the second incubation, however, indicate that P does not act on ultrastructure in the absence of F; i.e., although both F and P were present during the second incubation, no visible secretory proteins were synthesized and no polarization occurred until after the membranes were elaborated. Insofar as the IFP explants were at a lower level of development than the IF explants at the beginning of the second incubation in IFP, and
FIGURE 11 Schematic representation of some of the ultrastructural changes within the alveolar epithelial cells of explants from midpregnant mouse mammary glands cultured in “Medium 199” with added hormones. Asterisks indicate the hormone thought to be primarily involved in bringing about certain ultrastructural changes. The following abbreviations have been used: Nu (nucleoli), R (ribosomes), G (Golgi apparatus), M (mitochondria), P (protein granules), RER (rough endoplasmic reticulum).

since both F and P (rather than just P) had to perform their functions, it took the I explants longer than the IF explants to reach the same state of development. In other words, once F has had the opportunity to act on the I explants, their response to the IFP medium appears to be indistinguishable from that of the IF explants. The ability of the I explants to synthesize casein was not detected in earlier experiments because the second incubation periods were not long enough to allow the I explants to respond fully to the IFP medium, to synthesize, and to organize their synthetic apparatus.

The results of the present study have a bearing on the concept that differentiation of the epithelial cells is necessarily coupled to their proliferation. Several lines of evidence have been adduced (Topper, 1968) which strongly suggest this possibility. One line of evidence was derived from the observation that F2 explants did not synthesize casein within 24 hr after the addition of F and P, whereas IF2 explants synthesized casein within a day after the addition of P to the medium (Turkington et al., 1967). Since the epithelium within the I and IF explants underwent an equivalent amount of proliferation, and because only the IF explants differentiated (i.e. synthesized casein), it appeared that F could only act during the period of DNA synthesis and/or mitosis, and that this was one of the reasons for the proliferation requirement. The present results, however, cast doubt on the relation between the coupling of proliferation and differentiation, and the action of F. They suggest that F may be able to act postmitotically, since the I explants responded to the addition of F and P after the peak of proliferation; yet, because some mitosis did occur during the second incubation, we cannot exclude the possibility that F can act only during DNA synthesis and/or mitosis. In any case, the concept of coupling remains attractive even if it turns out to be unrelated to the action of F.

Some of the ultrastructural changes within the mammary gland epithelium after exposure to the hormones are depicted diagrammatically in Fig. 11.

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