FINE STRUCTURE OF DIFFERENTIATING MOUSE PANCREATIC EXOCRINE CELLS IN TRANSFILTER CULTURE

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ABSTRACT

Fine structural observations have been made in the 11-day embryonic mouse of exocrine cells in pancreatic epithelium developing in tissue culture transfilter from salivary gland mesenchyme of the 13-day embryonic mouse. After 2 days in culture, the exocrine cells show increased cytoplasmic density, abundant ribosomes in aggregate or "rosette" form, and expanded profiles of rough-surfaced endoplasmic reticulum. After 3 and 4 days in culture, the cells exhibit continued expansion of the profiles of endoplasmic reticulum, increased amounts of Golgi membranes, and large areas of light density (prozymogen granules). After 5 days in culture, dense zymogen granules are present in the most highly differentiated cells. In addition, at the filter-epithelial surface, at 2 days, small fibers can be discerned which, after 4 days in culture, show obvious periodicity and are thought to be collagen. The significance of these changes, in relation to the mesenchymal effect, to the onset of specific synthesis and to the stabilization of differentiation is discussed.

In recent years a number of studies have been carried out *in vitro* on the epithelio-mesenchymal interaction in embryonic organ rudiments in relation to cellular differentiation (Grobstein, 1953, 1955; Auerbach, 1960). These studies have involved both direct combination of epithelium and mesenchyme and interactions across membrane filters (Grobstein, 1956). The pancreas has been reported to yield to this kind of experimental analysis (Golosow and Grobstein, 1962) and it clearly has special advantages in view of the available detailed characterization from biochemical, cytochemical, and ultrastructural studies. Accordingly, studies of pancreatic epithelium responding transfilter to salivary gland mesenchyme have been extended to include fine structural observations, with the objective of obtaining the earliest clues to the nature and time-course of events which constitute cytodifferentiation. Attention has been given particularly to the first 48 hours of culture, since other evidence suggests that critical stabilizing changes occur in some cells during this period (Grobstein, 1962). The present report deals with these observations.

Pancreatic epithelium, taken from the 11-day embryonic mouse and separated from its mesenchyme by trypsin digestion, fails to differentiate in isolation, but it responds by producing zymogen granules, after 4 to 5 days in culture, if any of several different kinds of mesenchyme are placed on the opposite side of a filter membrane. Differentiation of pancreatic epithelium is thus presented for fine structure studies in reproducible cultures. This epithelium has the advantage of possessing an optically visible criterion of differentiation, the zymogen granules. Further, the fine structure of the normal development of the epithelium *in vivo* has already been extensively studied by others (Munger, 1958 *a*; Ferreira, 1959; Pipan, 1960).

We have found and report here that exocrine

differentiation of pancreatic epithelium is not manifest fine structurally during the 1st day in culture, except possibly in the prominence of aggregated ribosomes. By the end of the 2nd day, there may be an increase in the number of flat and expanded profiles of the endoplasmic reticulum. The number of such cisternae, and the quantity and complexity of the Golgi membranes and of the endoplasmic reticulum in general, increase during the 3rd day. After 3 and 4 days, particle-free, partially membrane-bounded prozymogen areas appear in the cytoplasm. These are followed (4 to 5 days) by the appearance of dense zymogen granules. These changes in cultured cells correspond in sequence and in time of appearance to similar changes in the same cells developing *in vivo*.

MATERIALS AND METHODS

Tissue Culture Procedures

The tissue culture procedures used in these experiments have been described previously (Grobstein, 1956; Golosow and Grobstein, 1962). The tissues used were pancreatic epithelia taken from 11-day mouse embryos and submaxillary salivary gland mesenchyme from 13-day mouse embryos. Age of the embryos was measured as the number of days following discovery of a vaginal plug in mature BALB/C females mated to C3H male mice. The embryos were removed aseptically from the uterus, rinsed in Tyrode's solution, and subsequently dissected in dishes containing horse serum and Tyrode's (1:1). When not under actual manipulation, the tissue was kept in an atmosphere of 5 per cent CO_2 in air. The 11-day embryonic dorsal pancreatic rudiment, which lies as a small outgrowth from the gut wall just behind the pyloric region of the stomach, was dissected by 2 cuts, the first one severing the mesenchyme lying between the pancreatic epithelium and the posterior aspect of the epithelium of the stomach, and the second one severing the rudiment from the gut wall. The epithelial portion of the rudiment was separated from its investing mesenchyme by trypsin-pancreatin digestion as previously described (Golosow and Grobstein, 1962).

At 13 days, the embryonic submaxillary salivary gland lies laterally beneath the base of the tongue. It consists of an epithelial portion and a highly condensed mesenchymal capsule, thickened proximally, beyond the bulging epithelium. The gland was dissected out and a piece of epithelium-free mesenchyme was cut from the distal region. Usually such a piece was used for a single culture although occasionally the mesenchyme provided by 1 rudiment was sufficient for 2 cultures.

Cultures were prepared in specially designed glass dishes[1] similar to small petri dishes but containing a central well-like declivity of sufficient size to contain 0.7 ml of medium (Grobstein, 1956). Culture platforms consisting of a disc of membrane filter[2] (THWP, 0.45 μ pore size), sealed to a Plexiglas ring, in turn glued to 2 glass rod supports, were prepared in advance (Grobstein, 1956). The pancreatic epithelium was cut into 4 pieces. Such pieces of epithelia were placed individually in the cup of filter assemblies. The cup is bounded by the filter at the bottom and by the Plexiglas ring at the sides. The epithelium was firmly stranded on the filter by draining excess fluid through the filter from beneath. Immediately subsequent to this, a clotting medium was added to the cup. After the clot was well formed, the assembly was turned over and a single piece of salivary gland mesenchyme was added to the upper side of the filter. Following this, 0.7 ml of medium was added to the well of the dish, thus wetting from beneath the filter assembly with attached tissues. The membrane filter was supported at the surface of the drop of medium by the glass rods and Plexiglas ring.

The culture medium consisted of Eagle's basal medium, horse serum (10 per cent), chick embryo juice (3 per cent), penicillin, streptomycin, and mycostatin. The clotting mixture consisted of 1 part 20 per cent embryo juice in Tyrode's solution and 2 parts chicken plasma. The cultures were incubated at 37.5°C in an atmosphere of 5 per cent CO_2, nearly saturated with water vapor. The medium was changed daily. Sterile procedures were followed throughout all manipulations.

Duplicate cultures, prepared as outlined above, were fixed at 0 time and at daily intervals up to 5 days in culture in several separate experiments. Some variability in the growth pattern of duplicate cultures frequently was noted (Grobstein, 1962). In these studies it was thought desirable to encourage compact growth and to eliminate the lateral extension of tubular growth. It was found that, by rigidly conforming to the conditions specified above, and by using only plasma that had an exceptionally short clotting time, it was possible to attain reasonably regular differentiation and growth. Any epithelia which failed to grow as an expanding compact mass were not included in samples taken for these observations.

Fixation and Embedding

The fixative used was 1 per cent osmium tetroxide buffered with potassium dichromate at pH 7.5 and stored at 4°C (Dalton, 1955). (Osmium tetroxide

[1] Available from Micro Instruments Specialty Company, Berkeley, Calif.

[2] Available from Millipore Filter Corporation, Bedford, Mass.

buffered in Veronal-acetate, with and without the addition of sucrose and calcium, was found to be less satisfactory for any of the tissues.) The time of fixation was 1 hour at 4°C.

Two schemes of dehydration were followed. In one, the tissues were placed directly from the fixative into graded alcohols, 50 per cent, 70 per cent, 95 per cent, and absolute alcohol, at 4°C, for 10 to 20 minutes each. Final dehydration was subsequently obtained with 3 changes of absolute alcohol at room temperature. The other scheme of dehydration was that of Parsons (1961) in which tissues were placed in ascending percentages of acetone (4°C) up to 90 per cent acetone containing $KMnO_4$ for 10 minutes each. After 3 subsequent changes of absolute acetone the tissues were ready for embedding in Epon 812.

Two types of embedding media were used. In one case, the tissue was infiltrated with 50 per cent alcohol and 50 per cent methacrylate (90 per cent butyl, 10 per cent methyl), followed by 2 changes in pure methacrylate mixture and final embedding in a prepolymerized hardening methacrylate mixture (90 per cent butyl, 10 per cent methyl, 2 per cent Luperco). Capsules were incubated for 18 hours at 38°C. Epon infiltration consisted of soaking the tissue in 50 per cent absolute alcohol (or acetone) and 50 per cent Epon 812 for no less than 2 hours. The tissues then were placed in the final Epon mixture with 2,4,6-tri(dimethylaminomethyl)phenol (DMP-30) added (Luft, 1961) and incubated for 18 hours in the capsules at 36°C. Subsequently, the capsules were placed at 58°C and allowed to harden.

Later changes of solution in the dehydration procedure were made by pipetting the fluid off from the culture. Infiltration procedures softened the glue holding the glass rods to the Plexiglas rings, thus leaving only the ring with filter attached for embedding. The ring could be placed vertically into a gelatin capsule of 00 size. To assure the maintenance of this vertical position, a wedge of hardened plastic was inserted adjacent to the assembly in the capsule.

Sectioning and Microscopy

Ultra-thin sections were cut on a Porter-Blum microtome with glass knives. Sections were mounted on Formvar- or Parlodion-coated grids. Subsequent staining procedures used were either uranyl acetate (Watson, 1958), or lead citrate (Reynolds, 1963). The sections were "sandwiched" by the addition of a thin Parlodion film or an evaporated carbon film. Polystyrene latex particles 260 mμ in diameter were sprayed on the sections to assist in evaluating the microscopy. Electron micrographs were taken on an RCA EMU 3 F with a 30 μ objective aperture.

Sections 0.5 μ thick were routinely cut serial to thin sections. These were mounted on glass slides, flattened by warming on a drop of underlying water, and stained by the Mallory mixture of Azure II and methylene blue (Richardson, 1960). A Zeiss photomicroscope was used for light photomicrography.

OBSERVATIONS

In the observations that follow, attention is concentrated on the development of exocrine tissue. Considerable variation exists in the level of differentiation among the cells of a given culture. In general, the descriptions given apply to the more advanced cells of the stage, but not to cells that are atypically advanced. Comparison is made between cells developing *in vitro* and those *in vivo* only when significant differences are observed. Similarly, although various preparative procedures were used, variations in appearance of structures will be emphasized only when these observations seem significant.

The appearance of the membrane filter varies with the two embedding procedures. In sections from methacrylate blocks, the substance of the filter appears as dense, interconnected, rounded profiles, with the space between them containing variable amounts of nondescript material. This material frequently is more abundant at the interface of filter substance and space. In Epon, after acetone dehydration, the profiles appear to be distorted in shape, and the density of the filter substance is vastly reduced as though considerable material has dissolved away. This difference in the filter, produced by the two embedding procedures, has no correlate in the overlying tissue.

Eleven-day Embryonic Pancreatic Epithelium at Explantation

THE INTACT RUDIMENT: A photomicrograph of a section parallel to the long axis of the 11-day embryonic mouse pancreas, consisting of an epithelial out-pocketing from the gut wall surrounded by condensed mesenchyme, is shown in Fig. 1. Although some variation among embryos is encountered, the epithelial portion of this typical example measures approximately 200 by 300 μ and is seen to consist of a short tubular "stalk" (*S*) and a bulging distal portion with an expanded lumen (*L*). The columnar cells lining the proximal part of the rudiment are arranged roughly in one layer and have basal nuclei. In the distal portions of the rudiment, the one layer arrangement of cells is less distinct in light microscope sections such as that portrayed in Fig. 1. Electron micros-

copy, however, indicates that the luminal cavity has many extensions and ramifications, and that each cell in the distal region in fact probably extends to and borders a lumen. Mitotic figures are frequently seen in "rounded-up" cells on the lumen.

Cells lining the large, expanded, distal lumen exhibit a few short microvilli with some amorphous dense materials at the luminal surface. The lateral boundaries of the cells exhibit terminal bars near the lumen, and the space between the plasma membranes narrows in this region. In the most distal portion of the epithelial rudiment the luminal cavities are highly compressed. Microvilli projecting into these most compressed lumina lie in close juxtaposition.

The junction between the longitudinally arranged mesenchymal cells and the underlying epithelium of the 11-day embryonic pancreas can be distinguished in light microscopy (*J*, Fig. 1) but is more difficult to discern in thin sections (Fig. 2). In terms of fine structure, this tissue junction is an irregular boundary varying from a few to several hundred Angstroms in width and is occupied by material of variable appearance. One consistent feature is a band of dense boundary material lying between the plasma membranes of the adjacent cells (between arrows in Fig. 2). This material corresponds to early basement membrane material seen in other forms (Salpeter and Singer, 1959). Lying within the plasma membrane of the epithelial cells at the mesenchymal junction is a distinct fine cortical material (*C*, Fig. 2), not exhibited by the mesenchymal cells in this area.

The cytoplasm of 11-day mouse embryonic pancreatic epithelial cells (Fig. 2) contains a few short profiles of endoplasmic reticulum with attached particles (ribosomes) which tend to be more abundant near the mitochondria. Dispersed ribosomes, some of which are in clumps (rosettes), smooth-walled, vesicle-like structures (*SV*), and a small Golgi region (not shown) are also present in the cytoplasm. Multivesicular bodies and lipid pools are occasionally seen. The nuclei of these cells are large, irregular in outline, and contain two or more prominent nucleoli and numerous nuclear pores (*P*, Fig. 2).

The only evidence of a differentiated cell type in the 11-day pancreatic epithelium is a few cells which stain densely with Azure II-methylene blue. They are observed in sections as single cells or as small clumps with no apparent consistent placement along the epithelium (arrow, Fig. 3). In fine structure they are found to contain dense

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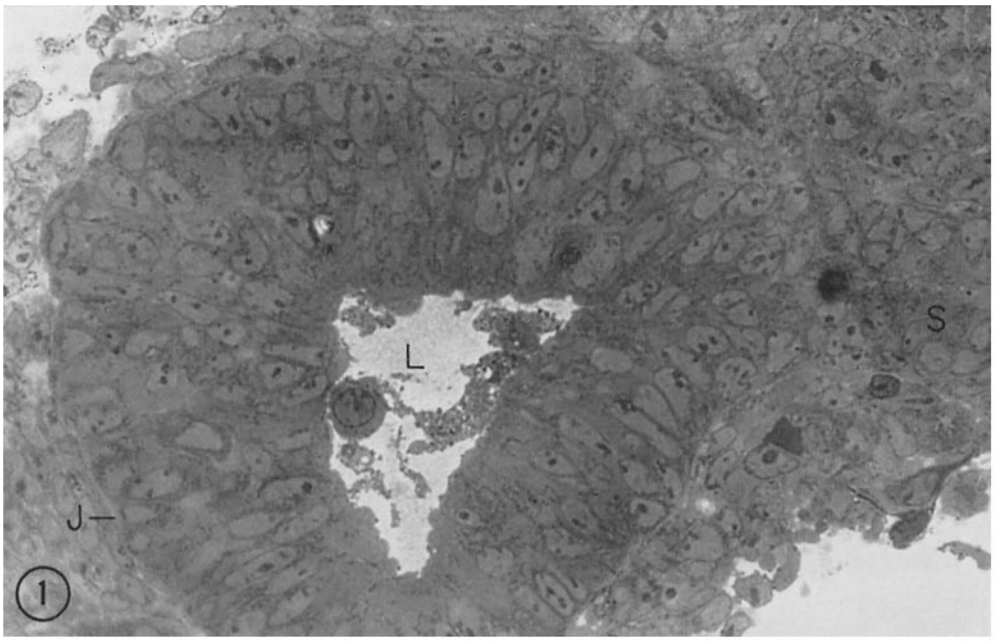
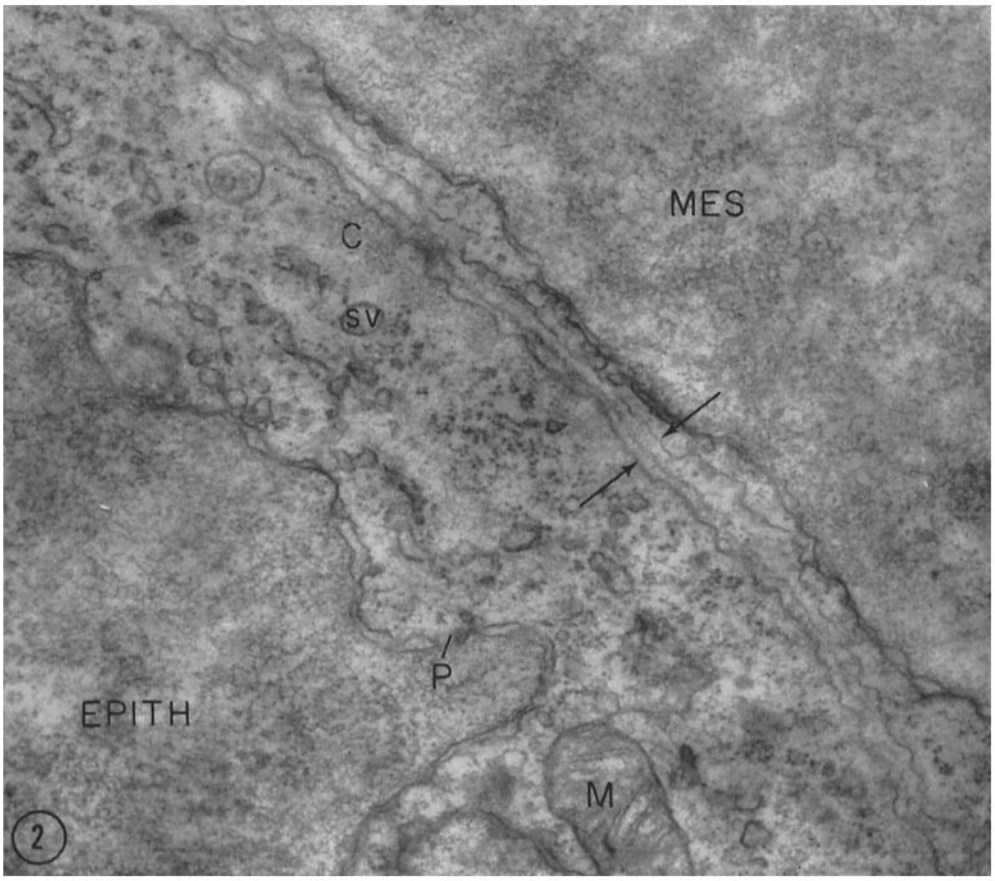
All figures are of tissues fixed in osmium tetroxide buffered with potassium dichromate, dehydrated in acetone-permanganate, and embedded in Epon. All light micrographs are of sections stained in Azure II-methylene blue.

FIGURE 1 A photomicrograph of a longitudinal central section through the pancreatic epithelium of an 11-day mouse embryo. To the right the constricted portion of the epithelium, comprising a "stalk" (*S*), lies close to attachment of the rudiment to the duodenal wall. The bulging distal portion with an expanded lumen (*L*) lies to the left. The junction (*J*) between condensed mesenchyme and epithelium is easily distinguished. At the upper left and lower right corners the mesenchyme appears to have been damaged during dissection and preparative procedures. × 450.

FIGURE 2 An electron micrograph of the junction between an epithelial cell (*EPITH*), bottom left, and a mesenchymal cell (*MES*), upper right. The plasma membranes of the two cells are denoted by the arrows. Lying irregularly, beyond the plasma membrane of the epithelial cell, is an intervening osmiophilic band of dense boundary material which appears in the region where basement membrane material will form. A nuclear pore in the nuclear membrane of the epithelial cell is seen at *P*. The cytoplasm of the epithelial cell contains particles (presumably ribonucleoprotein), some dispersed, some clustered in rosettes, a few bound to membranes. Mitochondria (*M*) are large with dense peripheral membranes and irregular cristae. Beneath the plasma membrane is a cortical area (*C*) free of dense particles but containing finely structured material of slight density. Membranous circular profiles free of particles are not infrequent in the cytoplasm (*SV*). Profiles of membranes with attached particles are few and are of short length. Uranyl acetate stain. × 30,000.





particles measuring 500 A or more in diameter, surrounded by a smooth-walled membrane. The cytoplasm contains an elaborate arrangement of particle-free membranous profiles, a small number of profiles of rough-surfaced endoplasmic reticulum, and elongate dense mitochondria with numerous well formed cristae. This appearance is consistent with what was described by others as early islet cells (Munger, 1958 *b*; Conklin, 1962). Since these cells are few in number in the pancreatic epithelium of the 11-day embryonic mouse, and since they do not increase in abundance in tissue culture, the observations which follow do not include details of changes in these cells.

Trypsin-separated Pancreatic Epithelium at Time of Explantation

Trypsin-pancreatin treatment of the 11-day pancreatic rudiment results in reduced adhesion at the epitheliomesenchymal junction. Subsequent gentle pipetting separates the mesenchyme and leaves the epithelial component with smooth contours, as viewed in the dissecting microscope. When this epithelium is quartered, the cut surface becomes more irregular in outline (Fig. 3). Dense blebs of damaged material may be seen adhering to the cut surface, although much of the intact surface appears to be regular in outline and the majority of the epithelial cells appear undamaged.

Fine structural examination of such one-quarter pieces of epithelium stranded on a membrane filter confirms the above observation. Except for those lying along the cut surface, the epithelial cells appear undamaged by the preparative culture procedures. The outer contours of the explant show small irregularities but are, in general, smooth. The plasma membrane of the peripheral cells (Fig. 8) does not show the dense boundary material, which we described, lying approximately 250 A beyond the plasma membrane in the intact rudiment. Occasional small pieces of seemingly damaged cytoplasm and patches of amorphous material may adhere to the epithelial surface (Fig. 8), but there is no consistent pattern of adhering material and no fibers.

The arrangement of epithelial cells with respect to the large lumen frequently is impossible to trace after the epithelium has been quartered and placed

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Figs. 3–7 represent portions of central cross-sections through 11-day embryonic mouse pancreatic epithelia cultured for varying lengths of time on filter membranes the opposite sides of which have upon them 13-day embryonic mouse salivary gland mesenchyme. In each case the sections are oriented with the filter toward the bottom of the picture and the overlying plasma clot at the top.

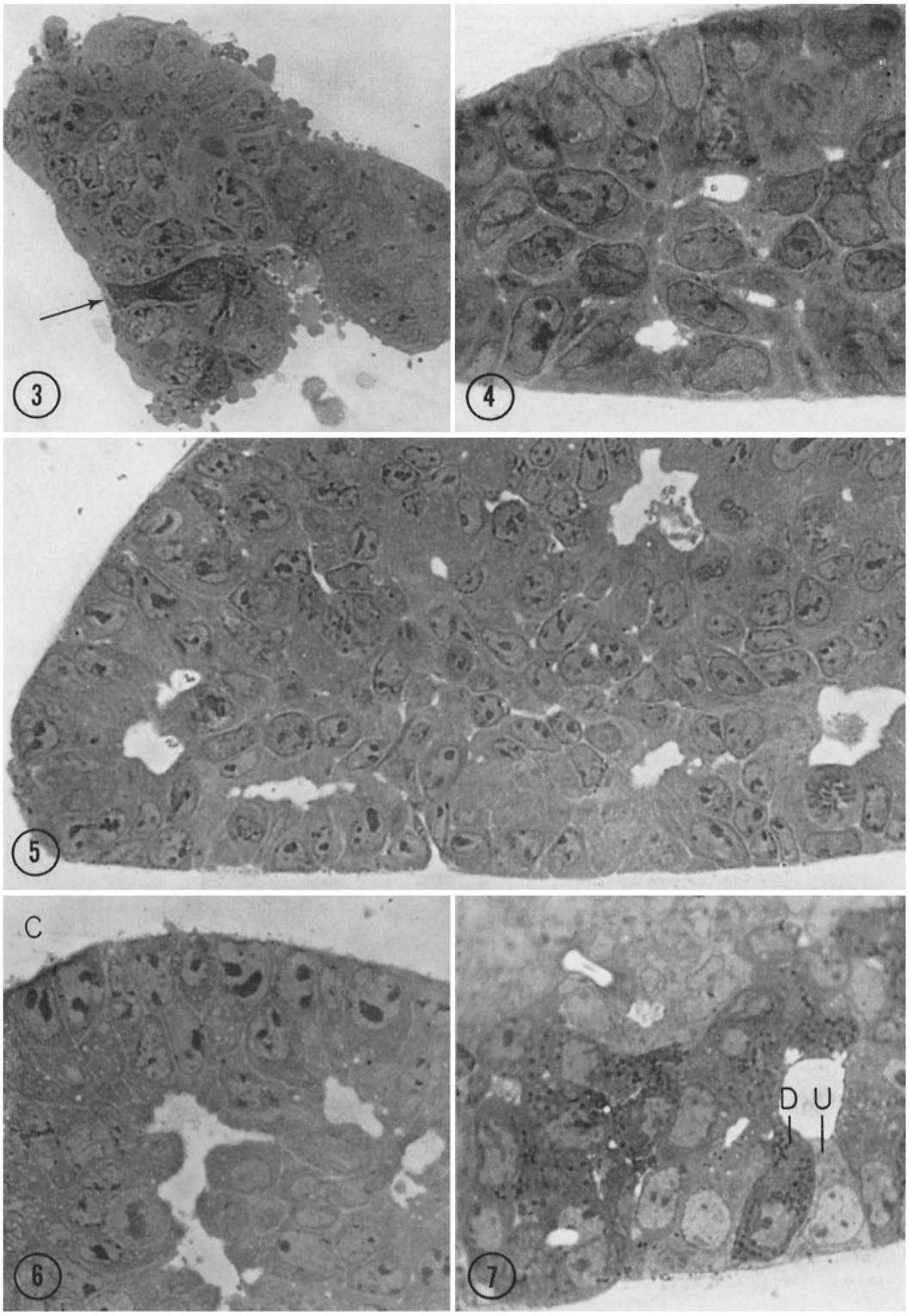
FIGURE 3 represents the appearance of a ¼ piece of pancreatic epithelium after separation from its surrounding mesenchyme by trypsin-pancreatin digestion. The lower right boundary is thought to represent the cut surface which is transverse to a small portion of the lumen evident at the indentation in this surface. The arrow indicates a distinctly different cell type seen in variable numbers in these early rudiments and which, when examined fine-structurally, appears to correspond to an early islet cell (Munger, 1958 *b*). × 500.

FIGURE 4 A 1-day pancreatic epithelial culture showing at least two luminal cavities in cross-section. The nucleocytoplasmic ratio appears high compared to that at later stages (see Figs. 5 to 7). × 1,300.

FIGURE 5 A 2-day pancreatic epithelial culture showing several mitotic figures and cross-sections through several luminal cavities. × 700.

FIGURE 6 A 3-day pancreatic epithelial culture showing cells with light staining vacuoles particularly abundant in the apical cytoplasm. × 1,000.

FIGURE 7 A 5-day pancreatic epithelial culture showing a differentiated cell *D* containing dark-staining zymogen granules. An apparently undifferentiated cell (*U*) is evident on this same acinus, as well as cells containing light bodies, presumably "prozymogen" granules, typical of an earlier stage of differentiation. × 1,000.



on the filter. It is rare for the luminal cavity to be bisected so as to expose the luminal surface of the cells on the periphery of the explant. Rather, compressed luminal cavities filled with microvilli are seen in the explant. The fine structure of the individual epithelial cells is indistinguishable from that of the epithelial cells of the intact rudiment.

Pancreatic Epithelium 1 Day in Culture

After 1 day in culture with salivary gland mesenchyme on the opposite side of the filter membrane (Fig. 4), the pancreatic epithelium is a small, compact, flattened mass somewhat greater in cross-sectional area than at the time of explantation. (In the absence of salivary gland mesenchyme, the epithelium is spread slightly more but is otherwise similar.) Peripherally, the contours are smooth, with very little adhering cytolyzed material, and the clot bounds the mass closely. The cells tightly adhere to the filter surface. Only rarely do short cell processes extend into the filter substance. Mitotic figures are frequent in the rudiment. The only consistent fine structural changes during the first day in culture are a small increase in the number of lipid inclusions, swelling of some mitochondria with an accompanying increase in the distance between cristae, and an expansion in the luminal cavities of the rudiment.

Pancreatic Epithelium 2 Days in Culture

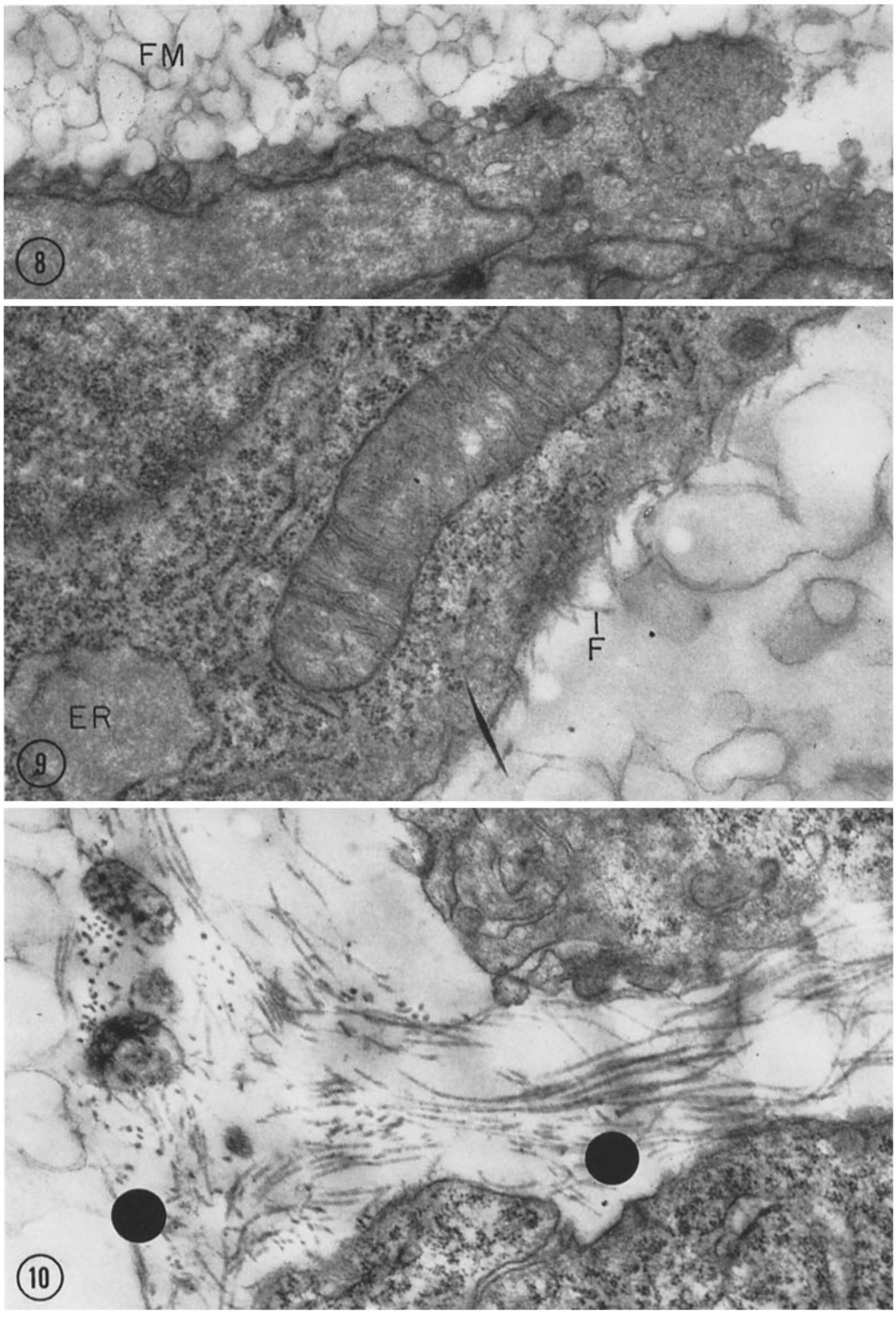
Pancreatic epithelia cultured for 2 days in the presence of salivary gland mesenchyme show a large increase in mass, accompanied by a high rate of mitosis and an increase in number and extent of the luminal cavities. Light micrographs of central sections of 1- and 2-day cultures may be compared in Figs. 4 and 5. The nucleocytoplasmic ratio has decreased in the 2-day cultures, and many of the epithelial cells appear more columnar. Although alterations in cell arrangement are evident, no distinctive cytological changes indicative of differentiation can be discerned with the light microscope. With the electron microscope, however, a significant percentage of cells bordering a lumen show the following features suggesting differentiation. The cytoplasm appears more dense with an increase in the amount of rough-surfaced endoplasmic reticulum, including both elongate and, occasionally, expanded profiles (*ER*, Fig. 9). Dense ribosomal particles are more uniformly arranged in rosettes. Mitochondria are elongate and have regularly arranged cristae. The Golgi region is expanded and some large single vesicles appear, largely on the luminal side of the cytoplasm.

In addition, at the filter interface, in somewhat irregular patches at the epithelial surface, there frequently are fibers (*F*, Fig. 9) which are not present at the time of explantation in culture and which are found infrequently after 1 day in culture. The fibers may be oriented parallel to the basal surface of the epithelial cells, but when present in short lengths they frequently seem to be almost normal to this surface (Fig. 9). The fibers measure

FIGURE 8 Electron micrograph of a section of cultured pancreatic epithelium fixed immediately after being placed on a filter membrane (FM). The epithelial surface is seen to be relatively free of adhering material, although there are some particles and vesicles which probably result from dissection and preparative procedures. The osmiophilic dense boundary material beyond the basal plasma membrane (seen in Fig. 2) has apparently been removed by the preparative procedures (trypsin digestion). Stained with uranyl acetate. × 10,000.

FIGURE 9 Electron micrograph of pancreatic epithelium fixed and sectioned after 48 hours in culture on a filter membrane, shown at the lower right. Fibers (*F*), without manifest periodicity, may be seen at the epithelial surface. An expanded cisterna of the endoplasmic reticulum (*ER*) is indicated, as well as an elongate profile of the endoplasmic reticulum between the nucleus and the mitochondrion. Stained with uranyl acetate. × 30,000.

FIGURE 10 Electron micrograph of the epithelium-filter interface after 4 days in culture. Elongate fibers showing a periodicity of approximately 600 A exist at the filter surface, extending up between the basal surfaces of two epithelial cells. Black spheres represent 260 mμ polystyrene latex particles. Stained with uranyl acetate. × 30,000.



up to 100 A in width and the periodicity is not marked either with phosphotungstic acid stain or with uranyl acetate staining of $KMnO_4$-acetone-dehydrated material embedded in Epon.

Pancreatic epithelium spreads widely on the surface of the membrane filter when cultured for 2 days in the absence of salivary gland mesenchyme. Luminal cavities become highly elongate, but are identifiable by the microvilli and the terminal bar densities of the flattened epithelial cells which border them. The cells do not show the internal changes outlined above as being indicative of differentiation, and no fibers form at the filter surface. Spreading of these cultures continues with further incubation but no fine structural changes of significance occur in them. Hence, further observation of the control cultures of later stages will not be detailed.

Comparison of the 2-day cultures and the pancreatic rudiment of 13-day embryos shows that the pancreatic epithelial cells differentiating *in vivo* are similar, fine-structurally, to their equivalent in culture. Fibers are not present at the basal border of acini of the intact epithelium of the 13-day embryo, but an osmiophilic dense boundary material surrounds the epithelium in this region where the basement membrane will form. The osmiophilic material is more evident than in the intact pancreatic rudiment of the 11-day embryo. No such material is demonstrable basally on the epithelium during the culture period, either at the filter surface or at the clot surface.

Pancreatic Epithelium 3 Days in Culture

Increase in mass continues, in epithelial cultures with transfilter salivary gland mesenchyme, and is accompanied by an expansion in the number and extent of luminal cavities. Peripherally there is a cell population which tends to be organized as a columnar epithelial layer, with the nuclei usually near the clot surface. Light micrographs of cross-sections of these cultures show an increase in the number of luminal cavities marking proacini (Fig. 6). Many of the cells lining these proacini contain small areas of reduced density which are particularly abundant on the apical side of the nucleus. These light-staining areas or vacuoles probably represent expanded Golgi vesicles and possibly other structures of reduced density in electron micrographs. Fig. 11 represents an electron micrograph of a portion of the cytoplasm bordering the lumen (*L*) of a proacinus. Of particular note is a considerable increase in the number of profiles of the endoplasmic reticulum, the expanded form of which is designated *ER* in Fig. 11. In the cytoplasm there are prominent vesicles of low density, which may well be derived from the Golgi membranes, and many particles, some of which are arranged in rosettes. Mitochondria in epithelia cultured for 3 days are elongate and not so swollen as previously. Fibers are present along the epithelial surface at the filter interface. Peripherally in these cultures there appear rounded inclusions, some as large in diameter as nuclei, but of variable content, which may be seen in both light and electron micrographs. These are less prominent at 4 days and may represent pools of cytolysed material.

The appearance of the most advanced acinar cells *in vitro* on the 3rd day does not differ markedly from that of their equivalent in the developing intact pancreas of the 14-day embryo.

Pancreatic Epithelium 4 Days in Culture

The pancreatic epithelial mass continues to enlarge between the 3rd and 4th days in culture. Proacini are increasingly abundant in cross-sections. The peripheral columnar cell layer continues to bound the mass. The cells of this peripheral layer are less dense than many of those lying interiorly. Mitotic figures are most frequent just below the surface layer. Some of these may represent nuclei of the cells of the peripheral layer which have shifted toward the interior of the culture. Those acini whose cells are the furthest differentiated have irregular luminal cavities. The cytoplasm of these cells stains more intensely blue at the luminal surface with Azure II-methylene blue. Deep within the epithelial mass there are a few cells whose nuclei are dense, or whose cytoplasm is highly vacuolated. In the same region occupied by these cells, which may be undergoing degenerative changes, are cells which appear to be the most highly differentiated.

As regards the fine structure of the differentiating acinar cells, it can best be summarized as an intensification of changes already outlined. The quantity and extent of the rough-surfaced endoplasmic reticulum is increased and the Golgi membranes and vacuoles are more abundant. There also appear in the cytoplasm numerous profiles of an organelle only occasionally present in 3-day cultures, undoubtedly the prozymogen granules described by Munger (1958 *a*). These have a smooth membrane, frequently incomplete and ir-

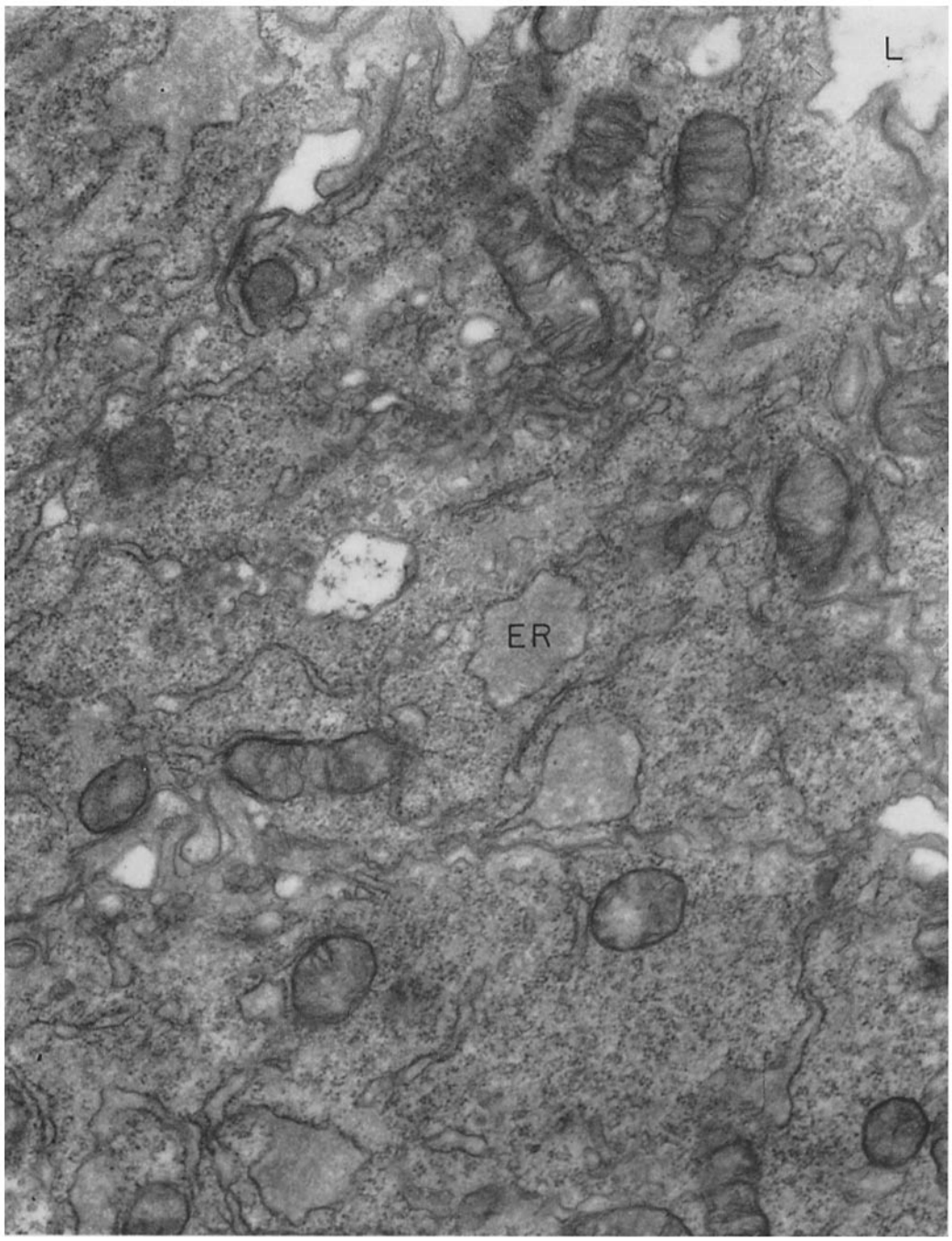


FIGURE 11 An electron micrograph of a section through several pancreatic epithelial cells fixed after 3 days in culture. The lumen of a proacinus (*L*) may be seen at the upper right. Several expanded cisternae of the endoplasmic reticulum are present, one of which is designated (*ER*). The cytoplasmic density and the number of cytoplasmic particles per cell appears greater than when the cultures were established. Lead citrate stain. × 40,000.

regular, surrounding a cytoplasmic area of reduced density which is free of particles but which does contain some indistinct osmiophilic material (*PZ*, Fig. 12). In addition, smaller, more dense structures are frequently seen in the cytoplasm at this time (*Z*, Fig. 12). It is not clear whether these are a small form of mature zymogen granule.

At the filter interface the epithelium shows reduced adherence to the filter, as judged by its separation from the filter surface at a number of points. Arrayed along the epithelial boundary are fibrils which have a periodicity appropriate for collagen (Fig. 10). These fibers are in greater abundance in the folds between adjacent acini, and they extend along such folds deeper into the mass of the epithelium. The fibers undoubtedly represent a further development of the aperiodic ones seen at 48 hours in culture. It will be noted (Fig. 10) that there is no suggestion of the dense boundary material at the basal surface of the epithelial cells.

Pancreatic Epithelium 5 Days in Culture

After 5 days in culture the pancreatic epithelium is a somewhat flattened, multi-layered mass. Light microscope sections reveal considerable diversity of differentiative state in the acinar cells (Fig. 7). Some contain clearly identifiable zymogen granules, others do not. Included in the latter category are masses of cells without distinct acinar arrangement, usually centrally located. Cells bordering a lumen may be highly differentiated (*D*), as judged by zymogen content, or completely undifferentiated (*U*), by this criterion.

With the electron microscope, acinar cells are seen to contain variable numbers of dense zymogen granules (*Z*, Fig. 13). The cells contain a well developed system of rough-surfaced endoplasmic reticulum, loosely stacked basally, and a prominent Golgi apparatus. Collagen-like fibers are present at the filter interface and, in some areas, in spaces within the tissue mass itself.

The appearance of the most highly differentiated cells at 5 days in culture is similar to that of the highly differentiated acinar cells in the intact rudiment of the 16-day embryo. The cell arrangement, however, is different. Among the less well characterized cells in culture are probably those which correspond to duct cells, centro-acinar cells, etc., *in vivo*. These were not studied in detail.

Salivary Gland Mesenchyme Opposite Pancreatic Epithelium

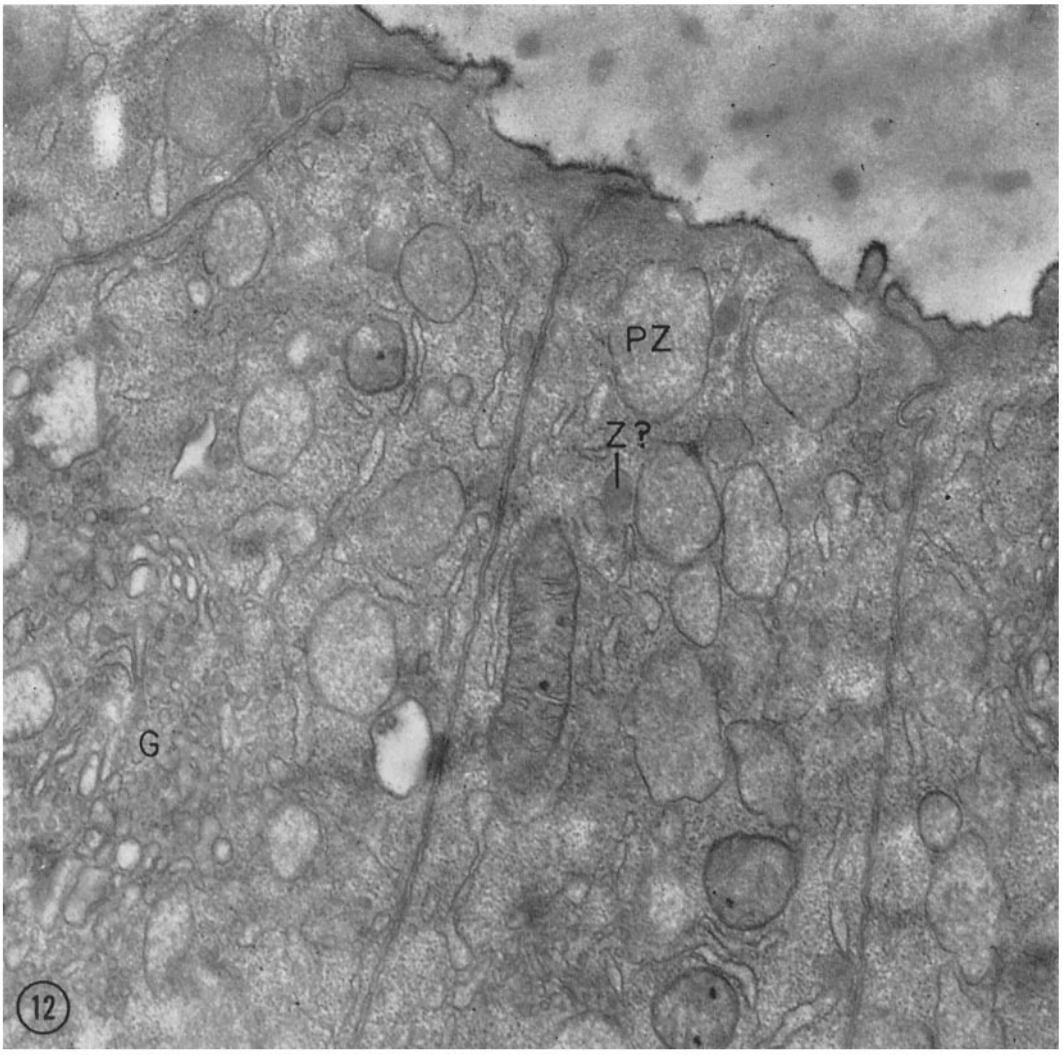
Salivary gland mesenchymal cells do not change importantly in fine structure during 5 days of culture. Initially the mesenchyme spreads rapidly on the filter surface, invading the filter only to a distance of about 1 to 2 microns. Intercellular fibers may be seen at this filter interface in cultures fixed immediately after preparation, but thereafter they are seen only between cells lying within the tissue mass. As the cells spread they send out longer processes laterally. Fibers showing periodicity are present at all times in the mesenchyme. Detailed observations of cytological changes during the culture period were not made.

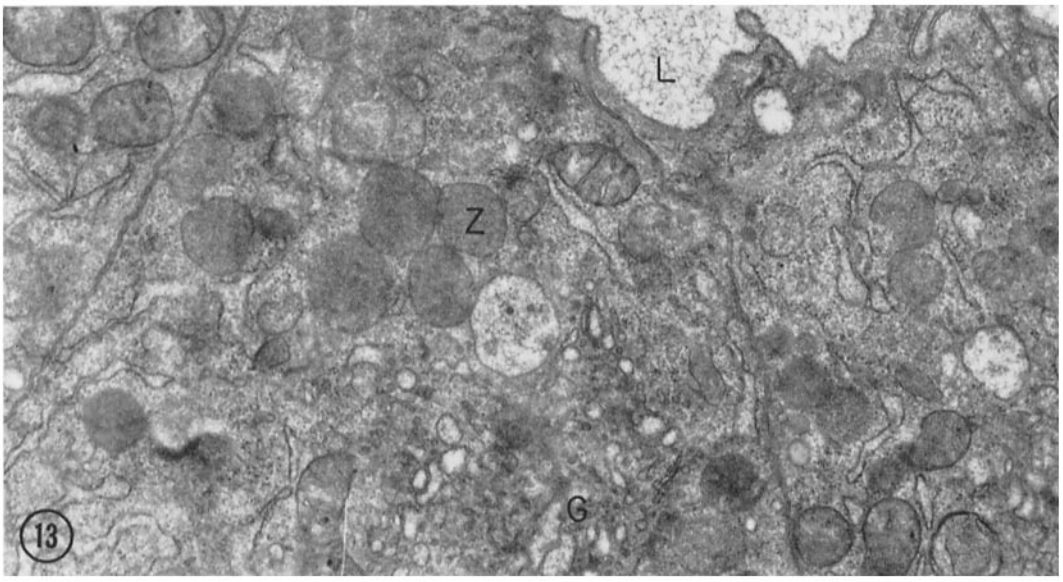
DISCUSSION

The observations described confirm the impression, derived from preliminary light microscope studies, that in their development the exocrine pancreatic epithelial cells *in vitro* proceed through the same sequence of cytological changes that they do *in vivo*. In earlier descriptions of the fine structure of differentiating epithelial cells of the mouse pancreas (Munger, 1958 *a*; Ferreira, 1957; Pipan, 1960), detailed changes were not followed in stages as early as the 11-day embryo. The importance of this early developmental period, from the eleventh

FIGURE 12 An electron micrograph of the luminal surface of epithelial cells cultured for 4 days in the presence of salivary gland mesenchyme. Various profiles of prozymogen granules (*PZ*) are typical for this stage. A dense, fibrous cortical area is seen beneath the luminal surface of the cells. Dense areas, possibly representing mature zymogen, are indicated by *Z?*. Uranyl acetate stain. × 31,000.

FIGURE 13 An electron micrograph of pancreatic epithelial cells organized around an acinar lumen *L*. Mature zymogen granules (*Z*), an increasingly large number of profiles of rough-surfaced endoplasmic reticulum, and an enlarged Golgi region *G* are typical of the stage. × 26,000.





to the thirteenth *in vivo*, or during the first 48 hours in culture, is emphasized by the finding (Grobstein, 1962) that associated mesenchyme is necessary for pancreatic epithelial differentiation during the first 30 to 36 hours in culture but can be removed thereafter without preventing the appearance of zymogen granules by the 5th day. Even as late as 48 hours in culture, however, no zymogen or prozymogen granules can be identified with the electron microscope. Thus, some change in the tissue occurs which stabilizes it on the path of exocrine differentiation prior to any ultrastructural sign of product accumulation in storage granules.

For this reason, ultrastructural changes occurring during the initial 48 hours of culture were scrutinized particularly carefully. The changes consist chiefly of the appearance of fibers at the basal surface of epithelial cells, particularly at the filter interface, an increase of cytoplasmic density, and an increase in the number of ribosomes and in the number and length of the profiles of the rough-surfaced endoplasmic reticulum. Subsequent morphologic changes, *i.e.* further increase in the quantity of endoplasmic reticulum and cisternae, expansion of the Golgi apparatus, and appearance of prozymogen and zymogen granules, presumably reflect heightened synthesis and accumulation of pancreatic enzymes as sequelae following the initiation or stabilization of the capability to produce these products.

Ultrastructural and cytochemical changes associated with enzyme synthesis in the adult are consistent with the finding of the present report that among the earliest developmental signs of exocrine cell differentiation are changes in the endoplasmic reticulum and its associated particles. Evidence from a series of morphologic and cell fractionation studies, as well as autoradiographic studies (Palade, Siekewitz, and Caro, 1961), indicates that new enzyme production in the adult pancreas is first manifest in granules associated with the endoplasmic reticulum; that product then moves into the cisternae of the endoplasmic reticulum, thence to the Golgi region, and finally to zymogen granules before being released into the acinar cavity. In the early development of the exocrine cell the comparable cytologic structures appear to become abundant in roughly the same sequence. The material within the expanded cisternae of the endoplasmic reticulum, seen as one of the first signs of exocrine differentiation, probably represents the counterpart of the intracisternal granules described by Palade *et al.* (1961). Beyond this stage, subsequent morphologic changes conform with those expected in the production of structure essential to accumulation and storage of specialized product.

It should be noted, however, that little direct evidence has been published that prozymogen and zymogen granules represent enzyme accumulation in the embryonic mouse pancreas. Unpublished data from an associated study (Rutter, unpublished data) indicate that amylase activity begins sharply to rise, both *in vivo* and *in vitro*, at about the time the expanded cisternae of the endoplasmic reticulum are noted, and continues to increase rapidly during the period of first appearance of prozymogen and zymogen. Whether amylase is typical of other enzyme products, and whether the several enzymes produced by acinar cells arise simultaneously or sequentially, are interesting questions whose answer must await further data.

If the stabilizing changes which precede the formation of the cisternae of the endoplasmic reticulum are reflected ultrastructurally, the available observations suggest two candidates. The first is the abundant ribosomes present in the cytoplasm of epithelial cells of the 13-day rudiments or 2-day cultures. The impression is that at this stage there may be an increased number of ribosomal aggregates, *i.e.* rosettes, and that there is an accompanying increase in the number of ribosomes fixed to membranes. Efforts to confirm this impression by counting have not yielded any greater validation of this observation. Were there to be a message and a message-reading stage in ribosomal aggregation (Warner, Knopf and Rich, 1963) for zymogen production, the rosettes might well represent its ultrastructural manifestation (Marks, Rifkind and Danon, 1963). Coming as it does, close to the time of stabilization of the zymogen-synthesizing property, the change of ribosome pattern could provide an explanation of stabilization in the appearance of a population of instructed ribosomes.

The second candidate for a significant controlling role relates to the changes noted at the surface of the epithelial cells. After 1 to 2 days in culture, fibers are seen at the basal surface of epithelial cells, particularly at the filter surface. Initially without any obvious periodicity, the fibers, by the 4th day in culture, are not only more abundant but also show an obvious periodicity consistent with that shown by collagen. Similar

fibers are seen in the salivary gland mesenchyme on the opposite side of the filter, but not at its interface with the filter, after even a brief period of incubation. Autoradiographic studies are in progress to determine whether the fibers associated with the epithelial surface have been synthesized by the epithelial cell or represent polymerization of soluble precursors which have crossed the filter from their synthetic source in the mesenchyme. Whichever the case, and whether or not the particular fibers are inductively active, changes of this general sort in surface-associated material may result from epitheliomesenchymal interactions and be the mediators to shift biosynthesis in cells on either side (Grobstein, 1961).

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REFERENCES

AUERBACH, R., Morphogenetic interactions in the development of the mouse thymus gland, *Devel. Biol.*, 1960, **2**, 271.

CONKLIN, J. L., Cytogenesis of the human fetal pancreas, *Am. J. Anat.*, 1962, **111**, 181.

DALTON, A. J., A chrome-osmium fixative for electron microscopy, *Anat. Rec.*, 1955, **121**, 281.

FERREIRA, D., L'ultrastructure des cellules du pancreas endocrine chez l'embrone et le rat nouveau-né, *J. Ultrastruct. Research*, 1957, **1**, 14.

GOLOSOW, N., and GROBSTEIN, C., Epithelio-mesenchymal interaction in pancreatic morphogenesis, *Devel. Biol.*, 1962, **4**, 242.

GROBSTEIN, C., Epithelio-mesenchymal specificity in the morphogenesis of mouse sub-mandibular rudiments *in vitro*, *J. Exp. Zool.*, 1953, **124**, 383.

GROBSTEIN, C., Inductive interaction in the development of the mouse metanephros, *J. Exp. Zool.*, 1955, **130**, 319.

GROBSTEIN, C., Trans-filter induction of tubules in mouse metanephrogenic mesenchyme, *Exp. Cell Research*, 1956, **10**, 424.

GROBSTEIN, C., Cell contact in relation to embryonic induction, *Exp. Cell Research*, 1961, Suppl. 8, 234.

GROBSTEIN, C., Interactive processes in cytodifferentiation, *J. Cell. and Comp. Physiol.*, 1962, suppl. 1, 60, 35.

LUFT, J., Improvements in epoxy resin embedding methods, *J. Biophys. and Biochem. Cytol.*, 1961, **9**, 409.

MARKS, P. A., RIFKIND, R. A., and DANON, D., Polyribosomes and protein synthesis during reticulocyte maturation *in vitro*, *Proc. Nat. Acad. Sc.*, 1963, **50**, 336.

MUNGER, B., A phase and electron microscope study of cellular differentiation in pancreatic acinar cells of the mouse, *Am. J. Anat.*, 1958 *a*, **103**, 1.

MUNGER, B., A light and electron microscope study of cellular differentiation in the pancreatic islets of the mouse, *Am. J. Anat.*, 1958 *b*, **103**, 275.

PALADE, G. E., SIEKEWITZ, P., and CARO, L. G., Structure, chemistry and function of the pancreatic exocrine cell, *Ciba Found. Symp. Exocrine Pancreas*, 1961, 23.

PARSONS, D. F., A simple method for obtaining increased contrast in Araldite sections using post fixation staining of tissues with potassium permanganate, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 492.

PIPAN, N., Licht und elektronenmikroskopische untersuchungen uber die differenenzierung des embryonalen pankreas der maus, *Z. Zellforsch.*, 1960, **52**, 291.

REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.

RICHARDSON, K. C., JARETT, L., and FINKE, E. H., Embedding in epoxy resins for ultra-thin sectioning in electron microscopy, *Stain Technol.*, 1960, **35**, 313.

RUTTER, W. J., unpublished data.

SALPETER, M., and SINGER, M., The fine structure of the adepidermal reticulum in the basal membrane of the skin of the newt, *Triturus*, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 35.

WARNER, J. R., KNOPF, P. M., and RICH, A., A multiple ribosomal structure in protein synthesis, *Proc. Nat. Acad. Sc.*, 1963, **49**, 122.

WATSON, M. J., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophys. and Biochem. Cytol.*, 1958, **4**, 475.