THE CIRCUMFUSION SYSTEM FOR MULTIPURPOSE CULTURE CHAMBERS

II. The Protracted Maintenance of Differentiation of Fetal and Newborn Mouse Liver in Vitro

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ABSTRACT

The circumfusion system is a complex in vitro pumping unit incorporating 12 multipurpose culture chambers through which a serum-supplemented fluid nutrient is recirculated at a rate of 4.5 ml/min per chamber. This system was used to study the differentiative responses of fetal and newborn mouse liver explants placed in the serum-free environment formed between the sheets of unperforated cellophane and cover glasses of the chambers. Hepatocytes (parenchymal cells) were discernible in 3–5 days. They retained many of their features of differentiation in the circumfusion system for more than 120 days of cultivation. The living morphological characteristics of the hepatocytes were studied by phase-contrast microscopy (direct viewing and time-lapse cinemicrography) and by special cytochemical staining. Electron micrographs were made of both fresh liver specimens and the cultured cells. Comparisons of the cultured parenchymal cells with their in vivo progenitors showed a remarkable preservation of their differentiated state.

INTRODUCTION

The circumfusion system[1] (36) was recently described as a self-contained tissue culture device which supports 12 interconnecting multipurpose culture chambers (32). Briefly, all chambers of the system are connected to two reservoir bottles by two 30 inch lengths of Teflon spaghetti tubing. In this way, the chambers receive a flowing supply of fluid nutrient from a common repository (the positive-pressure reservoir bottle) at a rate of 4.5 ml/min per chamber. Rapid recirculation is regulated by a peristaltic pump which moves the nutrient (400 ml total volume per system) through two polyvinyl chloride tubes from the negative-pressure reservoir bottle to the positive-pressure reservoir bottle at a rate of 54 ml/min. The negative-pressure bottle receives 4.5 ml/min from each of the 12 chambers; this completes the circulation pattern. The Teflon tubing and the polyvinyl chloride tubing are gas permeable. Since the oxygen tension of the atmosphere is higher than that of the nutrient, an ingression of oxygen occurs through the tubing so that the nutrient entering each chamber is oxygen satu-

[1] A 16 mm motion picture film describing the circumfusion system and showing the results obtained with it is available from the film libraries of the Tissue Culture Association, Pasadena Foundation for Medical Research, Pasadena, Calif. 91101, or the Armed Forces Institute of Pathology, Washington, D. C. 20305.

rated, namely, pO_2 = 160 mm Hg. Conversely, CO_2 is lost from the nutrient to the atmosphere through the tubing, but this loss is corrected by the immersion of an 8 foot long coil of Teflon tubing in the positive-pressure reservoir bottle through which pure CO_2 flows at a slow rate, the CO_2 diffusing through the Teflon into the fluid nutrient.

In circumfusion systems, the multipurpose culture chambers have been used with the cellophane sheet method (33, 35, 38). With this technique, a serum-free microenviroment is formed in each chamber between the cover slip and a sheet of unperforated cellophane cut from dialysis tubing. In earlier experiments, the effects of culturing in such thin compartments were found to be favorable for the maintenance of differentiation of a number of chick embryonic tissues for prolonged periods of time (34, 35, 37, 39, 40). The cellophane sheet method of cultivation coupled with the effects produced by the recirculation of nutrient in the circumfusion system were found to enhance the degree, as well as the longevity, of differentiation of chick embryo explants (36).

The purpose of this second paper is to report the resultant differentiation and maintenance of histotypic patterns observed in fetal and newborn mouse liver explants cultivated in the circumfusion systems for prolonged periods. The normalcy of cell structure and orientation of cells to one another, as well as their functional activity observed by serial phase-contrast micrographs, time-lapse cinemicrography, cytochemical staining, and electron microscopy, indicated a remarkable preservation of their differentiated state for more than 120 days.

MATERIALS AND METHODS

Mouse Colony and Specimen Procurement

Strain C_3H pregnant mice were obtained from a commercial source (Microbiological Associates, Inc., Bethesda, Md.). Caesarean sections were done after 16–20 days of gestation and the fetal mice were placed in sterile Petri dishes. Newborn mice were obtained 6–12 hrs after delivery. The mice were sacrificed by compressing the neck area with forceps and were washed with ethanol-impregnated sponges before dissection. Sterile procedures were used to make midline abdominal incisions for the dissection and removal of liver specimens. The ages of the various mouse broods used in this study are listed chronologically in Table I.

Preparation of the Explants for Cultivation

The liver specimens were placed in sterile petri dishes and washed with three changes of Waymouth's medium MB 752/1. After being washed, the organs were cut into smaller pieces, about 0.5 mm in diameter, and rewashed; 4–6 pieces were placed on the cover slips of 1–4 chambers. Glass cover slips (No. 1) were used for studies with the light microscope, and mica cover slips (Albert A. Henning and Co., New York) were used for procedures with the electron microscope. Sheets of unperforated dialysis cellophane, prepared as previously described (35, 38) and moistened with the Waymouth's medium, were gently laid over the explants. The chambers were fabricated in the manner described in the first publication on the circumfusion system (36). Other tissues and organs used in systems containing the liver were ovary, testis, adrenal, salivary gland, tooth germ, heart muscle, skeletal muscle, and thyroid.

TABLE I

Age of Mouse Broods Used in Study

M-1	16- to 17-day fetal mice
M-2	17-day fetal mice
M-3	18-day fetal mice
M-4	20-day fetal mice
M-5	Newborn mice

The Circumfusion System

The tissue fragments were placed in Petri dishes containing the MB 752/1 nutrient not supplemented with serum. Similarly, the cellophane was washed in this serum-free nutrient before its use in the chambers. After all chambers were fabricated, the two empty reservoir bottles attached to the circumfusion system unit during autoclaving were exchanged for bottles containing serum-supplemented nutrient; the positive-pressure bottle contained 250 ml of nutrient and the negative-pressure bottle 150 ml of nutrient. The polyvinyl leads were fed through the peristaltic pump, the chambers were placed in the stacking rack, and the pump was activated as illustrated and detailed in the first report (36).

Nutrient

The nutrient formula for mouse tissues was: Waymouth's medium MB 752/1, 75%; calf serum, 15%; whole egg ultrafiltrate, 5%; bovine ultrafiltrate, 5%; phenol red, 0.001%; and penicillin G, 1,000 units/ml. Polycarbonate centrifuge bottles

(250 ml capacity) were filled with this nutrient and stored in a refrigerator at 5°C. At specified intervals, the positive-pressure bottle of a circumfusion system was removed and replaced with a bottle containing fresh nutrient. At first, this was done semiweekly, but it was found later that weekly exchanges were equally effective.

Staining Procedures

For staining, chambers were detached from the circumfusion system, emptied of their nutrient, and injected with the indicated fixative. The fixative, with the exception of that used in the May-Grünwald–Giemsa method, penetrated the cellophane; after 10 min, the chamber was opened, the cellophane stripped away from the culture, and the slide washed and stained.

May-Grünwald–Giemsa: Fixation was accomplished by injecting methyl alcohol under the cellophane. When alcohol fixatives are used with a full sheet of cellophane, fixation in this way rather than through the cellophane is essential, because the alcohol removes water from the culture environment so rapidly that it effects a compression of the cells by the cellophane before the cells have been fixed, often severely distorting them. After removal of the cellophane, the staining procedure was done according to the method of Jacobson and Webb (18).

Hematoxylin and Eosin: Zenker's formol and routine staining

Acid Phosphatase: Cold (4°C) formol-calcium fixation and staining according to Gomori's lead-glycerophosphate procedure (12).

Alkaline Phosphatase: Cold (4°C) formol-calcium fixation and staining according to Gomori's lead-glycerophosphate procedure (12).

Adenosine Triphosphatase: Cold (4°C) formol fixation and staining according to both the Wachstein-Meisel (53, 54) and the Padykula-Herman (26, 27) procedures.

Glycogen: Cold (4°C) formol fixation and staining according to the PAS method of Hotchkiss (16).

Electron Microscopy Procedures

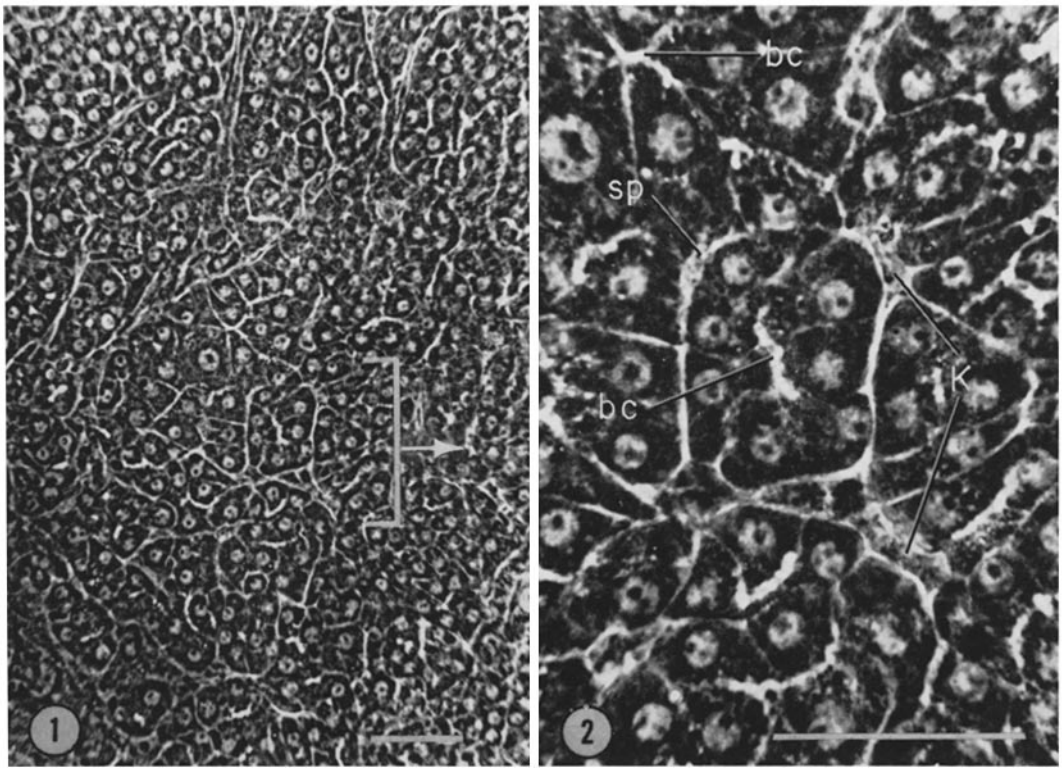
It is virtually impossible to remove polymerized epoxy resins from glass cover slips. However, Kumegawa et al. (20) found that mica cover slips (40), from which the resins can be removed, were a satisfactory substitute for the glass. In this study, tissue cultures designated for electron microscopy were cultivated on mica cover slips. The explants were fixed directly in the chambers with phosphate-buffered (0.15 M) glutaraldehyde (1%) and, after removal of the cellophane, phosphate-buffered (0.15 M) osmium tetroxide (1%). Some explants were then prestained with uranyl acetate (2%) and infiltrated with the Araldite resin in the chamber. After polymerization, a disc of the resin, approximately 1 inch in diameter by ⅛-inch thick, was removed from the chamber, inspected, encircled with a diamond objective marker, cut, and prepared for sectioning as recently reported in detail by Kumegawa et al (20). Fresh liver specimens also were similarly fixed and embedded.

The embedded specimens for electron microscopy were sectioned on a Porter-Blum II microtome provided with glass knives. The sections were stained with a 0.2 to 0.5% lead citrate and examined in an RCA EMU-3 electron microscope.

RESULTS

The cultivation of embryonic and fetal tissues in circumfusion systems is somewhat different from that generally found in reports of tissue culture studies. These explants may be thought of as tissue culture-organ culture hybrids. Even though explants growing on glass or mica cover slips and covered with unperforated cellophane membranes are thinly spread, many cell types are not as flat as those found in monolayers of cell strains growing on the walls of culture vessels or in outgrowths of primary explants under sheets of perforated cellophane. In circumfusion systems, many organ-specific characteristics of fetal explants are substantially preserved. Generally, the differentiated liver cells have considerable depth and often are multilayered so that focusing with the phase-contrast microscope seldom yields as sharp an image as is possible with flattened cells of strains growing directly on glass cover slips in monolayers. It should also be emphasized at the beginning that the liver explants were never the only tissues in one circumfusion system, each system always having male and female gonadal explants as well as some of the other tissues listed in the Materials and Methods section. The alternate condition of having 12 chambers containing only liver explants has not been examined at this time.

The cultivation of chick embryonic tissues in a V-614 supplemented media (11) was described in the first report of this series. Hepatocytes were shown after 20 days of cultivation, but rarely were many found to persist in histotypic patterns much longer than 3 wk. Sheets of hepatocytes broke up and the cells became difficult to recognize as unequivocal elements of the parenchyma by phase microscopy. Chick liver was not evaluated in the Waymouth's media. However, with the fetal and newborn mouse liver explants cultivated in the enriched media of Waymouth, differentiated hepa-



Abbreviations

Ac Ph, acid phosphatase stain	*L*, lipid
Alk Ph, alkaline phosphatase stain	*ly*, lysosome
bc, bile canaliculus	*M*, mitochondria
d, desmosome	*mb*, microbody(ies)
E, erythrocyte	*mv*, microvilli
f, cytoplasmic filaments	*N*, nucleus
g, glycogen	*rer*, rough-surfaced endoplasmic reticulum
Go, Golgi complex	*S*, sinusoid
hg, hepatic granules (also hg_1, hg_2, etc.)	*sp*, sinusoidal-perisinusoidal complex
K, Kupffer cells	*0′*, *15*, *73*, etc., zero minutes, *15* minutes, *73* minutes, etc.

FIGURES 1 and 2 Low- and medium-power (10 ×, 40 × objectives) views of parenchymal cells in a 12 day culture of liver from the 18 day fetal mouse brood (M-3). The phase-white sinusoidal-perisinusoidal complex (space of Disse) and bile canaliculi are not easily distinguished at low powers. The rectangular lobule in Fig. 2 appears to have a bile canaliculus (*bc*) in the center and to be surrounded by a sinusoidal-perisinusoidal complex (*sp*). Kupffer cells (*K*) are also shown as part of this complex. Magnification lines = 60 μ. Phase contrast. × 215 (10 ×); × 550 (40 ×).

tocytes were observed to persist 3–4 months, although there was a progressive waning of the total number and mitoses of parenchymal cells commencing after 3–4 wk of cultivation.

A low power view of a 12 day culture of hepatocytes from a liver of the 18 day fetal mouse brood (M-3) is shown in a lobular orientation in Fig. 1. These lobules are composed of 2–10 cells and, with the phase-contrast microscope, appear separated by a broad phase-white spacing. The central portion of this spread of hepatocytes is shown at higher power in Fig. 2, and the rectangular lobule of seven cells is shown again, with use of the oil-immersion 100 X objective, in Fig. 3. Many

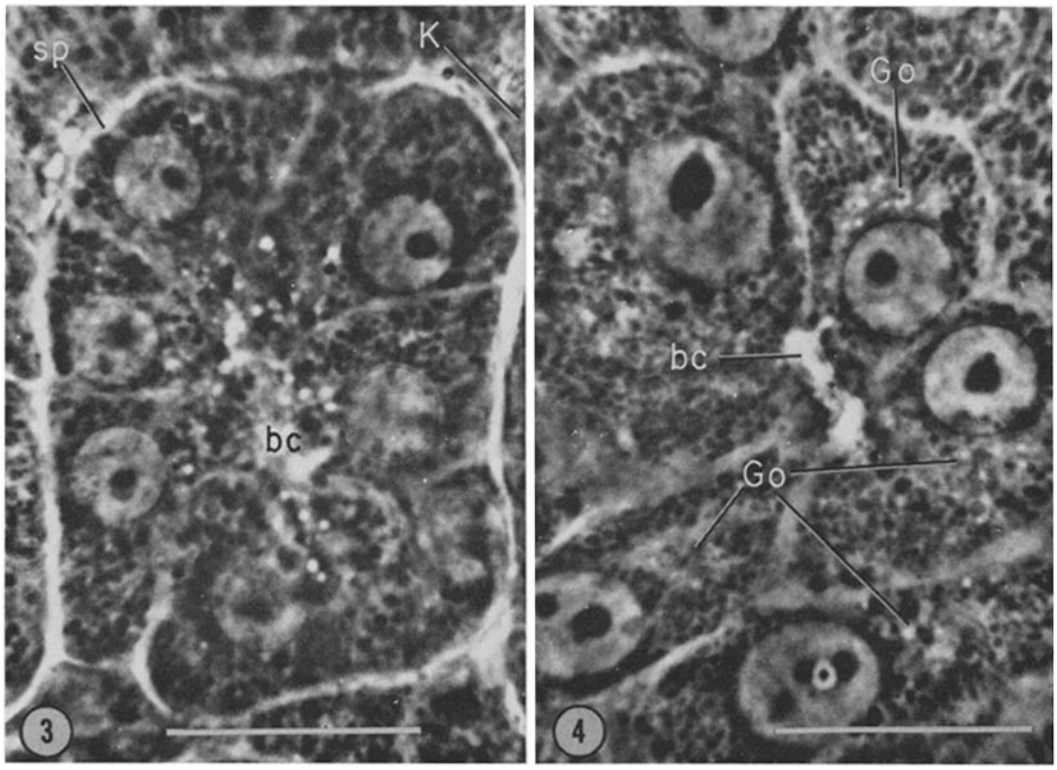
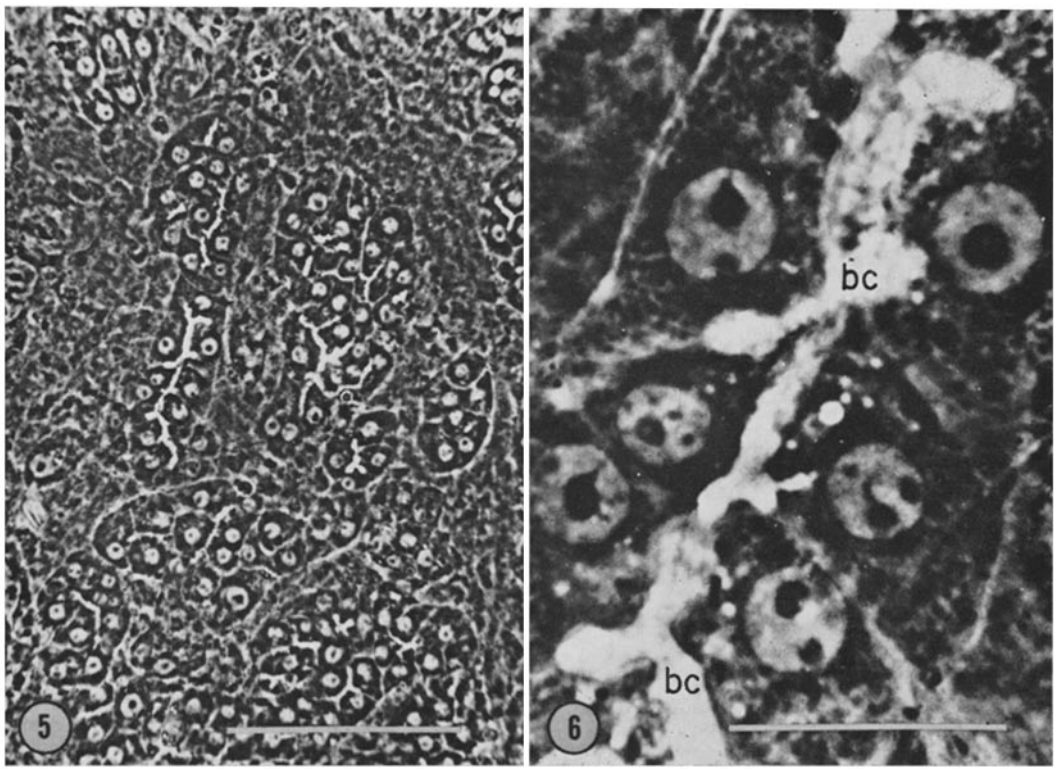


FIGURE 3 The central rectangular lobule of parenchymal cells from Figs. 1 and 2 shown at high-power (100 × objective). Phase-white droplets in the center of the lobule are indicative of secretory movement toward the centrally located bile canaliculus (*bc*). The Kupffer cell (*K*) is seen as part of the sinusoidal-perisinusoidal complex (*sp*) surrounding the lobule. Magnification line = 20 μ. Phase contrast. × 1,700.

FIGURE 4 High-power (100 × objective) view of parenchymal hepatocytes from the 18-day fetal mouse brood (M-3) after 12 days of cultivation. A small bile canaliculus (*bc*) occurred in the central portion of the cluster of hepatocytes which were spread more thinly in this area than in Figs. 1–3. The phase-gray agranular juxtanuclear areas contained small phase-white droplets representative of the Golgi complex (*Go*). Magnification line = 20 μ. Phase contrast. × 1,700.

phase-white vesicles are seen in the center of this lobule intermingled with phase-dark granules, and one would suspect this to be an area of secretion analogous to that of the bile canaliculi. Similarly, the surrounding space at the opposite poles of the cells appeared to be analogous to a sinusoidal-perisinusoidal complex. At this age of cultivation (12 days), most nuclei contained one or two prominent nucleoli, although some had more, and the cytoplasm contained large phase-black granules and often a clear juxtanuclear Golgi zone (Fig. 4) from which phase-white droplets emanated. Depending upon the tensions exhibited by various portions of the explant, the patterns of hepatocytic clusters (lobules) varied. Occasionally, the clusters were seen as multiple cords of rectangular cells.

One of the peculiarities of the cellophane-sheet method of cultivation with multipurpose culture chambers is that soft explants become flattened in a few days, varying, of course, according to the size and consistency of the explants and their positioning under the cellophane. This flattening occurs because of the compressive effect of the cellophane on the explant enforced by the removal of water from the culture environment by osmosis. In a week or two, many soft tissues become nearly uniformly compressed, although the individual parenchymal cells are not usually as flat as the peripheral elements of cell strains growing on a

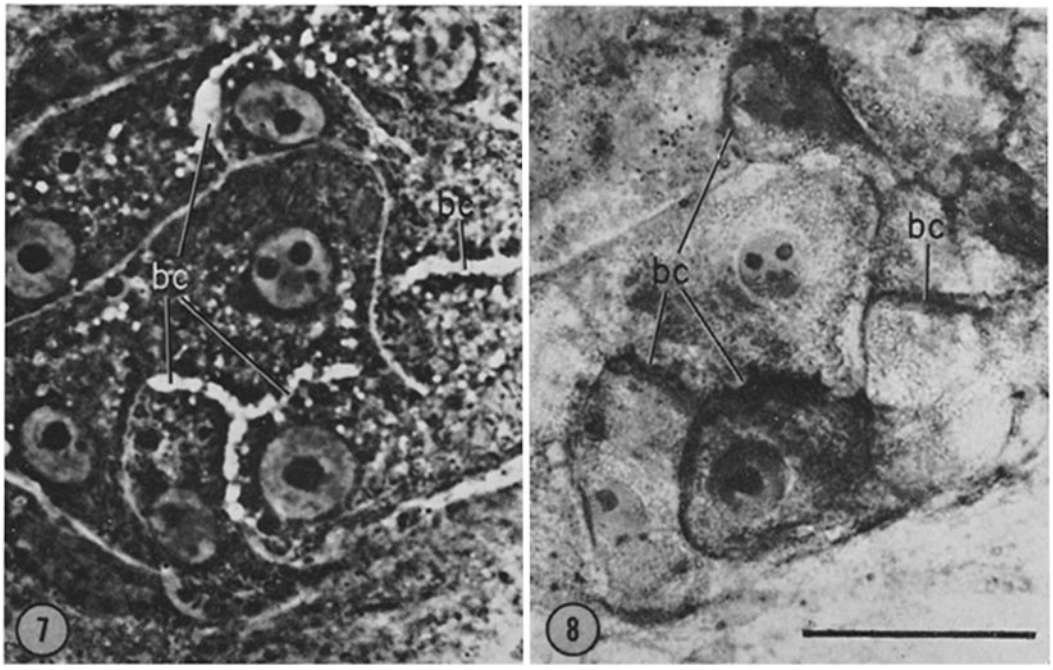


FIGURES 5 and 6 Low- and high-power (16 ×, 100 × objectives) views of a 12 day culture of liver parenchymal cells from the 18 day fetal mouse brood (M-3). In this culture, the hepatic clusters have become spread thinly, so that wide spaces of stromal tissue occur between the clusters. The vertically aligned bile canaliculus between the two rows of cells appears especially full of secretory accumulations. Magnification lines = 100 μ (16 ×); 20 μ (100 ×). Phase contrast. × 315 (16 ×); × 1,900 (100 ×).

glass substrate. In Fig. 5, for instance, the groupings of parenchymal cells after a 12 day cultivation period from another M-3 explant of the liver were compressed, resulting in a wider separation of the clusters than those seen in Figs. 1–3. The left central cluster of Fig. 5 is shown under higher magnification in Fig. 6, and the vertical phase-white intercellular zone can be seen between the two rows of cells. This zone is a distended bile canaliculus. Bile canaliculi in vivo have been shown to stain positively with the alkaline phosphatase and ATPase reactions. Cultures fixed in cold formol revealed strong positive responses for ATPase in areas which coincided with the phase-white intercellular spaces (Figs. 7 and 8). A positive alkaline phosphatase response was observed in these same intercellular areas (Figs. 9 and 10), and both reactions substantiated that these phase-white spaces between in vitro cells were analogous to the bile canaliculi of in vivo hepatocytes. Similarly, the parenchymal cells gave a positive response for acid phosphatase at the site of the large cytoplasmic granules located between the nucleus and the bile canaliculus (Figs. 11 and 12), indicating their lysosomal nature.

Generally, after 3–4 wk, hepatocytes contained large and pleomorphic cytoplasmic granules on the side of the cell with the prominent bile canalicular space (Fig. 11), whereas in the younger cultures smaller granules were more uniformly distributed throughout the cytoplasm regardless of the secretory activity (Fig. 4). These smaller granules were also seen in the older cultures with the large granules.

Fig. 13 is a montage of a group of low-power micrographs showing a portion of an M-3 liver explant and outgrowth after 35 days of cultivation. The montage represents about 5-10% of the total



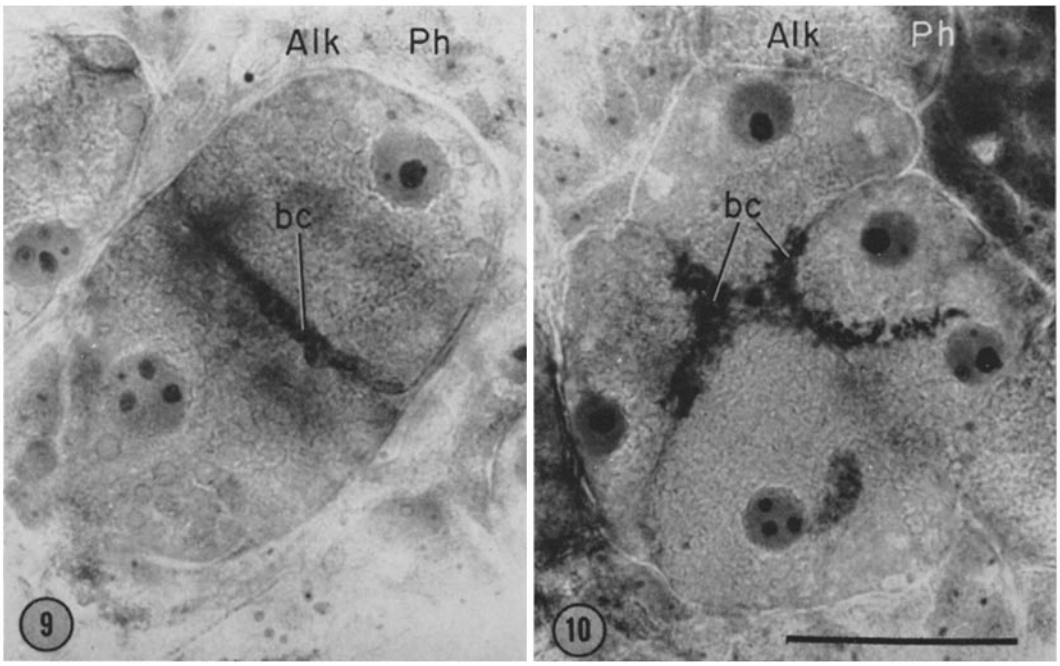
FIGURES 7 and 8 High-power (100 × objectives) views of a 22 day culture of parenchymal cells of the 17 day fetal mouse brood (M-2). Fig. 7 shows a cluster of hepatocytes before fixation by phase contrast, and Fig. 8 is the same cluster after staining for ATPase (Padykula and Herman). The phase-white bile canaliculi (*bc*) are seen to be the reactive sites. Magnification line = 30 μ. Phase contrast and bright field. × 1,050.

explant area, the center of the explant being to the lower left of the figure. By 35 days, stromal matrices had become less cellular and more collagenous. Clusters of macrophages (Kupffer cells) and other unidentified cells can be seen peripheral to the explant where they were growing either on the glass cover slip or on the cellophane.

Hepatic cords grown out from the M-3 liver explants were maintained in a circumfusion system for 128 days; the character of the hepatocytes remained essentially unchanged. Although the large cytoplasmic granules became progressively more numerous. A cluster of hepatocytes from an M-5 liver culture was photographed with the time-lapse cine phase system between days 82 and 115 of cultivation. An abstract of this time-lapse movie film is shown in Fig. 14. The recording shows a bile canaliculus increasing in size, undergoing a dispersion of droplets during and emptying phase, and then refilling again during a 263-minute segment of the recording.

The response of cultured hepatocytes to the general and special staining procedures listed in the section of Materials and Methods associated the cultured hepatocytes with those found in vivo.

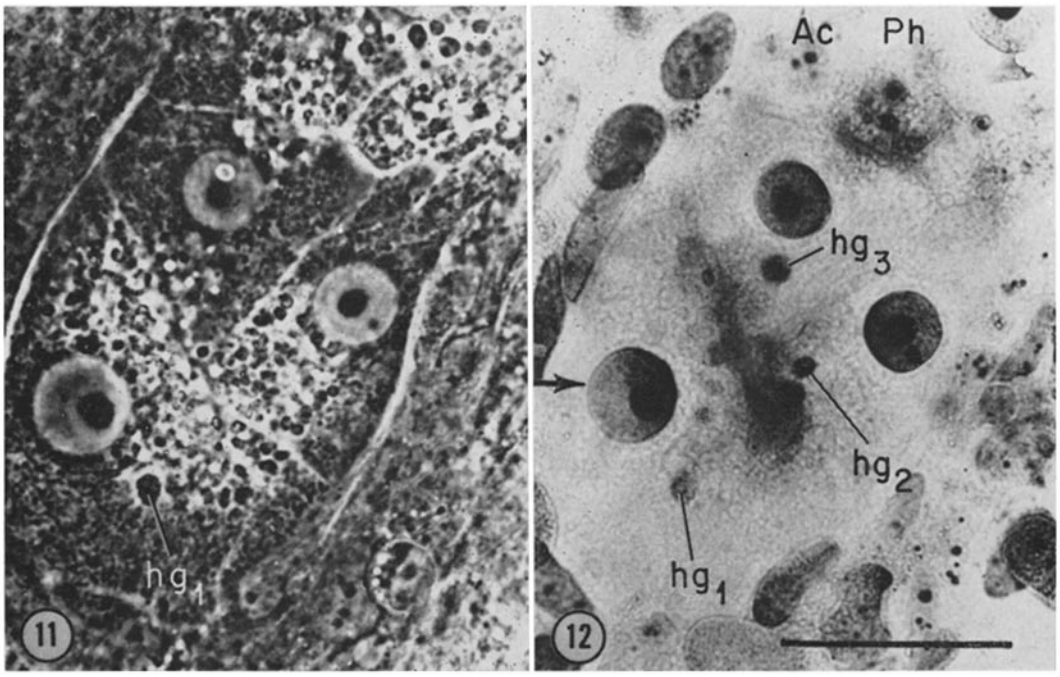
Electron micrographs of the in vivo fetal and newborn mouse liver are shown in Figs. 15-17 and of the cultures growing on the mica cover slips in Figs. 18-23. The prominent features of fetal liver parenchymal cells are the bile canaliculi with multiple microvilli projecting into them, aggregations of glycogen, and lipid bodies. Glycogen was also noted within mitochondria (Fig. 15). The lysosomes and microbodies, also characteristic of liver tissue, are not notable features of the fetal liver parenchymal cells. In the newborn mouse (Figs. 16 and 17) a common feature is the presence of lipid not only in the cytoplasm but in the nucleus as well, and small units of lipid often were found near the nuclear pores (Fig. 17). Glycogen usually was absent in liver parenchymal cells in the newborn. The presence of lipid in



FIGURES 9 and 10 High-power (100 × objectives) view of parenchymal cells from the M-4 fetal mouse liver stained for alkaline phosphatase after 33 days of cultivation. The reaction was strongly positive in the bile canaliculi (*bc*) and nucleoli. Magnification line = 30 μ. Bright field. × 1,035.

nuclei similarly was found in some cases of the cultured parenchymal cells (Fig. 18).

As in the in vivo specimens, the characteristic components of the cultured parenchymal cells were the bile canaliculi, lipid bodies, and glycogen. Generally, the mitochondria were slightly smaller than those found in vivo. After 1 wk of cultivation, microbodies and lysosomes commenced to appear in greater numbers. In Fig. 19, the abutting edges of two parenchymal cells after 1 wk of cultivation are shown with a distended bile canaliculus between them. In the lower left corner, there is an aggregation of single membrane-bound, smooth-surfaced, crystalloid-free small bodies which appear to be a cluster of immature microbodies. Aggregations of this sort were not found in the in vivo specimens but were found in the 1- to 2-wk-old cultures and rarely in the 4-wk-old cultures. In the right of this micrograph, an elongated mitochondrion also shows a small cluster of glycogen granules in a circumscribed crista, as described for the in vivo specimen in Fig. 15. In Fig. 20, a small area of aggregated immature microbodies is shown in a cell after a 2 wk cultivation. These single membrane-bound units appear identical with those described for microbodies in adult parenchymal tissue except that they do not contain crystalloids or nuclei. After 2 wk of cultivation, the microbodies were found not only in aggregates without crystalloids, but singly with crystalloids. By 4 wk (Fig. 21) they appeared mature as described for adult parenchymal cells and were found scattered in the cytoplasm among the mitochondria and increasing numbers of enlarging lysosomes. Several typical mature microbodies are shown at higher power near a rare bundle of wavy filaments in Fig. 22. These filaments usually were adjacent to, or surrounded by glycogen and were not found in parenchymal cells until after 4 wk of cultivation. The higher power view of one especially ordered bundle is shown in Fig. 23. There is a general periodicity to the waves and the spacing between these filaments, as shown in the drawing (insert, Fig. 23).



FIGURES 11 and 12 High-power (100 × objective) views of parenchymal cells from the M-1 fetal mouse liver after 3 wk of cultivation. Fig. 11 is a phase-contrast view of the living cells and Fig. 12 a bright field view after staining for acid phosphatase. The hepatic granules (hg_1, hg_2, and hg_3) stained strongly; other phase-black granules were negative. Magnification line = 30 μ. Phase contrast, bright field. × 1,035.

DISCUSSION

The first report on the circumfusion system (36) illustrated the efficacy of this technique with chick embryo tissues. The purpose of this second report is to demonstrate in detail the possibilities for growth of a mammalian cell type (liver cells) generally found difficult to cultivate. It was shown that the cells of the liver from the fetal and newborn mouse were well preserved in a differentiated state for long periods in the circumfusion system.

An old axiom from Doljanski's early studies of liver in vitro (3) was later amplified by Fell (10), Reisner (30), and Robineaux (31). It has direct bearing on this discussion. Doljanski concluded that cells which divide and emigrate rapidly lose their differentiation, whereas cells which do not divide nor emigrate rapidly are more likely to retain their differentiation. It seems that cells of nonproliferative organs, particularly in culture, cannot do both very long, for one excludes the other, and the present studies with the circumfusion system strongly support this axiom.

A primary reason for the difficulty in cultivating liver parenchymal cells in their differentiated form has been attributed to the inability of cultivating systems to provide sufficient oxygen requirements (8, 17) for aerobic glycolysis. Sandström (42–45) attempted to alleviate this problem by using the multipurpose culture chamber and a special cultivating condition. Chick embryo liver pieces were placed on an unperforated cellophane sheet lying on one of the cover slips and covered by a perforated sheet of cellophane. Using minimal amounts of nutrient and a gas phase, Sandström was able to cultivate chick embryo hepatocytes for 15 days and attributed this success to the greater availability of oxygen to the cells. This technique was similar to that of Trowell (50, 51) for organ culture as both procedures provided fluid and gas phases, although Sandström's used a compressed environment and Trowell's was an uncompressed organ culture environment. Stevens (49) calculated the minimal requirements of liver pO_2 in dogs and used the technique of Trowell to con-

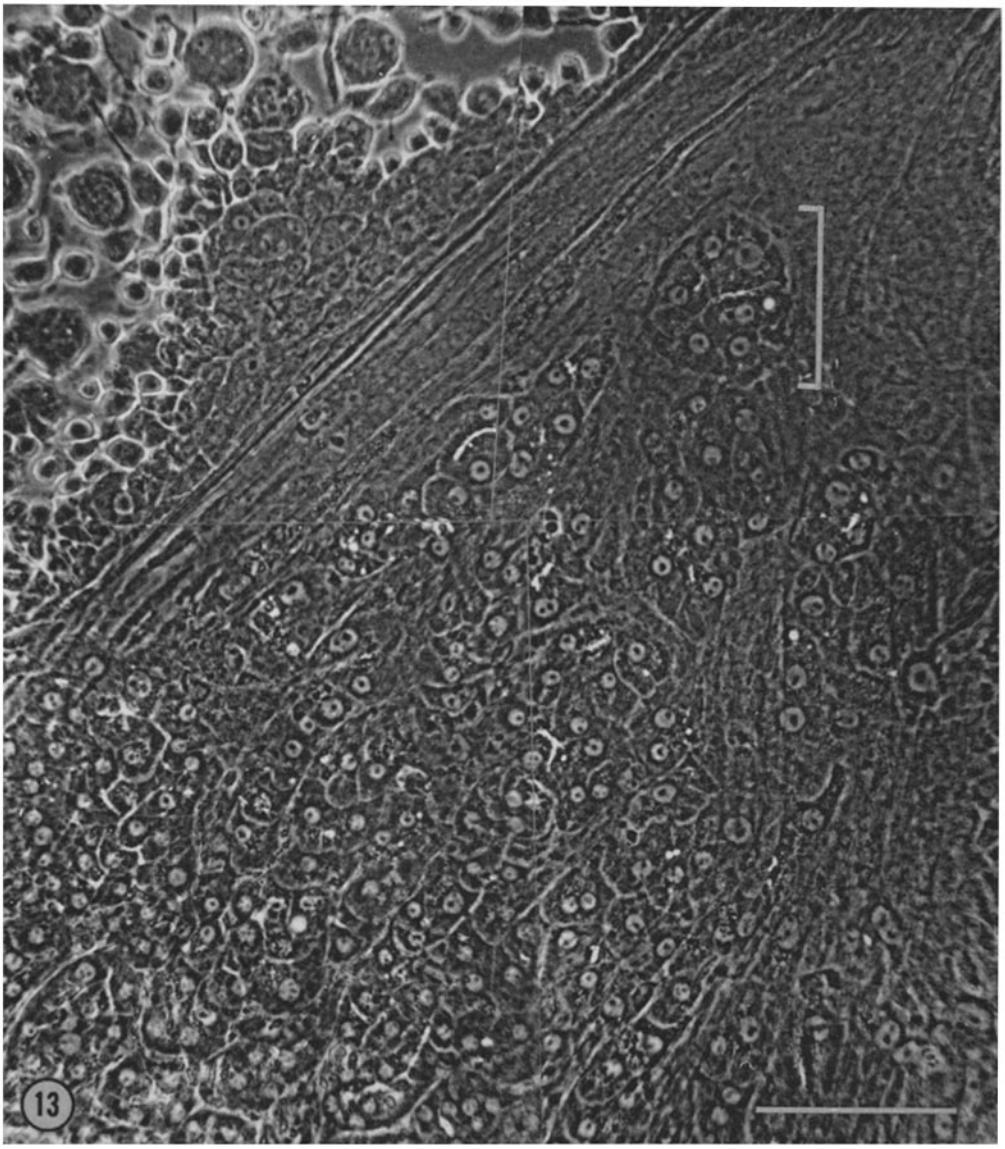


FIGURE 13 Low-power (16 × objective) montage of micrographs showing a 35 day cultivation of the liver parenchyma from the 18 day fetal mouse brood (M-3). The parenchyma is spread thinly on a stromal matrix and surrounded by Kupffer cells and other unidentified cells. The major portion of the explant is to the lower left of the micrograph. The hepatocytes are progressively flatter toward the upper right periphery. Magnification line = 100 μ. Phase contrast. × 230.

clude that liver cells could not obtain the energy needed for their maintenance and division in tissue culture from aerobic respiration alone. Since liver cells have a very low anaerobic glycolysis, Stevens stated that it was simply impossible for the liver to obtain energy in this way and that liver cells, therefore, would not survive; whereas cancer cells, fetal cells, and fibroblasts, having high anaerobic glycolysis, would survive. In our cultures of liver cells from the mouse, fetal cultures demonstrated a more uniform outgrowth of hepatocytes than the newborn explants; this is

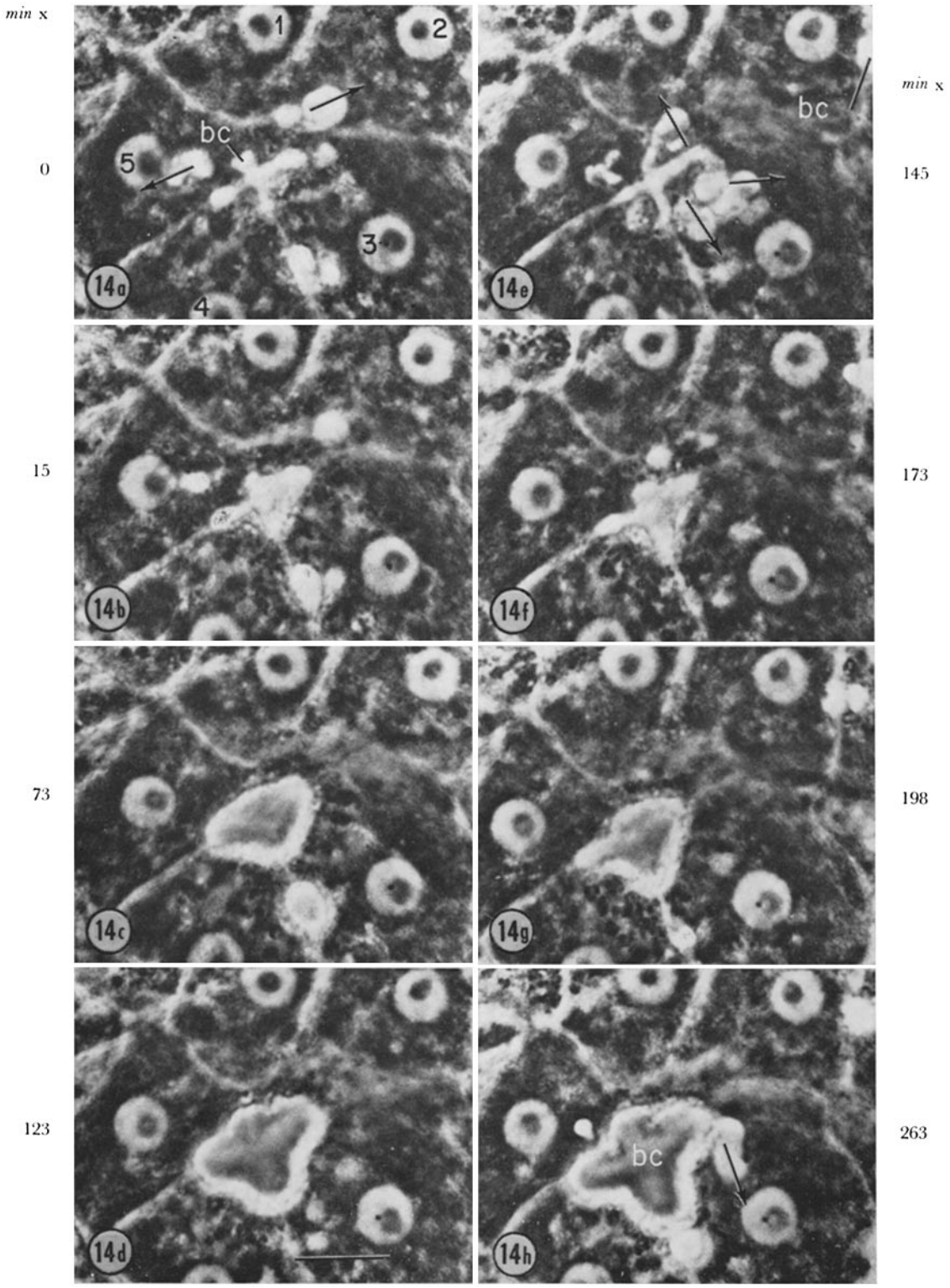


FIGURE 14 A series of time-lapse micrographs abstracted from a movie film. The cluster of five parenchymal cells was in a small group of 10 cells cultivated from the M-5 brood mouse liver. They were first observed and photographed after 70 days of cultivation. This series began after 82 days of cultivation and shows the secretory activity over a 263 minute period of filming. The bile canaliculus was filled twice during this observation period. Arrows indicate droplet movement, and the minute time of the sequence is noted in the lower right corner of each micrograph. Magnification line = 20 μ. Phase contrast. × 750.

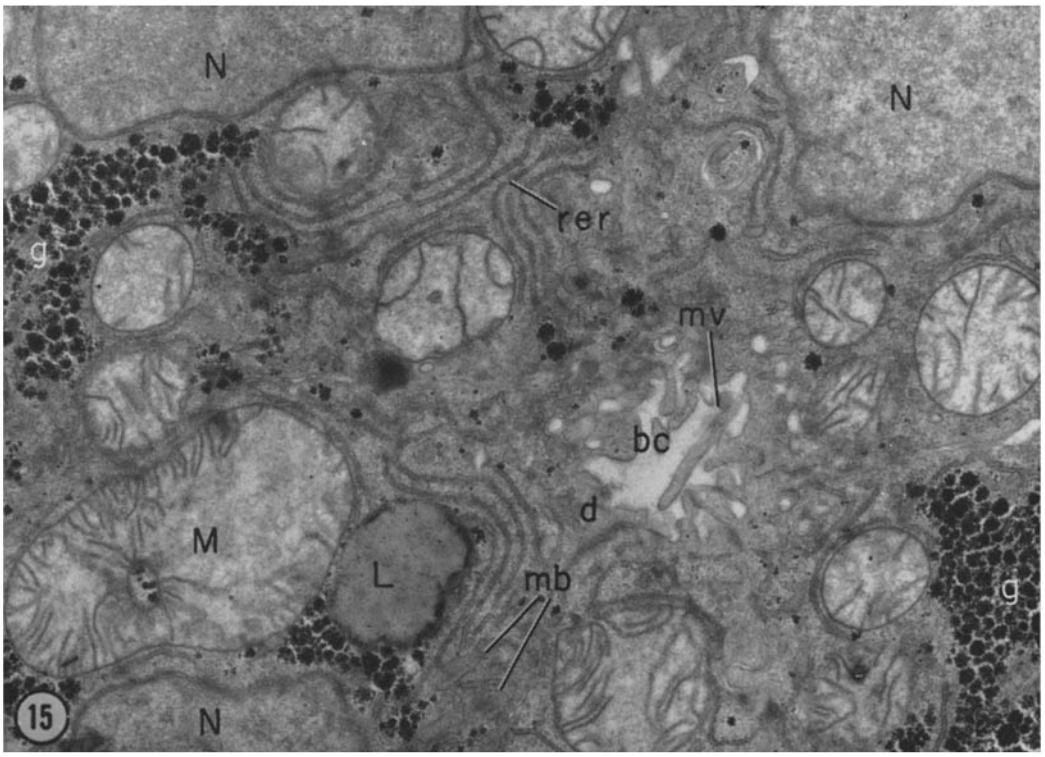
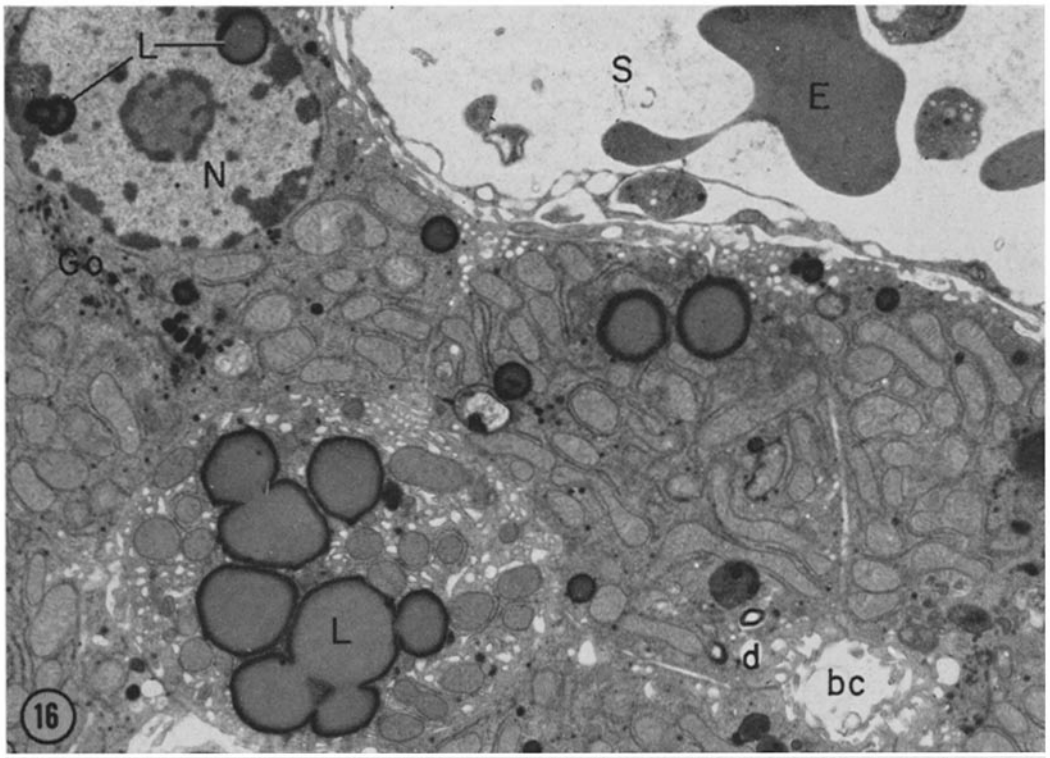


FIGURE 15 Electron micrograph of the 16-17-day fetal mouse (M-1) liver parenchymal cells (in vivo). The prominent features are the bile canaliculus (*bc*) with microvilli (*mv*), glycogen granules (*g*), lipid (*L*), and round or oval-shaped mitochondria (*M*). A desmosome (*d*) is also noted below the bile canaliculus. The mitochondrion in the lower left corner shows an accumulation of glycogen surrounded by one of the cristae. Faintly visible microbodies (*mb*) with crystalloids appear in the lower center. × 17,000.

compatible with Steven's conclusions about fetal tissues. However, hepatocytes were observed in newborn tissues as well, and the contribution of the circumfusion system to the technique of cultivation with the multipurpose culture chamber was shown to be of considerable advantge. The primary advantage to the liver cells quite likely is the high oxygen tension in the rapidly circulating nutrient. This was measured with the Beckman-Spinco gas analyzer (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) to be 160 mm Hg, and since the circulating fluid passes through each chamber at 4.5 ml/min, this oxygen tension is maintained. The uniformity of nutrient availability and excretory removal as well as the smoother temperature control in the circumfusion system obviously favor the total well-being of the cells being cultivated, as revealed by the morphology of the liver cells and the longevity of their differentiative characteristics when analyzed by phase-contrast viewing, time-lapse cinemicrography, cytochemical responses, and electron microscopy. An unexplored added component of circumfusion systems is the pulsatile pressure change in each chamber (108 pulses/min and a pulse pressure of 3 mm Hg).

However, there have been recent reports (13) on the cultivation of liver cells with the maintenance of parenchymal cells by using the reconstituted rat-tail collagen technique of Ehrmann and Gey (4). Bang and Warwick (1) report that it is possible to keep differentiated human fetal liver cells alive on the collagen for several months (100 days), although the multiplication of parenchymal cells was not observed after 3 wk (14). Conversely, Hillis and Bang (14) could keep mouse parenchyma differentiated on the collagen only for an average of 25 days. Wantanabe (55)



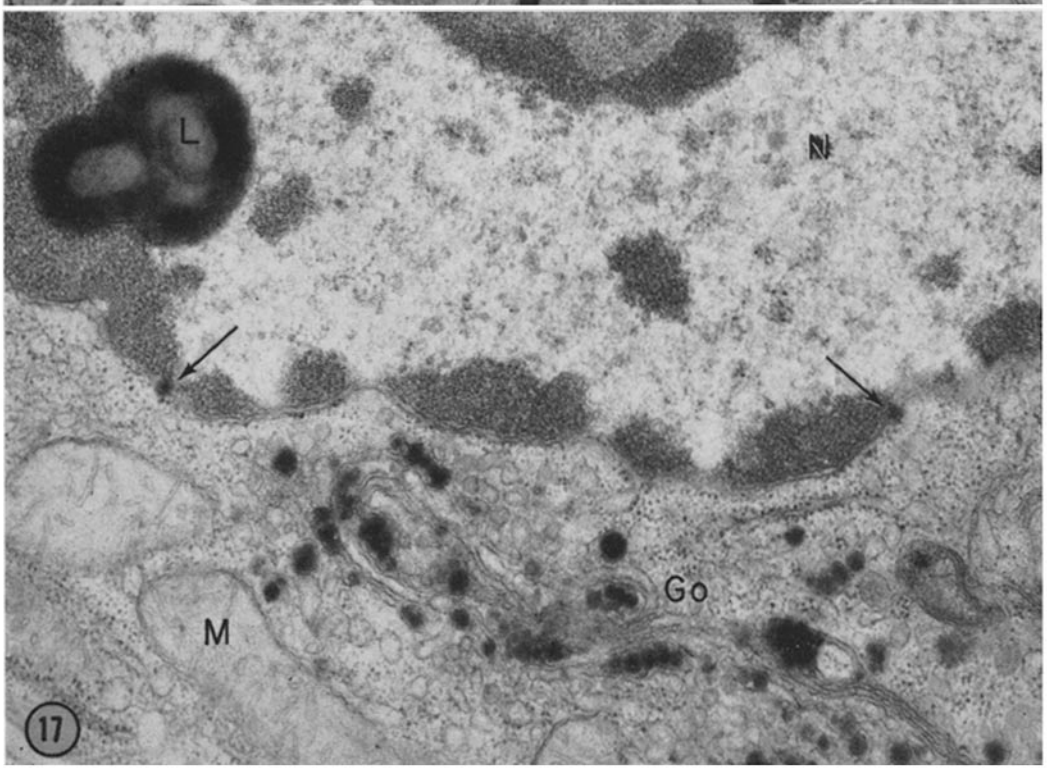


FIGURE 16 Low-power electron micrograph of the in vivo newborn mouse (M-5) liver parenchymal cells adjacent to a sinusoid (*S*) which contains an erythrocyte (*E*). A bile canaliculus (*bc*) is shown in the lower right and the cytoplasm of the cells contains large accumulations of lipid bodies (*L*). The nucleus in the upper left corner contains 3 lipid bodies. × 10,000.

FIGURE 17 A higher-power micrograph of the nucleus seen in Fig. 16, showing the lipid bodies (*L*) in the nucleus and two small lipid bodies near nuclear pores (*arrows*). Many other small lipid bodies are noted below the nucleus intermingled with elements of the Golgi complex (*Go*). × 50,000.

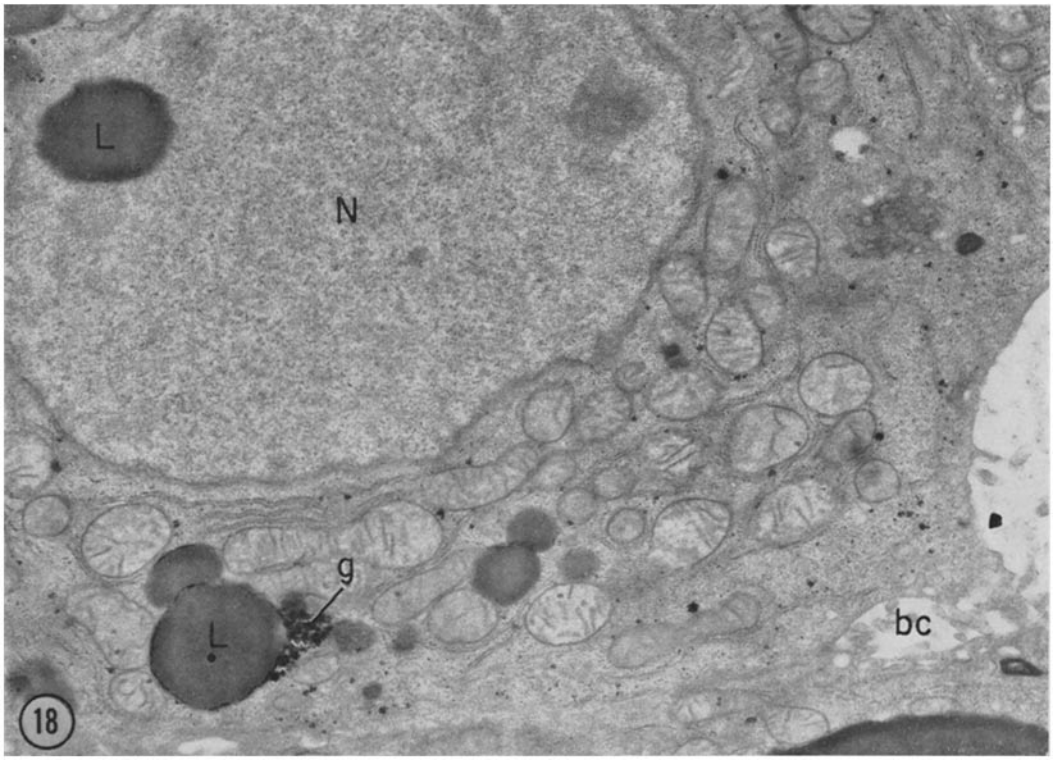


FIGURE 18 Electron micrograph of part of a mouse fetal liver (M-1) parenchymal cell after 1 wk of cultivation in the circumfusion system. A lipid body (*L*) is seen within the nuclear membrane and several lipid bodies are in the cytoplasm. Portions of distended bile canaliculi (*bc*) are visible in the lower right corner and the extreme right edge of the micrograph. × 17,000.

used electron microscopy to study the lateral views of newborn mouse liver cells growing on reconstituted rat-tail collagen. He described the parenchymal cells as remaining similar in size and appearance for more than one month. His 2-wk-old cultured hepatocytes, were reported to be ultrastructurally similar to the in vivo hepatocytes. However, neither Watanabe nor Hillis and Bang described in vitro bile canaliculi, which should be considered as a high index for the differentiation phase of hepatocytes. Similarly, Sandström (45) never found bile canaliculi in his rat liver cultures, although he did report their appearance in great abundance in 5-day cultures of chick embryo liver. Watanabe also noted a decrease in the size of mitochondria in his 2-wk cultured newborn mouse liver hepatocytes, as did Westman and Sandström (56) in their 3-day cultures of chick embryo liver. The hepatocytes in our cultures similarly had mitochondria generally smaller in diameter than those of the in vivo parenchymal cells.

Fetal, newborn, and young mice were found to have large lipid granules within both the cytoplasm and the nucleus of hepatocytes. Although lipid in the nucleus of the hepatocytes of neonatal mice has been reported (19), we have not found supporting published illustrations. These lipid bodies were found again in our cultured hepatocytes in the cytoplasm as well as in the nucleus, and their presence in the nucleus is currently being studied. Lipid in nuclei of hepatoma cells was shown to occur after thioacetamide poisoning (41). This was thought to result from an invagination of the nuclear membrane in which lipid was entrapped. After the invagination became detached from the nuclear membrane, a double membrane intranuclear inclusion resulted, which then reportedly lost its membrane, leaving the lipid free in the nucleoplasm. Although we did not see

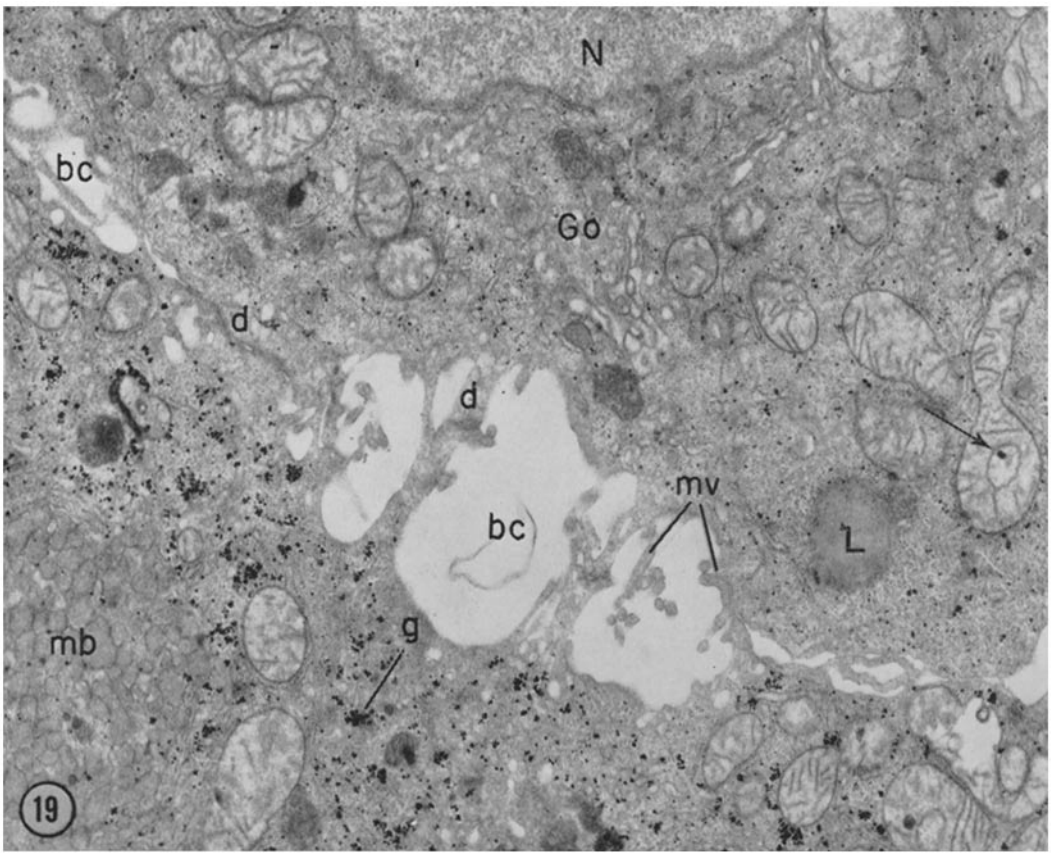


FIGURE 19 Electron micrograph of portions of two parenchymal cells from a fetal mouse liver explant (M-1) after 1 wk of cultivation in the circumfusion system. Distended bile canaliculi (*bc*) occurred between the two parenchymal cells. Between the nucleus (*N*) and the bile canaliculus of the upper cell are the sacs of the Golgi complex (*Go*), and in the lower cell in the same position is an aggregation of microbodies (*mb*). The mitochondrion in the right of the micrograph shows a small group of glycogen granules (*arrow*) circumscribed by a crista similar to that seen in the in vivo specimen in Fig. 15. × 17,000.

such a sequence, or even traces of this kind of entrapment, we did see small lipid masses in the nuclear pores which suggested but did not prove that their mode of entrance into the nucleoplasm was through these tiny orifices.

Sandström (43) concluded from his own observations and those of Sato et al. (47) that there is no reason to believe that cultivated liver cells, lacking the specificity of normal liver cells, were more than stromal in origin. Biberfeld et al. (2) agreed that fibroblast-like outgrowths were the usual results of long-term studies of liver, although they claimed to have cloned a parenchymal cell line (PAR-C1). Their studies of this line by electron microscopy, fluorescent antibody techniques, and immunoelectrophoresis strongly suggested an hepatic parenchymal origin. By another route of investigation, Paul and Pearson (28) supported Sandström's opinion. They found that the metabolism of chick embryonic liver in vitro was completely reversed in 24 hr and became identical to that of chicken heart fibroblast cultures.

In evaluating the differentiation of liver in tissue culture, one must have recognizable parenchymal and Kupffer cells. Presumably, the parenchymal liver cells can function properly only if they have (*a*) bile canaliculi whose villi stain positively and sharply with alkaline phosphatase and ATPase (7, 22–25, 46, 52), (*b*) positive

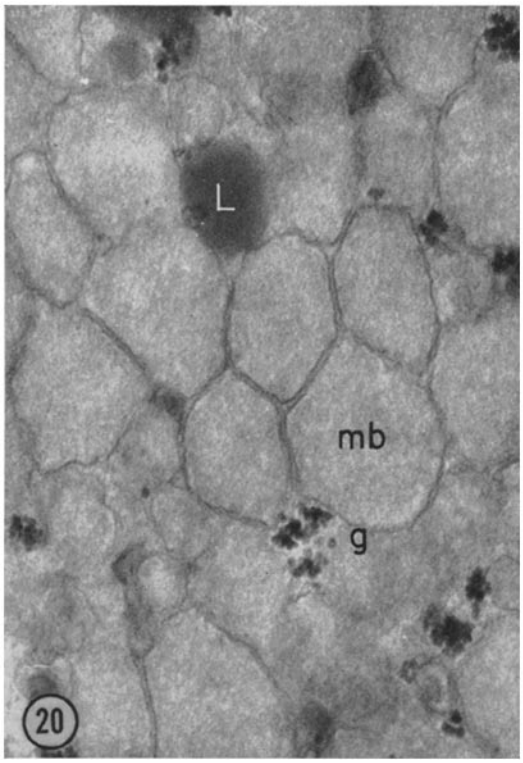


FIGURE 20 A high-power electron micrograph of a portion of an aggregation of microbodies (*mb*) observed in a fetal liver parenchymal cell (M-1) cultivated for 2 wk in the circumfusion system. An interspersion of lipid bodies (*L*) and glycogen granules (*g*) is seen between these single membrane-bounded crystalloid-free, and faceted units of closely packed microbodies. × 54,000.

acid phosphatase granules (lysosomes 6, 15) in the peribiliary and Golgi complex areas, (*c*) lipid, (*d*) microbodies, and (*e*) characteristic glycogen accumulations in the cytoplasm. These essential and somewhat specific criteria of liver cells were found in the mouse liver cultures observed in the circumfusion system. The large granules noted after about 1 month by phase-contrast microscopy possibly were related to the agglomerations of the smooth-surfaced endoplasmic reticulum (5, 9, 21, 23, 29, 41, 48) admixed with the enlarging lysosomes and lipid droplets or were, perhaps, in some instances, aggregates of microbodies. Obviously, they represented many forms or groupings of these cellular constituents rather than only one, for their staining reactions were extremely variable. The question of microbodies added considerable support to the evaluation of the differentiation of parenchymal cells in vitro. Isolated small sized crystalloid–containing microbodies rarely were found in the fetal and newborn in vivo sections. In the 1- and 2-wk-old cultures, large aggregates of crystalloid–free microbodies were found in the parenchymal cells and rarely after 4 wk. However, after 2 wk isolated and small sized crystalloid–containing microbodies commenced to appear among the mitochondria and by 4 wk (Figs. 21 and 22) full sized crystalloid–containing microbodies were scattered throughout the cytoplasm, indicating a progression toward maturity.

The rare observation of the bundles of filaments in the 1-month-old cultures was not comparable to findings of in vivo parenchymal cells. Bundles of filaments have been reported in biliary cells of cholangiomas (41) and in the PAR-C1 cell line of rat liver (2). However, the ordered state of the filaments shown in Fig. 23 was not mentioned in these reports.

The studies with the circumfusion system now seriously challenge Willmer's (57) opinion that all perfusion systems have an inherent defect in that they wash away important cellular metabolites. The circulating qualities of the circumfusion system prevent a complete washing away since that which has been washed from one chamber will in part, go back to the chamber of origin. Secondly, the cellophane membrane itself prevents a considerable washing away from the microenvironment except for dialyzable materials. The circumfusion system may appear to be a complex and impractical device, yet when we attempt to cultivate and maintain the functioning units of life, i.e. cells and their complex associations, as tissues and organs in a condition analogous to their origin, it seems necessary that simple techniques must give way to technological complexities. In retrospect, it now seems foolish to have attempted to find economical and simple methods for the cultivation of cells in a state of differentiation, although it would have been more foolish not to have done so. The circumfusion system is much more complex than the flask, test tube, or hanging drop preparation, yet the results as described in this report are much more complex than those obtained with these simpler techniques.

Mouse liver cells have been shown to retain their functioning state for at least 4 months. This means

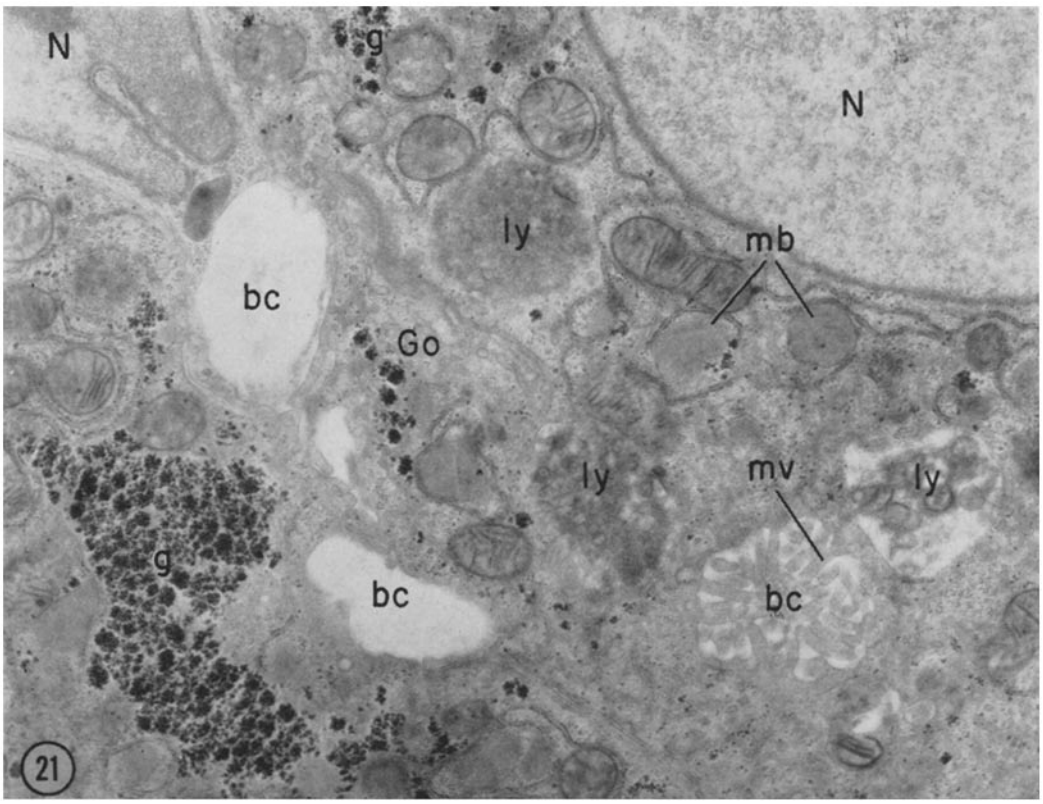
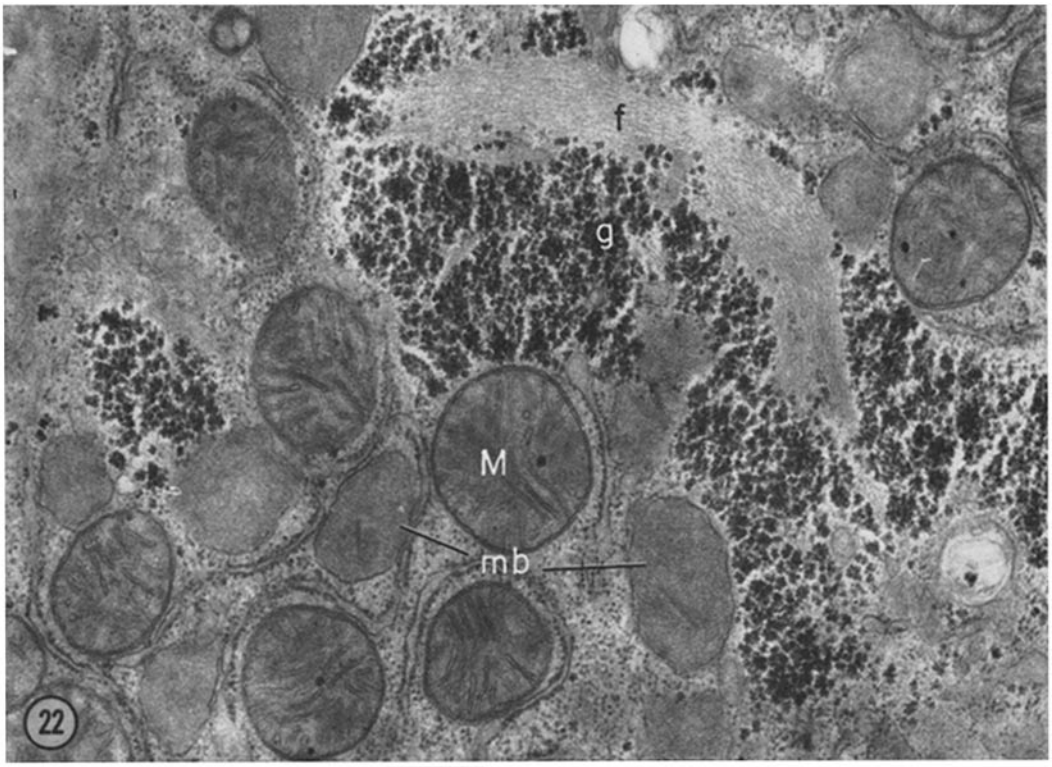


FIGURE 21 Electron micrograph of a portion of three parenchymal cells in a fetal mouse liver (M-1) cultivated in the circumfusion system for 4 wk. The distended bile canaliculi (*bc*) show no microvilli, whereas the bile canaliculus in the lower right is full of microvilli (*mv*). Lysosomes (*ly*), crystalloid-containing microbodies (*mb*), and glycogen (*g*) are conspicuous features in these cells. × 17,000.

that many in vitro conditions which were used in the past favored dedifferentiation, longevity of one cell type, and autonomy. The circumfusion system, however, is unfavorable to multiplication but favors longevity through a retention of many cell types functioning in close association with each other, that is, as histotypic or organotypic cellular complexes. The circumfusion system emphasizes a longevity of function and a loss of replication, whereas culturing in the less restricted environments of test tubes and flasks emphasizes a loss of function and a longevity through replication. The former, of course, favors differentiation of cells; the latter favors cell strains and neoplastic-like results.

Nevertheless, the circumfusion system is not perfect. It is now a new tool which is incomplete but potentially very valuable. It allows the study of in vitro normal cells in a functioning state which more closely resembles that of cells found in vivo than has been possible with simpler methods. This does not mean that cells in the circumfusion systems are fully normal, for they can never be as active or function as well as in the in vivo evironments, even though they may appear to do so with our limited means of detection. What has been attained with the circumfusion system is that the gap between the in vitro and in vivo environments has been shortened. This means that the tissue culturist has some maneuverability and that he is not hampered by the expected death and dedifferentiation of the originally cultivated cell population. For instance, the responses of liver and other tissues to intentional alterations of the environment may be fully visualized and scrutinized both by optical and electron microscopic techniques. The secretory activity of the parenchymal cells was viewed in quite old cultures with



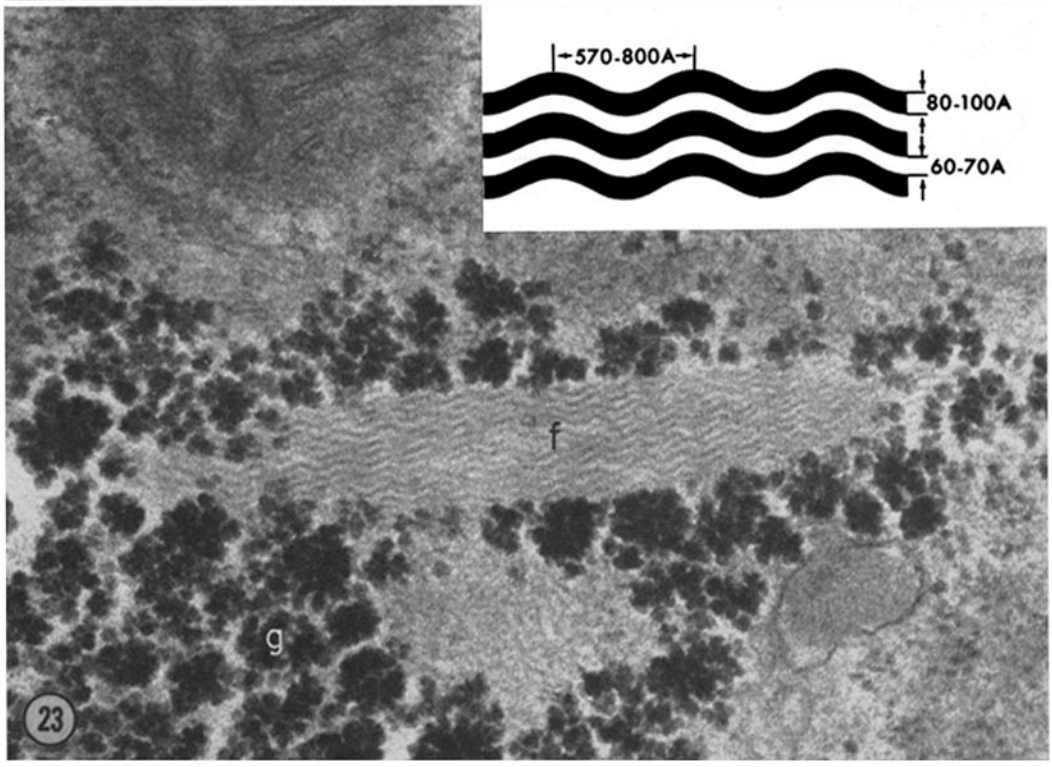


FIGURE 22 Electron micrograph of a portion of an M-1 mouse liver parenchymal cell cultivated in the circumfusion system for 4 wk. Prominent crystalloids now appear in the dispersed microbodies (*mb*). An elongated aggregate of filaments (*f*) is in the cluster of glycogen (*g*). × 34,000.

FIGURE 23 A high-power view of another filamentous unit also found in a parenchymal cell near the one shown in Fig. 22. The filaments are wavy and highly ordered in their interrelationship. The inserted diagram gives the measurements of the cytoplasmic filaments. × 89,000.

time-lapse cinemicrography, and their analogy to their in vivo counterparts also was shown by their cytochemical responses. This gives us assurance that much more can be done with normal tissues and cells in vitro than has been possible in the past. The great shortcoming of the circumfusion system at this point of development appears to be that of cellular or tissue atrophy, i.e. a cell mass plateau is reached after 3–4 weeks, rarely longer. Cultivation methods, nutrient formulas, and technical procdures are all being varied in an attempt to prevent this atrophy and thereby extend the life of these functioning cell cultures. Other tissues now being studied (principally the pancreas, male and female gonads, tissues of the oral cavity, and cell strains) will be reported subsequently. Moreover, upgrading of the entire circumfusion system is being pursued in an effort to marshall as many of the in vivo regulatory mechanisms as possible into in vitro environments.

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REFERENCES

1. BANG, F. B., and A. C. WARWICK. 1965. Liver. *In* Cells and Tissues in Culture. E. N. Willmer, editor. Academic Press, Inc., New York. **2**:607.
2. BIBERFELD, P., J. L. E. ERICSSON, P. PERLMANN, and M. RAFTELL. 1965. Increased occurrence of cytoplasmic filaments in *in vitro* propagated rat liver epithelial cells. *Exptl. Cell Res.* **39**:301.
3. DOLJANSKI, L. 1930. Glycogene dans les cultures de foie. *C. R. Soc. Biol. (Paris).* **105**:504.
4. EHRMANN, R. L., and G. O. GEY. 1956. The growth of cells on a transparent gel of reconstituted rat-tail collagen. *J. Natl. Cancer Inst.* **16**:1375.
5. EMMELOT, P., and E. L. BENEDETTI. 1960. Changes in the fine structure of rat liver cells brought about by dimethyl-nitrosamine. *J. Biophys. Biochem. Cytol.* **7**:393.
6. ESSNER, E., and A. B. NOVIKOFF. 1961. Localization of acid phosphatase activity in hepatic lysosomes by means of electron microscopy. *J. Biophys. Biochem. Cytol.* **9**:773.
7. ESSNER, E., A. B. NOVIKOFF, and B. MADEK. 1958. Adenosine-triphosphatase and 5-nucleotidase activities in the plasma membrane of liver cells as revealed by electron microscopy. *J. Biophys. Biochem. Cytol.* **4**:711.
8. EVANS, V. J., W. R. EARLE, E. R. WILSON, H. K. WALTZ, and C. J. MACKEY. 1952. The growth in vitro of massive cultures of liver cells. *J. Natl. Cancer Inst.* **12**:1245.
9. FAWCETT, D. W. 1955. Observations on the cytology and electron microscopy of hepatic cells. *J. Natl. Cancer Inst.* **15**:1475.
10. FELL, H. B. 1957. Discussion. *In* Gaillard, P. J. Morphogenesis in animal tissue cultures. *J. Natl. Cancer Inst.* **19**:601.
11. FISCHER, A., T. ASTRUP, G. EHRENSVARD, and V. OEHLENSCHLAGER. 1948. Growth of animal tissue cells in artificial media. *Proc. Soc. Exptl. Biol. Med.* **67**:40.
12. GOMORI, G. 1951. Histochemical staining methods. *In* Methods in Medical Research. M. B. Visscher, editor. Year Book Medical Publishers, Inc., Chicago. **4**:1.
13. HILLIS, W. D., and F. B. BANG. 1959. Cultivation of embryonic and adult liver cells on a collagen substrate. *Exptl. Cell Res.* **17**:557.
14. HILLIS, W. D., and F. B. BANG. 1962. The cultivation of human embryonic liver cells. *Exptl. Cell Res.* **26**:9.
15. HOLT, S. J., and R. M. HICKS. 1962. Combination of cytochemical staining methods for enzyme localization with electron microscopy. *Symp. Intern. Soc. Cell Biol.* **1**:193.
16. HOTCHKISS, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.* **16**:131.
17. INGRAM, R. L. 1962. Maintenance of organized adult liver tissue in vitro. *Exptl. Cell Res.* **28**:370.
18. JACOBSON, W., and M. WEBB. 1952. Nucleoprotein during mitosis. *Exptl. Cell Res.* **3**:163.

19. JEZEQUEL, A.-M., K. ARAKAWA, and J. W. STEINER. 1965. Fine structure of the normal neonatal mouse liver. *Lab. Invest.* **14**:1894.
20. KUMEGAWA, M., M. CATTONI, and G. G. ROSE. 1968. Electron microscopy of oral cells in vitro. III. In situ embedding of cultures in chambers of the circumfusion system. *Texas Rept. Biol. Med.* In press. Summer, 1968.
21. LAFONTAINE, J. G., and C. ALLARD. 1964. A light and electron microscope study of the morphological changes induced in rat liver cells by the AZO dye 2-ME-DAB. *J. Cell Biol.* **22**:143.
22. NOVIKOFF, A. B., J. DRUCKER, W.-Y. SHIN, and S. GOLDFISCHER. 1961. Further studies of the apparent adenosinetriphosphatase activity of cell membranes in formol-calcium-fixed tissues. *J. Histochem. Cytochem.* **9**:434.
23. NOVIKOFF, A. B., and E. ESSNER. 1960. The liver cell. Some new approaches to its study. *Am. J. Med.* **29**:102.
24. NOVIKOFF, A. B., E. ESSNER, S. GOLDFISCHER, and M. HEUS. 1962. Nucleosidephosphatase activities of cytomembranes. *Symp. Intern. Soc. Cell Biol.* **1**:149.
25. NOVIKOFF, A. B., D. H. HAUSMAN, and E. PODBER. 1958. The localization of adenosine triphosphatase in liver: In situ staining and cell fractionation studies. *J. Histochem. Cytochem.* **6**:61.
26. PADYKULA, H. A., and E. HERMAN. 1955. Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. *J. Histochem. Cytochem.* **3**:161.
27. PADYKULA, H. A., and E. HERMAN. 1955. The specificity of the histochemical method for adenosine triphosphatase. *J. Histochem. Cytochem.* **3**:170.
28. PAUL, J., and E. S. PEARSON. 1957. Metabolism of chick embryonic liver explants during transition from in vivo to in vitro conditions. *Exptl. Cell Res.* **12**:223.
29. PORTER, K. R., and C. BRUNI. 1959. An electron microscope study of the early effects of 3'-Me-DAB on rat liver cells. *Cancer Res.* **19**:997.
30. REISNER, E. H., JR. 1959. Tissue culture of bone marrow. *Ann. N. Y. Acad. Sci.* **77**:487.
31. ROBINEAUX, R. 1964. Cellular studies in inflammation and immunity with time-lapse microcinematography with special reference to the spleen cultivated by Rose's technique. *In* Injury, Inflammation and Immunity. Miles Laboratories, Inc., Elkhart, Ind. 94.
32. ROSE, G. G. 1954. A separable and multipurpose tissue culture chamber. *Texas Rept. Biol. Med.* **12**:1074.
33. ROSE, G. G. 1957. Special uses of the multipurpose tissue culture chamber. *Texas Rept. Biol. Med.* **15**:310.
34. ROSE, G. G. 1961. The Golgi complex in living osteoblasts. *J. Biophys. Biochem. Cytol.* **9**:463.
35. ROSE, G. G. 1966. Cytopathophysiology of tissue cultures growing under cellophane membranes. *In* International Review of Experimental Pathology. G. W. Richter and M. A. Epstein, editors. Academic Press, Inc., New York. **5**:111.
36. ROSE, G. G. 1967. The Circumfusion System for multipurpose culture chambers. I. Introduction to the mechanics, techniques, and basic results of a 12-chamber (in vitro) closed circulatory system. *J. Cell Biol.* **32**:89.
37. ROSE, G. G., M. CATTONI, and C. M. POMERAT. 1962. Tissue culture study of human gingiva. I. The morphological diversity of cells when using the cellophane membrane techniques. *J. Dental Res.* **41**:997.
38. ROSE, G. G., C. M. POMERAT, T. O. SHINDLER, and J. B. TRUNNELL. 1958. A cellophane-strip technique for culturing tissue in multipurpose culture chambers. *J. Biophys. Biochem. Cytol.* **4**:761.
39. ROSE, G. G., and T. O. SHINDLER. 1960. The cytodifferentiation of osteoblasts in tissue culture. A description of cellular emigrations from embryo chick leg bones. *J. Bone Joint Surg.* **42-A**:485.
40. ROSE, G. G., and T. O. SHINDLER. 1964. The mineralization of emigrating bone cells in tissue culture. *Texas Rept. Biol. Med.* **22**:174.
41. ROUILLER, CH., and A.-M. JEZEQUEL. 1963. Electron microscopy of the liver. *In* The Liver. Ch. Rouiller, editor. Academic Press, Inc., New York. 195.
42. SANDSTRÖM, B. 1964. A method of cultivating morphologically highly differentiated liver epithelium in vitro. *Acta Soc. Med. Upsalien.* **69**:209.
43. SANDSTRÖM, B. 1965. Studies on liver cells in tissue culture. I. Influence of the culture method on the morphology and growth pattern of the cells. *Exptl. Cell Res.* **37**:552.
44. SANDSTRÖM, B. 1965. Studies on liver cells in tissue culture. 1. The origin and mutual relationship of the cells. *Exptl. Cell Res.* **37**:558.
45. SANDSTRÖM, B. 1966. Liver parenchymal cells in tissue culture. A morphological study on foetal rat and chicken liver cells. *Acta Soc. Med. Upsalien.* **71**:21.
46. SANDSTRÖM, B. 1966. Cytochemistry of cultivated chicken embryonic liver. *Acta Soc. Med. Upsalien.* **71**:65.
47. SATO, G., L. ZAROFF, and S. E. MILLS. 1960.

Tissue culture populations and their relation to the tissue of origin. *Proc. Natl. Acad. Sci. U.S.* **46**:963.

48. SIMON, G., and CH. ROUILLER. 1962. Akute intoxikation durch thioacetamia (erste ergebnisse). *Mikroskopie.* **17**:50.
49. STEVENS, K. M. 1965. Oxygen requirements for liver cells in vitro. *Nature* **206**:199.
50. TROWELL, O. A. 1959. The culture of mature organs in a synthetic medium. *Exptl. Cell Res.* **16**:118.
51. TROWELL, O. A. 1961. Problems in the maintenance of mature organs in vitro. *In* La Culture Organotypique. *Colloq. Intern. Centre Natl. Rech. Sci.* (*Paris*) **101**:237.
52. WACHSTEIN, M. 1963. Cyto- and histochemistry of the liver. *In* The Liver. Ch. Rouiller, editor. Academic Press, Inc., New York. 137.
53. WACHSTEIN, M., and E. MEISEL. 1957. Histochemistry of hepatic phosphatases at a physiologic pH with special reference to the demonstration of bile canaliculi. *Am. J. Clin. Pathol.* **27**:13.
54. WACHSTEIN, M., E. MEISEL, and A. NEIDZWIEDZ. 1960. Histochemical demonstration of mitochondrial adenosine triphosphatase with the lead-adenosine triphosphate technique. *J. Histochem. Cytochem.* **8**:387.
55. WATANABE, H. 1966. A fine structural study of liver culture. *Exptl. Cell Res.* **42**:685.
56. WESTMAN, J., and B. SANDSTRÖM. 1966. Electron microscopy of organic and cultivated foetal chicken liver. *Z. Zellforsch.* **71**:271
57. WILLMER, E. N. 1965. Introduction. *In* Cells and Tissues in Culture. E. N. Willmer, editor. Academic Press, Inc., New York, **1**:7, 13.