ADULT RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE

Ultrastructural Characteristics of Intercellular Contacts and Cell Membrane Differentiations

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ABSTRACT

Primary monolayer cultures were obtained in 60-mm petri dishes by incubating 3 $\times$ 10^6 isolated hepatocytes at 37°C in Dulbecco's medium supplemented with 17% fetal calf serum. The ultrastructure of monolayer cells was examined after various incubation periods. Within 4 h of plating, the isolated spherical cells adhere to the plastic surface, establish their first contacts by numerous intertwined microvilli, and form new hemidesmosomes. After 12 h of culture, wide branched trabeculae of flattened polyhedral cells extend in all directions. Finally, after 24 h of culture, bile canaliculi are reconstituted, and a biliary polarity is recovered: the Golgi elements, which are scattered throughout the cytoplasm in the isolated cells, are reassembled in front of the newly formed bile canaliculi, symmetrically in the adjacent cells; lysosomes are concentrated in that region, and microtubules reappear. Concomitantly, plasma membrane differentiations, namely desmosomes and tight junctions, develop. Tight junctions sealing the bile ducts constitute a barrier to the passage of ruthenium red and horseradish peroxidase. *De novo* formation of these junctions was studied by the freeze-etching technique: 10-nm particles compose a network of anastomosed linear arrays in the vicinity of the bile canaliculi; in the next step of differentiation, the particles fuse, form short ridge segments and finally continuous branched smooth strands, characteristic of the mature tight junction.

KEY WORDS   isolated hepatocytes · cultured hepatocytes · intercellular contacts · membrane differentiations · biliary polarity

Isolation of adult rat liver parenchymal cells by the enzymatic perfusion technique leads to high yields of morphologically well-preserved and function- ally active hepatocytes (6, 14, 23, 38). Freshly isolated cell suspensions have been used in many laboratories to prepare primary monolayer cul- tures of hepatocytes (1, 2, 8, 9, 12, 35). This experimental model composed of a homogeneous population of cells appears suitable for study in vitro of the ultrastructural events related to the

process of aggregation of cells and their reassociation in cell cords, similar to those present in the original liver tissue. Moreover, metabolic, biochemical, and pharmacological studies have been performed on cultured cells, which display a viability of several days (7, 9, 35) and which are able to repair in a few hours functional injuries caused by the isolation procedure (7).

Numerous different culture media were used, containing inorganic or organic buffers (7) supplemented or not with different types of sera (10, 35) in the presence or absence of a collagen support (31, 35). These different culture conditions have been proposed in an attempt to maintain adult hepatocytes in the best physiological condition and to retain as long as possible the morphological and functional properties of the cultures. The addition of insulin, as will be described in the accompanying paper (5), improves cell plating, induces precise ultrastructural modifications in the monolayers, and stimulates glycogenesis.

The purpose of the present investigation is to analyze under well-defined conditions of isolation and cell culture the ultrastructural characteristics of the process of cell aggregation. The development of cell membrane differentiations, tight junctions and desmosomes, will be illustrated, and the recovery of a biliary polarity at the level of newly formed bile canaliculi will be described.

MATERIALS AND METHODS

Cell Isolation and Culture

Hepatocytes were isolated from adult Sprague-Dawley rats, weighing 200–250 g, according to the enzymatic perfusion technique described in a previous paper (14) and summarized as follows: (a) a continuous recirculating perfusion of the liver was performed by means of a Ca^{++}-free Hanks' solution, containing 0.05% collagenase CLS (Worthington Biochemical Corp., Freehold, N. J.) and 0.10% hyaluronidase (Sigma Chemical Co., St. Louis, Mo.); (b) the dissociation of hepatic cell cords into perfectly isolated cells was obtained by mild mechanical treatment, i.e., by rolling the cell aggregates in a siliconized flask; and (c) finally, the cell suspension was filtered on Perlon (Perfect Thread Co., Valley Stream, N. Y.) (Polyamide PA6) of 28-μm pore size in order to eliminate cell aggregates. The isolated cells were then washed by centrifugation at 50 g for 2 min (MSE Mistral 6L, Rotor 62303, Crawley, Sussex, England), resuspended in the culture medium, and counted after appropriate dilution in a hemocytometer. All the manipulations were carried out under the best conditions of sterility.

3–4.5 × 10^6 hepatocytes suspended in 3 ml of medium were incubated in 60-mm plastic petri dishes (Falcon

Plastics, Division of BioQuest, Oxnard, Calif.) in Dulbecco's modification of Eagle's medium (Gibco-Biocult Ltd., Paisley, Scotland; or Flow Laboratories, Irvine, Scotland) buffered with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) and supplemented with 4 mM glutamine, 20 mM glucose, 17% fetal calf serum (Gibco-Biocult Ltd.), penicillin 100 U/ml and streptomycin 100 μg/ml. The petri dishes were placed in a humidified incubator at 37°C under air and CO_2 (in proportion of 95:5). The medium was changed every day.

Preparation of Monolayer Cells for Light and Electron Microscopy

Cultured hepatocytes were examined at different time intervals with a Reichert inverted phase-contrast microscope and were photographed with a Zeiss photomicroscope after the medium was discarded and a glass cover slip was placed on the preparation. After incubation periods of 4 and 12 h, 1, 2, and 3 days, cells were fixed for electron microscopy, according to the following methods. The cells were washed with culture medium and fixed in situ in 2.5% chilled, distilled glutaraldehyde, buffered with 0.1 M Na cacodylate, pH 7.2, at 4°C for 5–10 min and postfixed for 1 h in 2% cacodylate-buffered osmium tetroxide. En bloc staining in a 2% uranyl acetate solution buffered with 0.05 M maleate, pH 6.0, was performed for 2 h at 4°C in darkness, after osmium tetroxide postfixation, when specified.

RUTHENIUM RED STAINING: In order to visualize the cell coat, ruthenium red (tetra-amino-ruthenium hydroxide chlorochloride from Alfa Div., Ventron Corp., Danvers, Mass.) was added to the glutaraldehyde fixative at a final concentration of 0.1%. After thorough rinsing with cacodylate buffer, the cells were postfixed for 3 h in 2% osmium tetroxide in 0.1 M cacodylate buffer containing 0.1% ruthenium red, according to Luft (29). Cells were then dehydrated in absolute methanol and embedded in Epon, directly in the culture dishes. Blocks of selected areas of the plastic disk were cut out and sectioned in a plane parallel to the surface of the culture; in some cases, however, sections were cut in a plane perpendicular to the bottom of the petri dishes in order to study the mono- or plurilayer arrangement of the adult hepatocytes. Sections were stained with lead citrate according to Reynolds (37) for 5 min or with a 4% aqueous uranyl acetate solution for 1 h followed by lead citrate in order to study the cell membrane differentiations. Observations were made in Philips EM 200 and EM 301 electron microscopes.

HORSERADISH PEROXIDASE ACTIVITY: The tracing technique of Graham and Karnovsky (20) was slightly modified and applied to our cultures. Horseradish peroxidase (type II, Sigma Chemical Co.) was dissolved in Dulbecco's medium at a final concentration of 6 mg/ml and put into contact with cultures for 5 min at 37°C. The cultures were then washed, fixed briefly (1 min) with 2.5% cacodylate-buffered glutaraldehyde, and

incubated for 30 min at 37°C in a 0.05% solution of 3-3'-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.02% H_2O_2. The cells were then washed, postfixed, and dehydrated and embedded as described above.

FREEZE-ETCHING: For freeze fracturing, the cells were fixed in fresh 2.5% chilled distilled glutaraldehyde buffered with 0.1 M cacodylate at pH 7.2 for 30 min. After one or two brief washings in 0.1 M cacodylate buffer, the fixative was replaced by 25% glycerol diluted in the same buffer. The cells were impregnated for 1 h at 4°C, peeled off with a rubber policeman and centrifuged at 1,300 g for 5 min (International Centrifuge PR 1, Rotor 269, International Equipment Co., Boston, Mass.).

Small pieces of pellets were frozen in solid nitrogen (−210°C), fractured at −115°C, etched for 1 min at 10^{-6} mm Hg and replicated in a Balzers BAF 301 apparatus (Balzers AG, Balzers, Liechtenstein).

Replicas were cleansed in a sodium hypochlorite solution for 2 h, rinsed three times in distilled water, mounted on bare copper grids, and examined with a Philips EM 301 electron microscope.

RESULTS

Monolayers of Hepatic Parenchymal Cells

Hepatocytes, isolated by continuous recirculating perfusion of the rat liver in the presence of collagenase and hyaluronidase, appear more or less spherical in shape and well dissociated under the phase-contrast microscope (Fig. 1). Nearly all the isolated cells are refringent while few altered cells present a granular cytoplasm (Fig. 1, arrow).

Within 4 h after plating, hepatocytes adhere to the plastic surface and aggregate in groups of 2-10 cells. Straight and apparently rigid cell contacts (Fig. 2, arrows) are detected between the reassociated hepatocytes. After 12 h of incubation, hepatic trabeculae are reconstituted, extending in all directions. They progressively enlarge after 24 h (Fig. 3). At this time, the hepatocytes present a polygonal shape and show straight contacts with three to six neighboring cells. These refringent contacts between hepatocytes show focal round or elongated enlargements (Fig. 3, arrows) which correspond to newly formed bile canaliculi. No doubt exists about the de novo formation of these canaliculi, because much care was devoted to dissociating fully the liver tissue and to filtering the cell suspension on a fine Perlon filter. Trabeculae subsist intact for 3 days and afterwards degenerate into round granular cells which lose their adherence to the plastic. During this survival period of the parenchymal cells, no fibrocytes grow in the cultures.

Primary Cell Contacts

Isolated hepatocytes fixed in glutaraldehyde and osmium tetroxide solutions containing 0.05% ruthenium red reveal, on their surface, in close contact with the external leaflet of the plasma membrane, the presence of an electron-dense cell coat or glycocalyx. This continuous dense layer covers the short microvilli and thus resists the enzymatic and mechanical treatments imposed during the isolation procedure (39). We have noticed that the entire surface of well preserved cells is covered with microvilli and that, consequently, the large plane areas of contact where hepatocytes in situ are joined, at both sides of the bile canaliculi, have disappeared. Similarly, the characteristic junctional complexes of the plasma membrane which separate the bile canaliculi from the space of Disse are apparently absent.

Within 4 h of incubation, the hepatocytes establish their first contacts, as mentioned in the previous section. These areas of contact which appeared in the phase-contrast microscope as straightened surfaces marked by a refringent rim (Fig. 2, arrows) consist, in corresponding electron micrographs, of closely set microvilli arranged in such a way that the cytoplasmic extensions of one

FIGURES 1-3 Phase-contrast micrographs of isolated and cultured hepatocytes.

FIGURE 1 Isolated hepatocytes from adult rats. The dissociated cells appear round and refringent. Few altered cells present a granular cytoplasm (arrow). × 450.

FIGURE 2 Hepatocytes 4 h after plating. The cells are flattened on the bottom of the petri dishes and reaggregate in groups of 2-10 cells. The junctions between the cells are straight and rigid (arrows). × 450.

FIGURE 3 Hepatocytes 24 h after plating. Large trabeculae of polyhedral cells run in all directions. Intimate cell contacts are detected between the adjacent hepatocytes. At some places, local enlargements of the intercellular space are observed (arrows) corresponding to newly formed bile canaliculi. × 450.
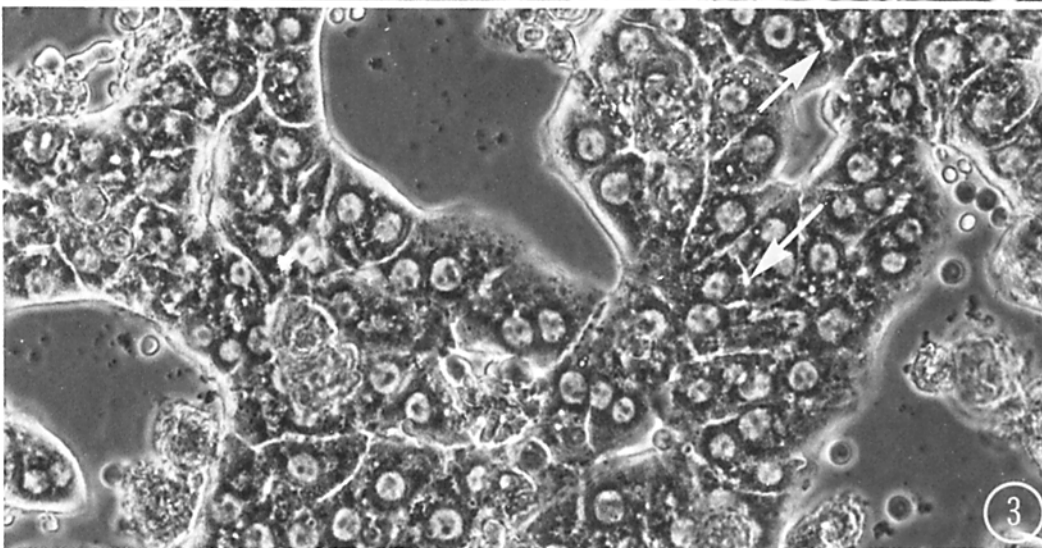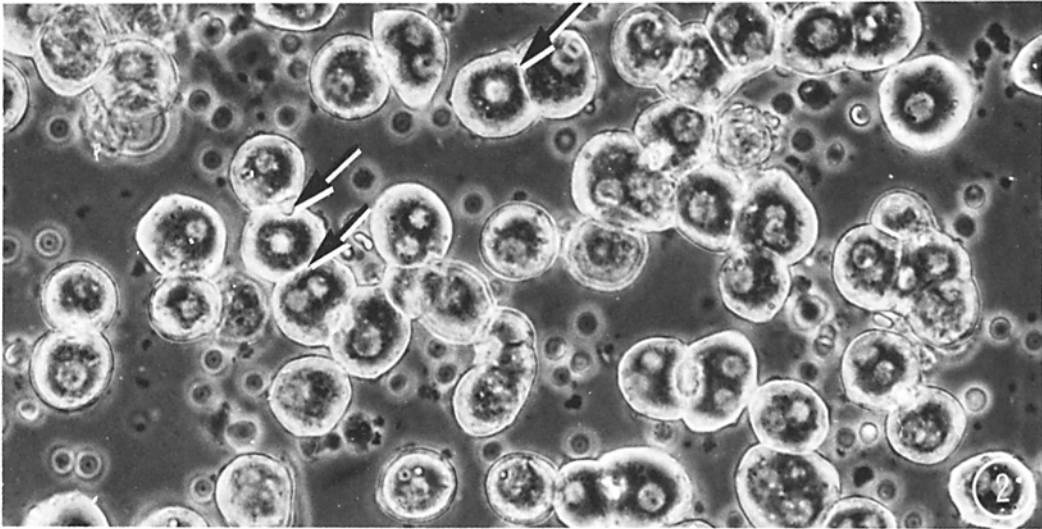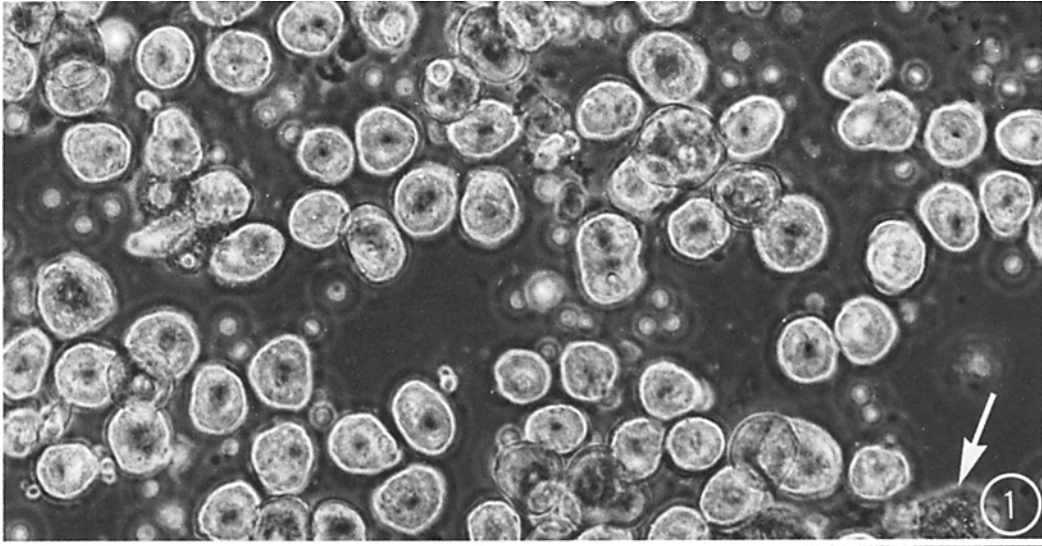
cell alternate with those of the adjoining cell and sometimes intertwine (Fig. 4, arrows). The cell coat, present at the level of the closely apposed villi, is detectable by the ruthenium red stain which is still able to penetrate into the narrow intercellular space. With uranyl acetate staining, numerous newly formed hemidesmosomes are detected on the plasma membrane of joined cells. Thin tonofilaments insert on the electron-dense, single desmosomal plate. The corresponding symmetrical plate, typical of the mature desmosome, is totally absent on the adjacent plasma membrane.

Trabeculae composed of polyhedral hepatocytes are observed within 12 h of plating. In sections, these polygonal cells exhibit close relationships with their neighbors. Two types of cell contacts are distinguished: (a) linear segments characterized by the juxtaposition of two parallel plasma membranes (Fig. 5, a) separated from one another by a constant space of 150 Å; (b) loose villous segments, characterized by accumulations of intertwined microvilli mainly grouped at the angles of the polyhedral-shaped cells (Fig. 5, b). The intercellular spaces are still permeable to the ruthenium red stain. The preferential localization of cell organelles in the region of the bile canaliculus, typical of the in situ hepatocyte, is still completely lost at this time of hepatocyte culture: the Golgi complex and the lysosomes have left their usual site and are dispersed in the perikaryon. Numerous mitochondria with transverse cristae are evenly dispersed in the cytoplasm. The rough-walled cisternae of the endoplasmic reticulum form large stacks or else meander in between the mitochondria. Lipoprotein particles (Fig. 5, lp) are detected in the cisternae of the Golgi vesicles (Go) of some cells.

Differentiation of Bile Canaliculi and Development of a Cell Polarity

The hepatocytes cultured for a longer period, 24 h and more, show important morphological changes which correspond to differentiations taking place within the cytoplasm around the Golgi complex and concomitantly on the plasma membranes. Because of the rapidity of the events, it is difficult to determine which one has precedence in the process of differentiation and would influence or eventually induce other subsequent changes. It seems obvious from our observations that the development of a Golgi complex at the periphery of the cell is closely related to the differentiation of a

bile canaliculus. This latter transformation includes the local formation of grouped microvilli in the two adjacent cells and the differentiation of junctional complexes which finally seal the intercellular bile canaliculus.

The structure of a hepatocyte trabecula obtained after 24 h of culture is illustrated at low magnification in a section-plane parallel to the surface in Fig. 6, and in a section perpendicular to the plastic surface in Fig. 7. Large areas of the culture are formed of monolayered cells, but in some regions cell debris may be superimposed. Numerous small microvilli (Fig. 7, mv_1) are present on the upper surface of the hepatocytes, and also on the lower surface (mv_2) which is the site of attachment to the plastic. All the classical cell organelles present the same morphological features and topographic relationships that have been reported for hepatocytes in situ. The rough endoplasmic reticulum is composed of associated parallel undilated cisternae (Fig. 6, rer), mainly distributed at the periphery of the cultured cells; isolated cisternae appear frequently to be related to round or oblong mitochondria, apparently increased in size (Fig. 6 and 9, mi), which present a matrix of normal density and numerous transverse cristae. Peroxisomes and lysosomes (Figs. 6 and 7, Pe, Ly) appear to be normal. The smooth endoplasmic reticulum is not proliferated, and few glycogen particles are detected under the conditions of culture.

A biliary polarity is reconstituted after 24 h of culture: numerous Golgi complexes, seeming to arise from the perinuclear region, accumulate in front of the newly formed bile canaliculi (Fig. 8, Go). This type of picture was systematically encountered in our experimental material, in more than forty different culture assays. The Golgi cisternae appear to be very active as suggested by the extensive budding of small vesicles. In some of them, lipoprotein particles are present, and we see that a few of them seem to be extruded into the bile capillary (Fig. 8, lp). The extruded particles are of the same size and electron opacity as lipoprotein particles. However, this tentative identification should be subject to final evidence concerning their chemical nature. This observation, which is frequently made on this material, suggests a transport of substances from the Golgi region to the compartment where normally bile is excreted. Also, numerous smooth-walled vesicles originating from the Golgi complex or from the smooth endoplasmic reticulum seem to be directed to that region.
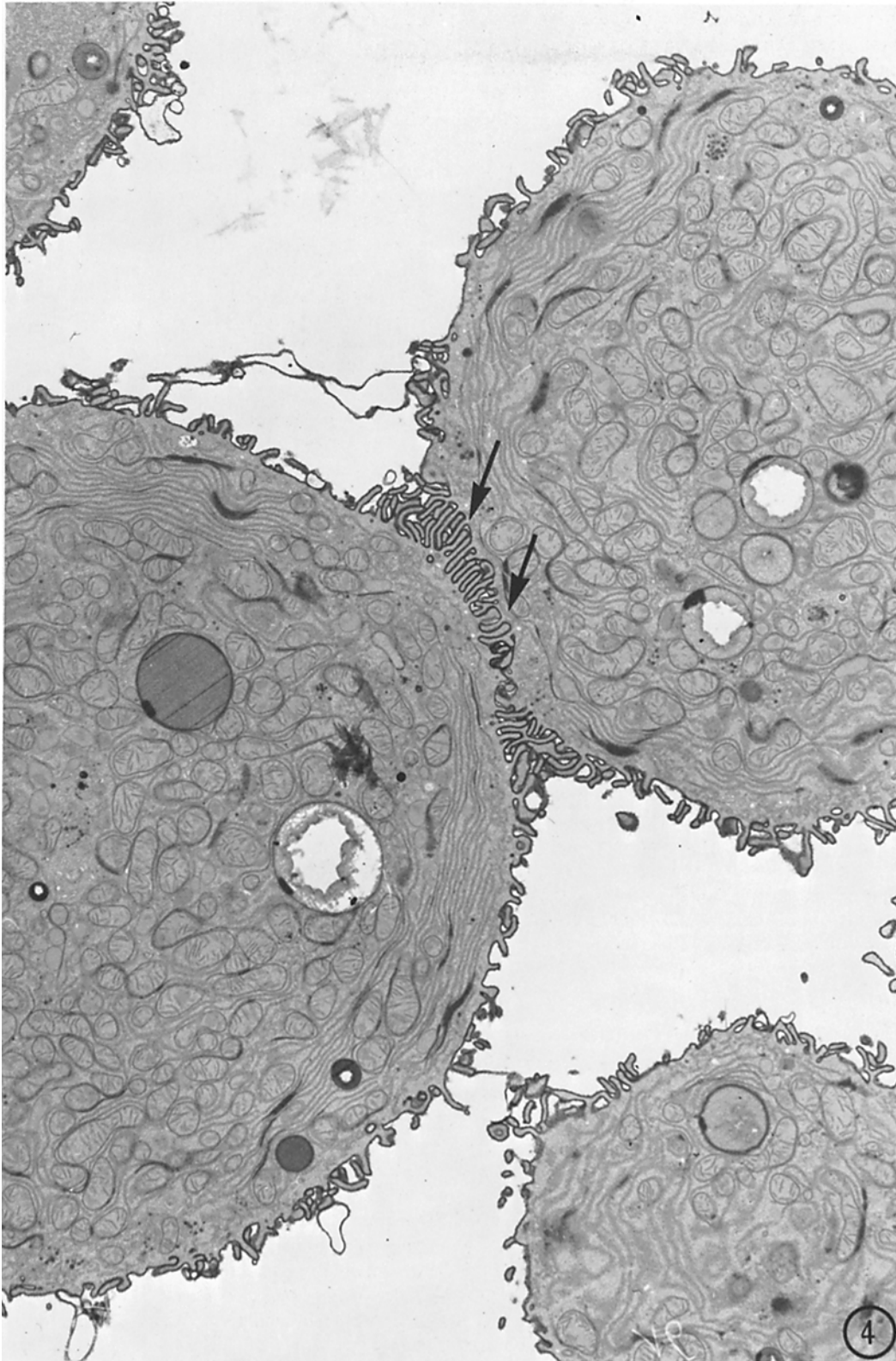
FIGURE 4 Electron micrograph of hepatocytes incubated for 4 h. The first cell contacts are established, consisting of numerous closely apposed intertwined microvilli (arrows). Ruthenium red intensely stains the cell coat covering the plasma membranes and penetrates into the narrow intercellular space existing between the adjacent cells. × 7,000.

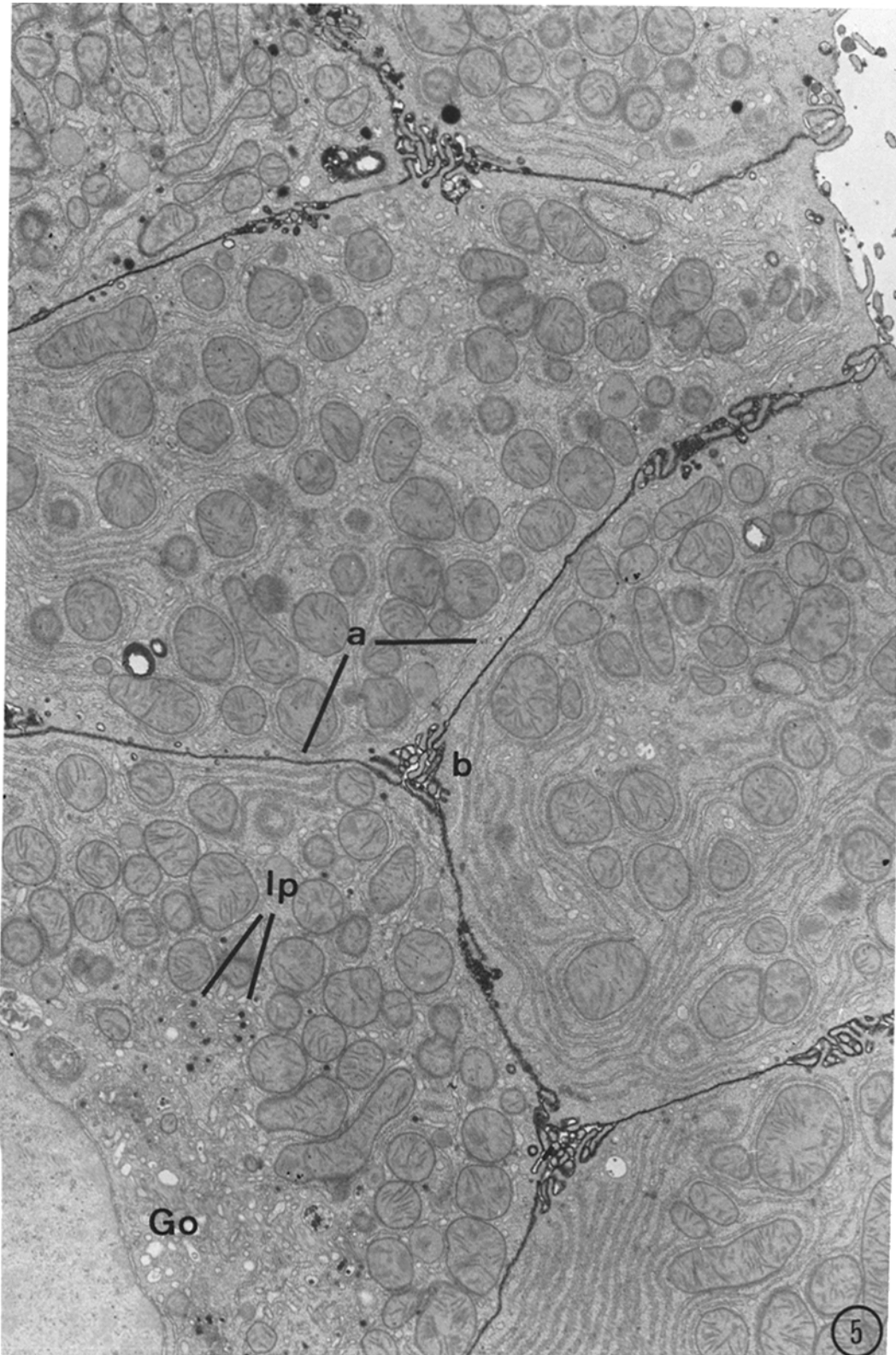FIGURE 5   Electron micrograph of hepatocytes incubated for 12 h. Trabeculae of polyhedral and flattened hepatocytes are observed. Linear cell contacts (*a*) alternate with loose segments composed of microvilli (*b*). Intercellular spaces are permeable to ruthenium red, which stains the continuous cell coat. Mitochondria are evenly distributed in the cytoplasm. Single cisternae of rough endoplasmic reticulum encircle mitochondria, while cisternae running parallel are mainly detected at the periphery of the cells. Notice the presence, in the perinuclear region, of Golgi complexes (*Go*) containing osmiophilic lipoprotein-like particles (*lp*). × 9,000.

At the same time, microtubules (Figs. 8 and 10, *tu*) and microfilaments, which are practically absent in the earlier culture stages, reappear in the vicinity of the bile duct. The objection might be raised that at least some of the bile canaliculi are remnant structures of cells that were not originally dissociated. This possibility can easily be excluded, since in these rare cases the bile canaliculi have a characteristic appearance due to a considerable enlargement of their lumen. Finally, paraplasmic smooth cisternae (Fig. 8, *pp*) reappear in close relation to the linear segments of the adjacent apposed plasma membranes.

Newly Formed Differentiations of the Plasma Membrane

The glycocalyx, which forms a continuous cell coat intensely stained with ruthenium red, is interrupted in the region of the newly formed bile canaliculus (Fig. 8, arrowheads). At both sides of the canaliculus, the closely apposed plasma membranes of the adjacent cells do not allow the ruthenium red to penetrate the bile duct. This sealing, as will be demonstrated later with freeze-etching techniques, is due to the differentiation of tight junctions which extend on relatively long portions of the plasma membranes. By using horseradish peroxidase as tracer, it was confirmed that a junction tightens the intercellular space and constitutes, thus, a barrier to the passage of the tracer (Fig. 9, arrowheads). In exceptional cases, bile canaliculi with their usual morphological characteristics show a persistent permeability to electron-dense tracers.

In order to better characterize tight junctions and other possible newly formed membrane differentiations, uranyl acetate staining and freeze-etching techniques were applied to the 24-h cultures of hepatocytes.

With the complementary technique of heavy metal staining, the large differentiated segments of the plasma membranes in the vicinity of the bile ducts are clearly shown (Figs. 10 and 11), but it remains difficult to determine to which type of junction some of them belong. The differentiations which line the bile ducts (Fig. 10, ti_1 and ti_2), containing focal fusions of the external lamellae of the adjacent plasma membranes, are undoubtedly related to tight junctions because it can be demonstrated that in that region structures are present which form a barrier to electron-dense tracers. With double uranyl acetate staining, a fine border of fibrillar material of unknown nature is also

systematically observed in the newly formed tight junctions (Fig. 10), at least at the beginning of their differentiation. Desmosomes (Fig. 11, *D*), which are not difficult to identify, are also encountered: tonofilaments insert on the electron-dense desmosomal plates, and a fine granular material constitutes the intercellular cement.

Freeze-etching applied to the monolayer cultures supplies further complementary observations which contribute to the identification of the tight junctions and, at the same time, offer the opportunity to follow the different stages in the *de novo* genesis of this junctional differentiation. We selected three stages in its evolution (Figs. 12–16) and propose a sequence of events, which is in accordance with that postulated by recent studies on the in vivo assembly of tight junctions (16, 32).

In a first stage, the 10-nm particles, visible on the A face of large areas surrounding the forming bile canaliculi, become ordered so that chains of particles appear and delineate polygonal areas (Figs. 12 and 13, arrows). In these figures, only this stage of differentiation is present on the entire surface extending 2 μm or more from the bile duct limits. Notice that in this picture, as in many others taken in similar areas, the fracture remains in one plane and does not pass from one membrane to the other. Another particularity observed at higher magnification (Fig. 13, *inset*) is the presence, within the polyhedral areas limited by the arrays of particles, of a fine granularity which seems not to be artifactual. The protruding small particles about 2 nm in diameter form either fine ridges or, occasionally, a hexagonal pattern (*inset* of Fig. 13, arrowheads).

These particles have dimensions very close to the limit of resolution usually attained in shadowed replicas and must be taken with the necessary caution. At this developmental stage, it is probable that the paracellular permeability is not yet impaired and that this morphology corresponds to that in situations described in sectioned material where the electron-dense tracer still penetrates the intercellular space in spite of the development of a duct.

In the next step of differentiation, the 10-nm particles fuse and form discontinuous branched smooth ridges, 10 nm in width (Figs. 14–16). The transformation of individual particles lying in chains into smooth ridges or segments is confined to a narrow region (Figs. 14 and 15), close to the duct. In the more peripheral regions, most of the particles seem to regress, leaving large areas of the
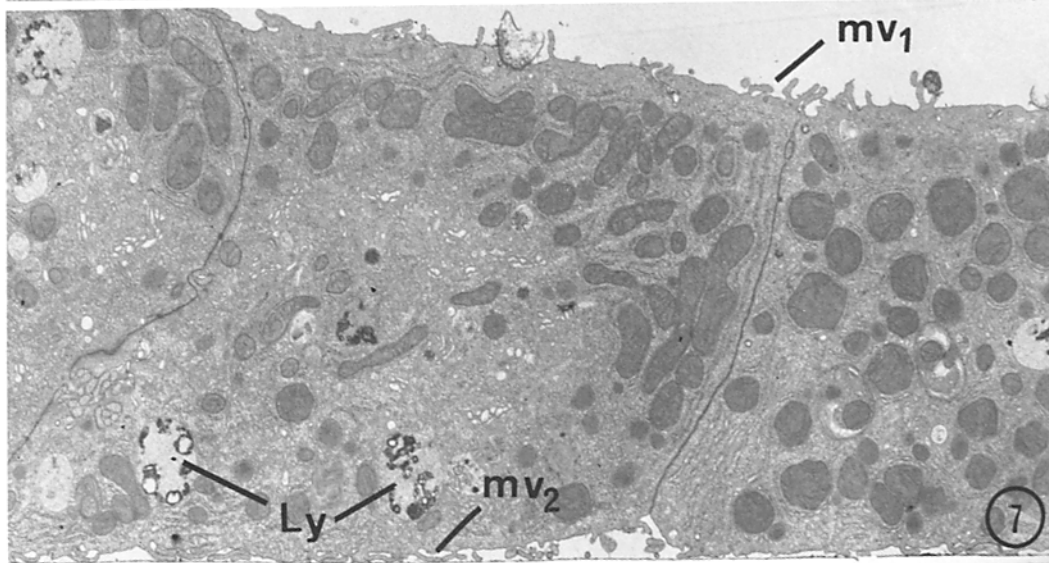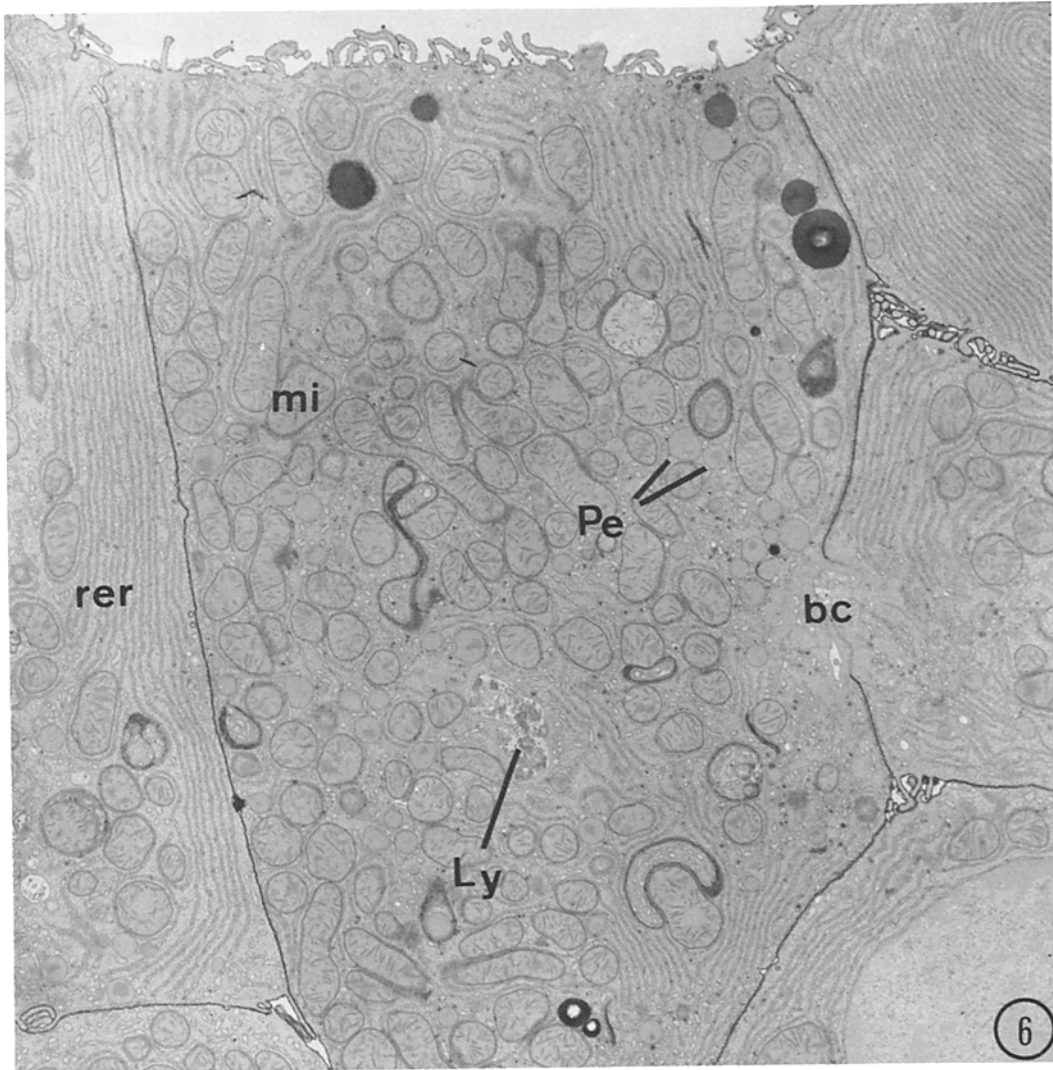
A face with few particles (Fig. 14, arrowheads). In contrast with what was observed in the very early stages of junctional differentiation, these nearly mature occluding junctions show frequent transitions from one membrane to the adjacent one, exposing either the A or B face (Fig. 14, *A* and *B*). On the exoplasmic B face, smooth grooves are detected (Figs. 14-16); occasionally, they are in continuity with the ridges of the cytoplasmic A face (Fig. 16, arrowheads). A few particles lying in the grooves were probably detached from the opposing face. Compared to the structures observed in sections, these mature junctions correspond to most of the junctions called tight on the basis of their sealing effect against tracers.

Morphological Characteristics of the Cultures in the Late Survival Periods

After 48 h of incubation, trabeculae composed of flattened cells remain attached to the bottom of the petri dishes. Well developed bile canaliculi (Fig. 17, *bc*) and cell membrane differentiations are still present. The most striking feature is the accumulation of numerous cytolysomes in the cytoplasm of the hepatocytes. These autophagic vacuoles appear partially empty, but contain some cell debris, membranes, and glycogen particles (Fig. 17, *AV*). These vacuoles increase in number as a function of time, and after 3-4 days of incubation the cytoplasm of nearly all the cultured hepatocytes appears to be filled with numerous autophagosomes.

At the periphery of the cells, large membrane-bounded cytoplasmic extensions develop that are devoid of cell organelles such as mitochondria, peroxisomes, Golgi complexes, or cisternae of the rough and smooth endoplasmic reticulum. These undulating cytoplasmic extensions are filled with a considerable number of parallel microfilaments

(Fig. 18, *fi*) and also contain microtubules (*tu*). After 3-4 days of incubation, the hepatocytes become granular and undergo a rapid involution.

DISCUSSION

Much effort has been devoted in the last few years to obtaining cultures of adult liver cells (1, 2, 8, 9, 12, 21, 24, 31, 36). Monolayers of hepatocytes from adult rats are now frequently used as an experimental model particularly suited to the study of metabolic functions, specifically of liver parenchymal cells: albumin synthesis and secretion (8), gluconeogenesis (8), glycogen synthesis (5, 8), glycogenolysis (8), lipogenesis (18), and numerous enzymatic activities related to the synthesis, conjugation, and secretion of bile salts, heme synthesis, and drug metabolism (7, 8, 10). Nearly all these characteristic hepatic functions are relatively stable for several days, except for some specific microsomal functions in which cytochrome P-450 is involved (7): low enzymatic levels reflect phenotypic changes in liver cell culture, which are reversible by appropriate modifications of the culture conditions (7). At the submicroscopic level, Chapman et al. (12) described for the first time the detailed ultrastructure of hepatocytes established in monolayers. These authors illustrate the process of cell plating and aggregation and the behavior of the different subcellular organelles during the first days of culture.

Optimal Culture Conditions

Successful culture of hepatic parenchymal cells requires that the investigator starts with a population of well preserved cells (6, 14, 23, 38) and controls several parameters related to the culture conditions. High yields of isolated intact hepatocytes are obtained by using an appropriate combination of enzymatic treatment, consisting of a con-

FIGURES 6-7 Longitudinal and transverse sections of 24-h-cultured hepatocytes.

FIGURE 6 Numerous microvilli are present on the free surfaces of flattened hepatocytes. The intercellular boundary is interrupted in one place by a structure resembling a bile canaliculus (*bc*). Mitochondria (*mi*), peroxisomes (*Pe*), lysosomes (*Ly*), and *rer* exhibit a normal aspect and their distribution within the cytoplasm is similar to that reported for hepatocytes *in situ*. × 7,000.

FIGURE 7 This section, perpendicular to the bottom of the petri dish, reveals the monolayer dispersion of hepatocytes, which is representative of large areas of the culture. Note that microvilli are present on the upper (*mv₁*) and lower surfaces (*mv₂*) which are the sites of attachment to the plastic. *Ly*: lysosomes. × 7,000.
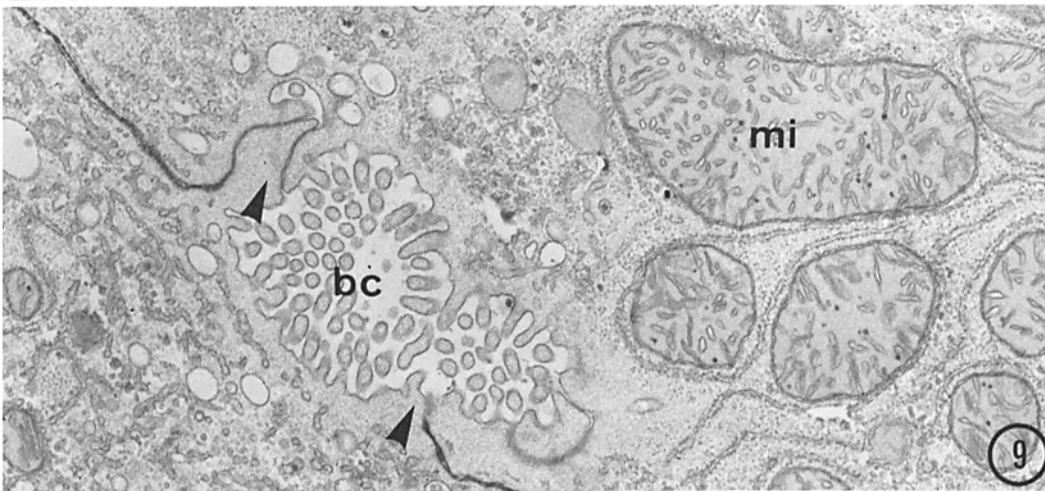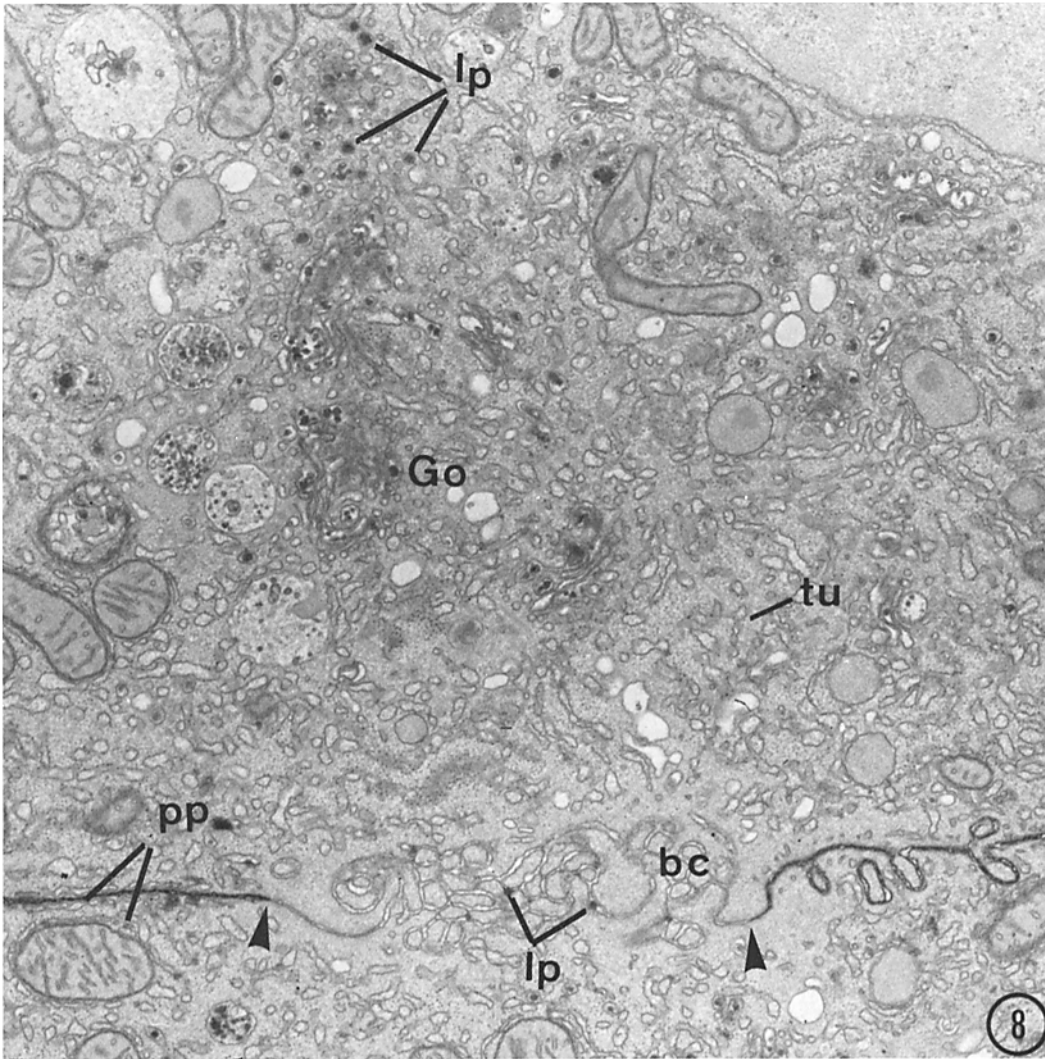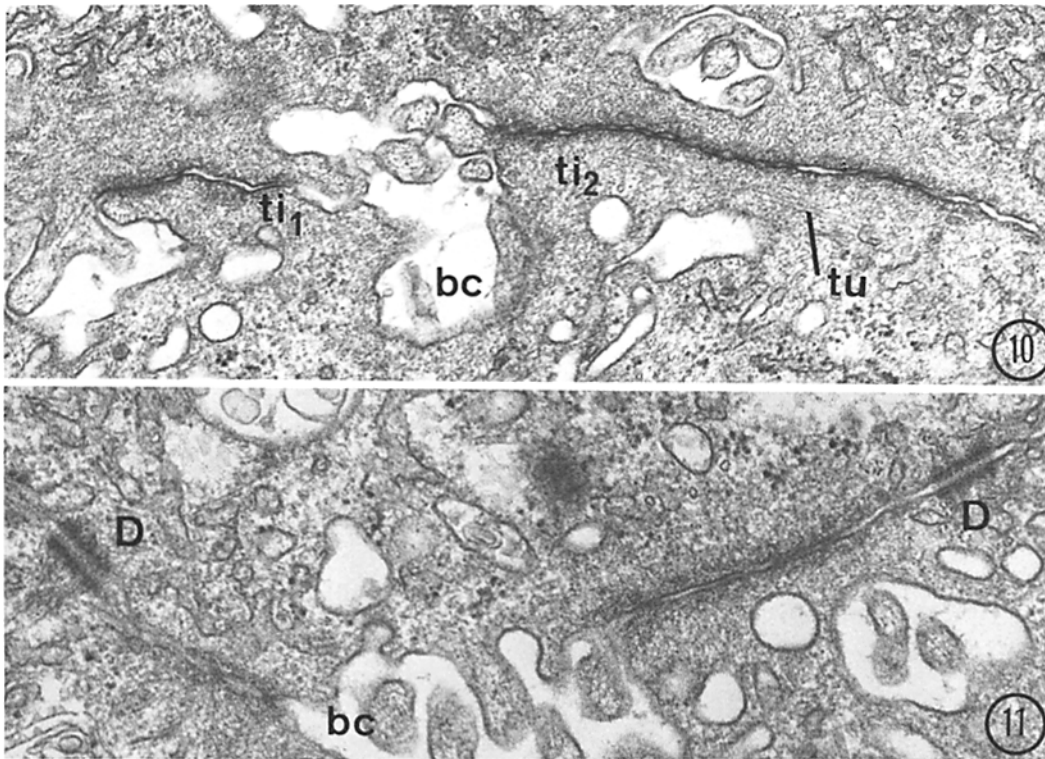
FIGURES 10–11   24-h cultures. Examples of intercellular contacts and cell membrane differentiations after uranyl acetate impregnation.

FIGURE 10   Typical tight junctions (ti_1) are detected near the bile canaliculus (bc). In some cases, numerous focal fusions between membranes of adjacent hepatocytes extend on long segments of the plasma membranes (ti_2). Notice the presence of microtubules (tu) in the peripheral cytoplasm. × 32,000.

FIGURE 11   Desmosomes (D) are reconstituted after 24 h of incubation. Thin tonofilaments insert on both electron-dense desmosomal plates, and a granular material forms the intercellular cement. In the center of the figure, a bile canaliculus (bc) is seen. × 57,000.

tinuous Ca^{++}-free recirculating perfusion of collagenase and hyaluronidase, and gentle mechanical action (14). Multiple factors play a role in the process of cell adhesion and cell plating and appear favorable to prolonging the viability of the monolayers. Optimal cell attachment was obtained when Ham's F 12 medium was supplemented with 10% rat serum or 15% fetal calf serum (10). Only a restricted number of cells adhere to the plastic surface in the absence of

FIGURES 8–9   Portions of 24-h-incubated hepatocytes examined at higher magnification.

FIGURE 8   A typical biliary polarity is reconstituted, characterized by the presence of numerous Golgi complexes (Go) in the vicinity of the newly formed bile canaliculi (bc). Numerous lipoprotein-like particles (lp) are detected in the Golgi cisternae and also in the lumen of the bile capillary. Microtubules (tu) reappear in the peripheral cytoplasm and paraplasmic smooth cisternae (pp) in relation to the linear segments of the apposed plasma membranes. Sealing of the newly formed bile duct does not allow the ruthenium red stain to penetrate into the duct lumen (arrowheads). × 17,000.

FIGURE 9   Exogenous horseradish peroxidase activity. Arrowheads indicate the presence of a barrier to the passage of the tracer at the level of a newly formed bile canaliculus (bc). Mi: mitochondria. × 16,500.
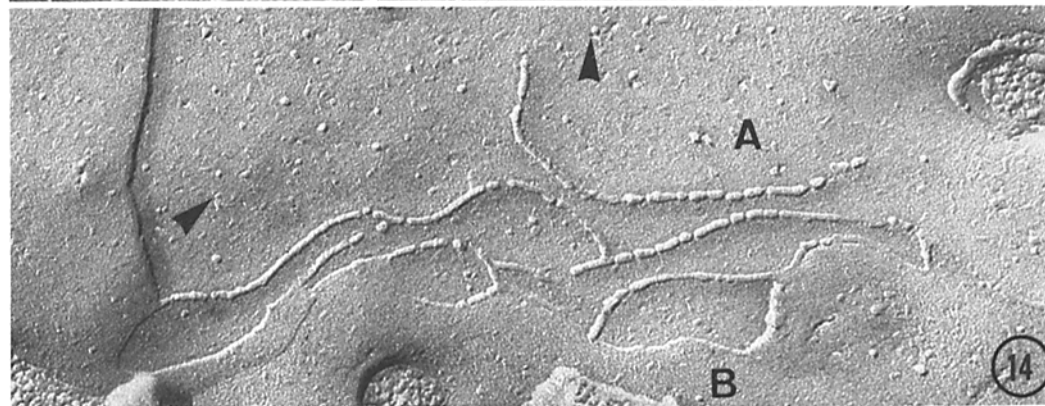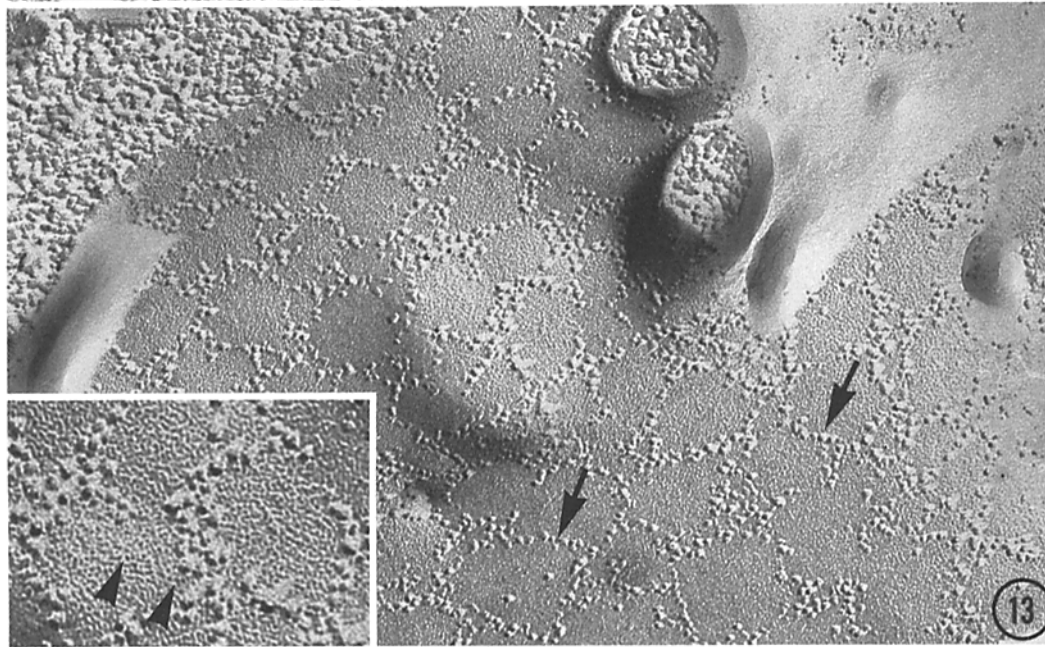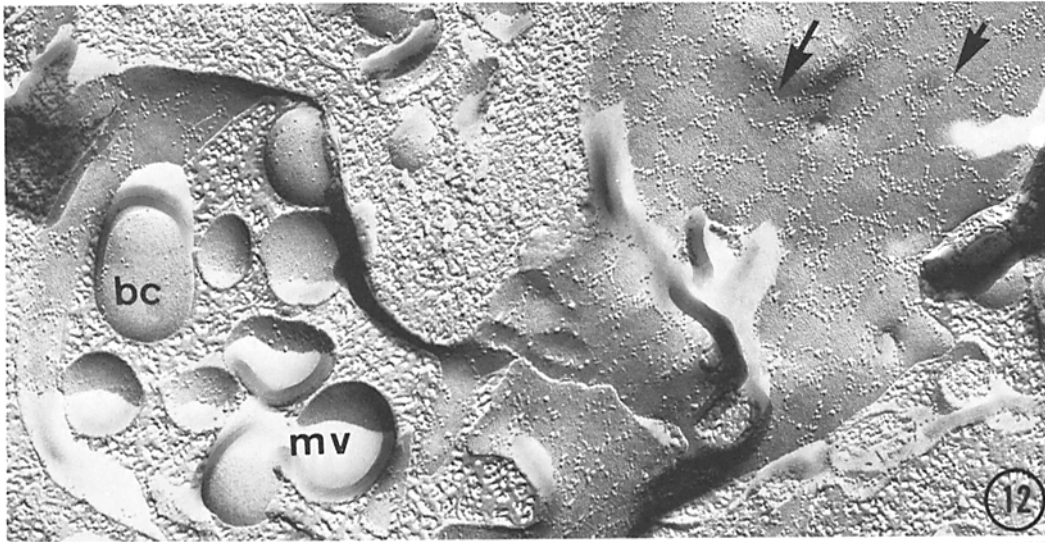
serum, unless collagen-coated plates or collagen gels are used (31). A similar percentage of adhesion is obtained by partial hepatectomy, 4 days before cell isolation, as proposed by Bissell et al. (8). However, partial hepatectomy produces important metabolic modifications which could still remain 4 days later, even though the cells seem to present no alterations of their morphological characteristics. In our assays, the following parameters were rigorously controlled: (a) fetal calf serum was added to the incubation medium, at a final concentration of 15-20%; (b) constancy of the pH during the culture was obtained in the culture medium by using HEPES buffer and incubating the hepatocytes under a constantly renewed air-CO_2 atmosphere; (c) optimal cell concentration was determined: 1.10^6 cells/ml gave the best results when 3 ml of cell suspension were transferred to 60-mm Falcon petri dishes; low concentrations, such as 5.10^5 cells/ml, prevent the formation of trabeculae due to the absence of cell contacts, and too high concentrations favor the development of multilayers which rapidly degenerate; and (d) finally, the use of hormones constitutes an important way to improve cell viability. The effect of insulin on isolated and cultured hepatocytes under our experimental model conditions was studied by Bernaert et al. (5). As reported in the accompanying paper, the degree of cell plating is considerably enhanced, and the cell survival is twice as long. A higher survival period of 8-12 days was reported for hepatocytes cultured in the presence of 10^{-5} M hydrocortisone (21). However, careful attention has to be paid to specific hormonal modifications, such as an increase in mitochondrial volume (4)

and, perhaps, some degree of dedifferentiation of hepatocytes.

Development of Cell Membrane Differentiations

Incubation of perfectly dissociated hepatocytes in plastic petri dishes offers the opportunity to analyze the different steps of cell aggregation and the development of cell membrane differentiations.

Reaggregation has been described for growing cultures of embryonic frog liver cells (3) and chick liver cells (27) and for adult rat hepatocytes in monolayers (2, 12). This process requires, as proposed by Moscona (33) for sponge cell suspensions, the presence of intact specific cell ligands localized at the cell surface, which allow the linking of cells into multicellular systems. These specific cell-binding constituents may probably be involved in intercellular attachments in higher organisms. Reaggregation implies that cells retain their potentiality of reciprocal recognition and of preferential association. This potentiality allows the reconstitution in vitro of the in vivo architecture of the liver, i.e., the reformation of classical cell trabeculae.

Isolated hepatocytes from adult rats are able to reestablish many of the cell membrane differentiations typical of the liver in vivo, i.e., desmosomes and tight junctions. These junctional elements disappear at the surface of isolated cells, either by internalization (6) or by dislocation. In our experimental conditions, their regular absence during the first steps of reaggregation argues in favour of a process of de novo formation. Hemidesmosomes

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Figures 12-16   Freeze-cleave replicas of 24-h-incubated hepatocytes. Analysis of the de novo formation of tight junctions.

Figure 12   The cleavage plane reveals an A face of the plasmalemma close to the bile canaliculus. Short arrays of particles form a network of anastomosed linear chains (arrows), in the vicinity of the bile canaliculus (bc). Notice the presence of fractured microvilli (mv). × 62,500.

Figure 13   Detailed view of the network present in the preceding figure. Protruded particles, 10 nm in diameter, are either disposed in linear branched rows (arrows) or in aggregates. No fusions of particles are observed at this step of differentiation. × 112,500. Inset: high magnification view of anastomosing chains composed of 10-nm particles, which reveals in the meshes of the network the presence of anastomosed small particles, ordered in round or hexagonal patterns (arrowheads). × 200,000.

Figure 14   Particles fused in smooth ridges, which appear still discontinuous, composed of alternating short and long segments. The cleavage plane reveals the A and B faces of the cell membrane. Note the continuity of ridges of the A face through the grooves of the B face. Isolated particles, frequently associated with short, thin cristae (arrowheads), are detected on the A face. × 112,500.

bc
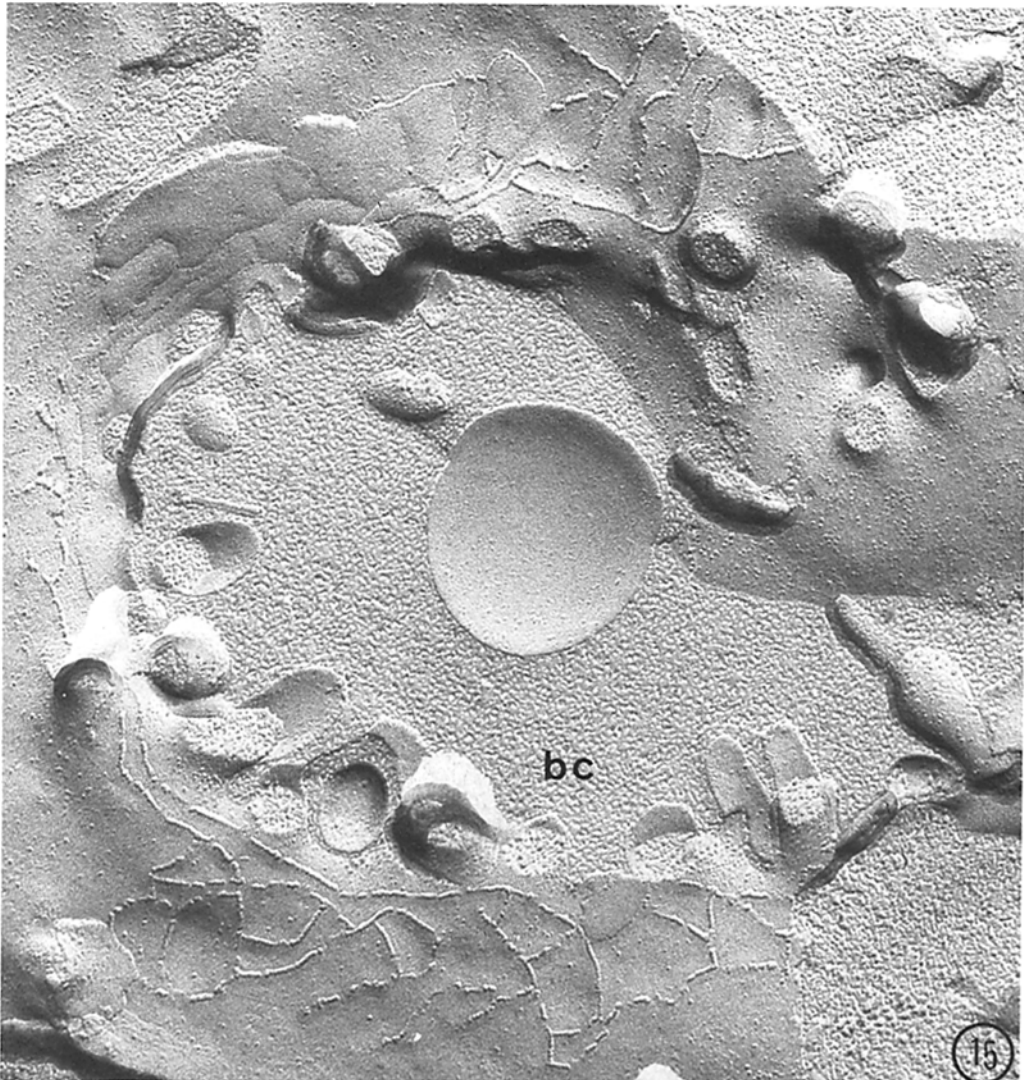
develop after 4 h of incubation and induce, in the adjacent, intimately associated cells, the differentiation of a symmetric structure. Numerous, randomly distributed desmosomes are formed on the same interface and frequently alternate with newly formed paraplasmic smooth cisternae.

Tight junction-like structures appear at their classical location near the bile canaliculi, but differ from the corresponding in vivo structures by their unusual length of 2-3 μm and by the particular development of an electron-dense cytoplasmic border (17). It seems possible that, along the linear contacts established between the liver cells, a large region starts to differentiate by aligning the intramembranous particles according to a characteristic polygonal pattern. Within this area, a defined limited region will serve as building place for a new bile canaliculus, with reciprocal agreement of both adjacent cells. This location could be determined by intracellular factors, among which is the positioning of the Golgi complexes at both sides of that particular region. The plasma membrane particles undergo, there, close to the bile duct, a new localized rearrangement that induces them to join and form the typical smooth ridges or fibrils. Only a few proximal strands of particles are transformed into ridges, and the remaining more peripheral arrays of particles may regress. The possibility of regression or degradation of parts of the membrane differentiations was clearly taken into consideration in the work of Montesano et al. (32). The fine framework of round and hexagonal units which were described with due caution may also be related to temporary structures which regress with time or are considered as precursors of other types of junctions.

Biliary Polarity

Spaces resembling bile canaliculi, frequently dilated, have been observed in monolayers of adult rat hepatocytes by many authors (1, 2, 21, 28). Different criteria have been chosen in order to define and recognize typical newly formed bile ducts, namely: the presence of microvilli, limiting the lumen; the development of mature tight junctions, visualized by the freeze-etching method and by the use of permeability tracers such as horseradish peroxidase; the existence of a granular zone, devoid of cell organelles, surrounding the lumen. Our liver cell preparations, obtained after enzymatic treatment, by mechanical disruption of liver cell cords into perfectly isolated cells, exclude any possible confusion with remnants of canaliculi still present between undissociated cells.

The general structure and location of the Golgi apparatus of rat hepatocytes has been described by Bruni and Porter (11) and Novikoff and Shin (34). The Golgi complexes are polarized with respect to either the bile capillaries or the cell center. During the isolation procedure, important ultrastructural modifications occur, namely the loss of the classical peripheral location of the Golgi complexes together with the disappearance of the bile capillaries. One of the most striking features of the 24-h-cultured hepatocytes is the recovery of the Golgi biliary polarity, similar to the situation in vivo, which reappears simultaneously with the development of cell membrane differentiations. Are these two events intimately related or not? Treatment with antimicrotubular compounds, which interfere with the intracellular distribution of cell organelles, would be an interesting approach for an analysis of the sequential interrelationships between these two events.

Formation and Secretion of VLDL-Like Particles

Osmiophilic, dense lipoprotein-like particles were detected in the cisternae of the Golgi complexes after 24 h of culture. These membrane-bounded particles, which have the same size, shape, and electron opacity as those described in the liver in situ (13, 15) or in the isolated perfused rat liver (22, 26, 30), were identified (22, 26) with very low density lipoproteins (VLDL).

The VLDL are transported intracellularly from

FIGURES 15-16   Further steps in the de novo assembly of tight junctions.

FIGURE 15   The zonula occludens is seen as a network of strands and grooves running around the bile canaliculus (bc). × 66,000.

FIGURE 16   This freeze-fracture demonstrates the complementary, extended meshwork of anastomosed and branched grooves, detected on the B face. The grooves are, occasionally, in continuity with the ridges of the cytoplasmic A face (arrowheads). This aspect is typical of the mature junction. × 62,500.
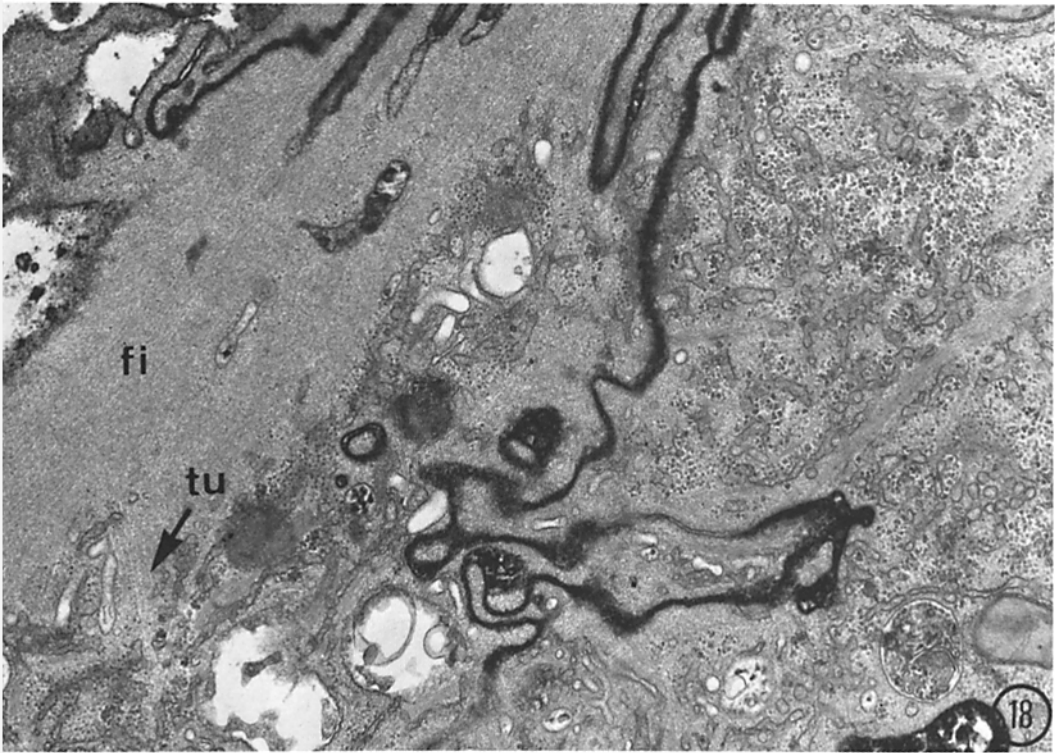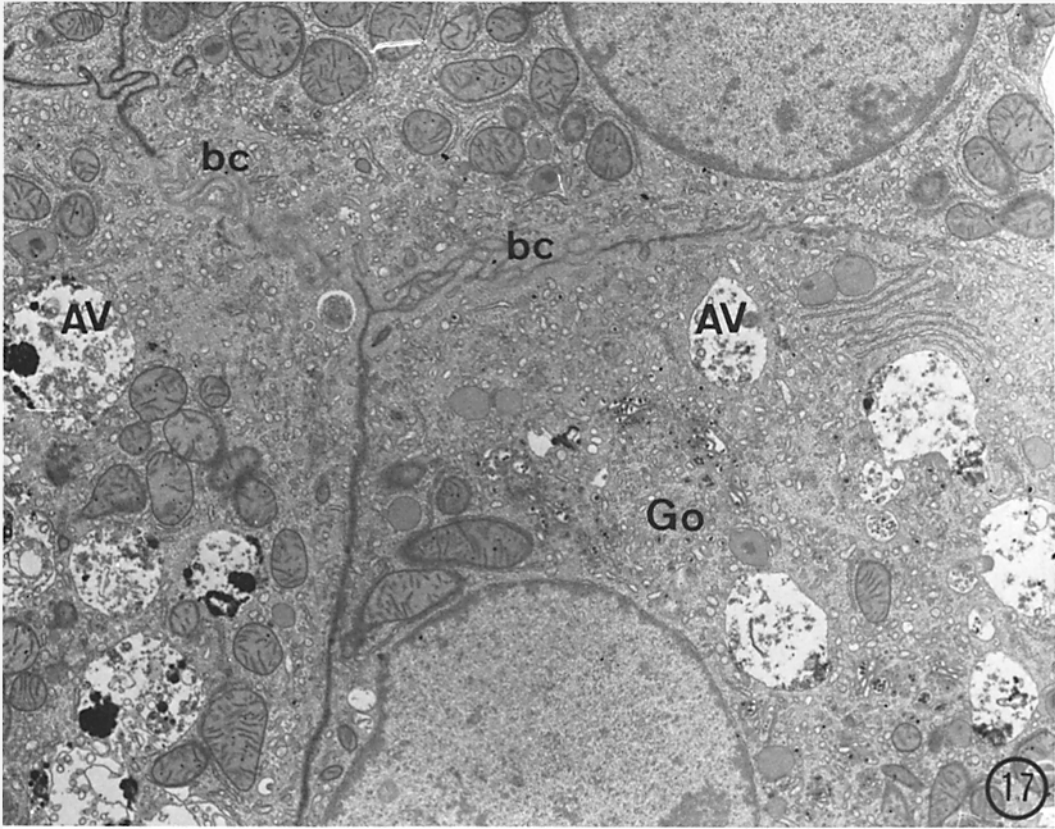
the RER to the SER and the Golgi cisternae. This transport appears to be associated with changes in size and in lipid composition, characterized by a progressive enrichment in triglycerides and phospholipids and a decrease in protein concentration (19). Finally, VLDL particles are extruded into the space of Disse. The synthesis and secretion of VLDL have been previously observed in isolated cell suspensions (25). In our experimental cell culture model, newly formed VLDL particles are detected, after 24 h of incubation, either in the smooth endoplasmic reticulum or in the Golgi cisternae. These particles are then principally extruded into the extracellular space, but some of them are also detected in the lumen of the bile canaliculi. Biochemical evidence of the lipoprotein nature of the particles discharged into the bile ducts would confirm the possible flow of substances from the Golgi areas to the bile canaliculi, which is never observed *in situ* but which appears to be clearly shown under these particular conditions of cell culture.

We may raise, here, the question as to whether the functional recovery of the biliary polarity is completed. The formation of an extracellular bile compartment delineated by tight junctions does not necessarily imply that the selectivity of the secretion into this compartment is effective on the second day of culture. Further investigation of the hepatocytic secretory processes will be necessary to demonstrate the functional integrity of the bile secretion.

Adult hepatocytes cultured under our conditions retain their ultrastructural characteristics for at least 3 days. The cell membrane differentiations and the biliary polarity subsist intact during this period of time. The trabeculae remain well attached to the bottom of the petri dishes. However, after 3 days in culture, the hepatocytes involute unless insulin is added to the incubation medium. The effects of the hormone on cultured hepato-

cytes are illustrated in detail in the accompanying paper.

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FIGURE 17 Hepatocytes incubated for 48 h in the presence of Dulbecco's medium supplemented with 17% fetal calf serum. An important accumulation of autophagic vacuoles (*AV*) occurs within the cytoplasm of nearly all the cultured hepatocytes. The vacuoles contain membrane derivatives and few glycogen particles. Notice the presence of a well developed Golgi complex (*Go*) and mature bile canaliculi (*bc*). × 7,800.

FIGURE 18 Detailed view of the peripheral cytoplasm of adult hepatocytes incubated for 48 h. Numerous microfilaments (*fi*) and microtubules (*tu*) run parallel to each other and delineate a cytoplasmic border devoid of other cell organelles. × 23,000.

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