

FACTORS CONTROLLING DEVELOPMENT OF CHICK EMBRYO LIVER CELLS DURING ORGAN CULTURE

CAMILLO A. BENZO and ANDREW M. NEMETH

From the Anatomy Department, Medical School, The University of Pennsylvania, Philadelphia, Pennsylvania 19104. Dr. Benzo's present address is the Anatomy Department, State University of New York, Upstate Medical Center, Syracuse, New York 13210

ABSTRACT

The 5 day chick embryo liver cell still lacks many of the ultrastructural features of the adult liver cell. During organ culture on rafts over Eagle's medium, it develops electron-opaque mitochondria with granules, biliary microvilli, and compact Golgi complexes containing what appears to be secretory material. Rough ER proliferates and free ribosomes become bound to membrane. Thus, the 5 day cell, exposed only to simple nutrients (glucose, amino acids, vitamins) develops the general appearance of the adult liver cell except for the continued absence of smooth ER and glycogen. The significance of this incomplete differentiation and the factors controlling development are discussed in the light of accompanying metabolic changes.

The simultaneous appearance of new enzymes and new structures in a cell during development suggests the operation of coordinated regulatory processes. The appearance of new enzymes and structures might be linked through a common induction mechanism, or through the balanced growth of the structural unit in which the enzymes reside. Development of specific liver enzymes at birth may reflect the former situation (1), development of mitochondrial enzymes the latter (2, 3). In our recent investigations, we have attempted to uncover possible links among developmental processes by studying changes in immature embryo cells during culture in a simple medium, believing that a limited environment might permit the appearance of some, but not necessarily all, adult features. Such an approach could also suggest the requirements for initiation of developmental changes.

Previous work (4) had shown that UDP-glucuronyltransferase activity in chick liver increases from zero to adult levels on hatching at 21 days of egg incubation. Subsequently (5),

it was found that this development could be produced precociously by culturing embryo liver tissue in a liquid medium containing serum. Several questions (6) were then investigated with grafts, and organ and cell cultures with various media: (a) what substances are needed by cultured embryo liver cells to develop UDP-glucuronyltransferase activity? (b) is induction of enzyme on culture due to escape from repressive embryonic influences or to stimulation by culture conditions? and (c) in what period of the cell cycle does enzyme induction occur? It was found: (a) that cultured embryo liver requires only the simple nutrients found in Eagle's medium to develop adult levels of UDP-glucuronyltransferase activity; (b) that enzyme development in culture is very likely due to removal of the liver from an inhibitory embryonic environment rather than to incidents of culture; and (c) embryo liver cells can develop enzyme activity during culture without first passing through the S, G₂, or M periods of the cell cycle.

The early chick embryo liver cell lacks many of

the ultrastructural features of the adult liver cell. The present work inquires whether early embryo liver during organ culture develops adult ultrastructural features concurrently with UDP-glucuronyltransferase activity, and whether the appearance of enzyme activity is necessarily associated with any particular structural development.

METHODS

Ten livers or liver segments (totaling 10 mg in weight) from 5- and 11-day chick embryos, respectively, were placed on Millipore filter rafts (25 μ thick; 0.45 μ pore size) supported by nylon screen (Nitex; 65 μ grid size) over the center well of an organ culture dish (Falcon Plastics, Los Angeles, Calif.) containing 1.2 ml of Eagle's medium and Tyrode's solution (1:1, v/v) with phenol red (Microbiological Associates Inc., Bethesda, Md.), 2 mM l-glutamine, and 50 units penicillin, 0.25 μ g Fungizone and 50 μ g streptomycin/ml medium. Tissue was incubated in CO₂-air (1:19, v/v) at 37.5°C, atmospheric pressure, and 100% humidity and media were replaced every 2 days (6).

Fresh and cultured tissues were fixed for 2 hr at 4° in 1% osmium tetroxide buffered at pH 7.4 with Veronal acetate, dehydrated by increasing concentrations of ethanol, and embedded in Araldite 502 by standard procedures. The resin blocks were cut into sections showing silver-gray to pale-gold interference colors, which then were stained with uranyl acetate followed by lead citrate and examined with an RCA EMU-3C electron microscope.

Upwards of five embryos were examined for evaluating each experimental group, and the normal stages. Representative examples are presented in the figures.

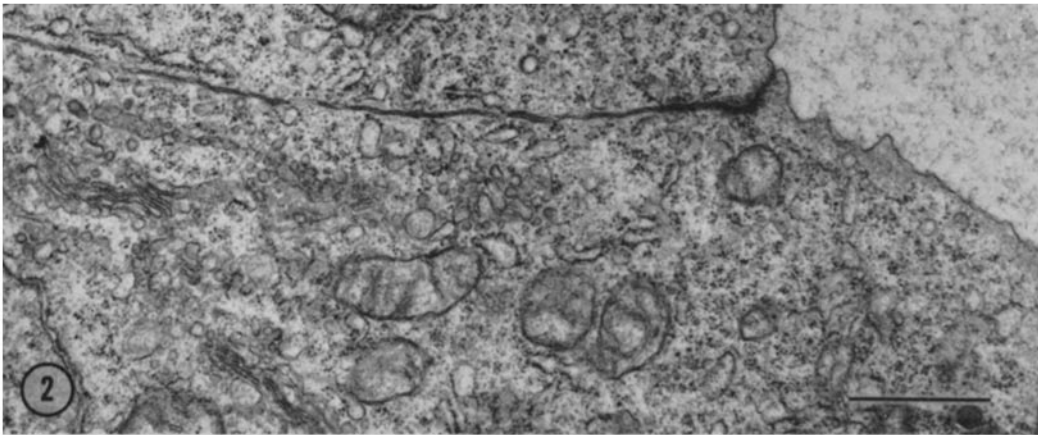
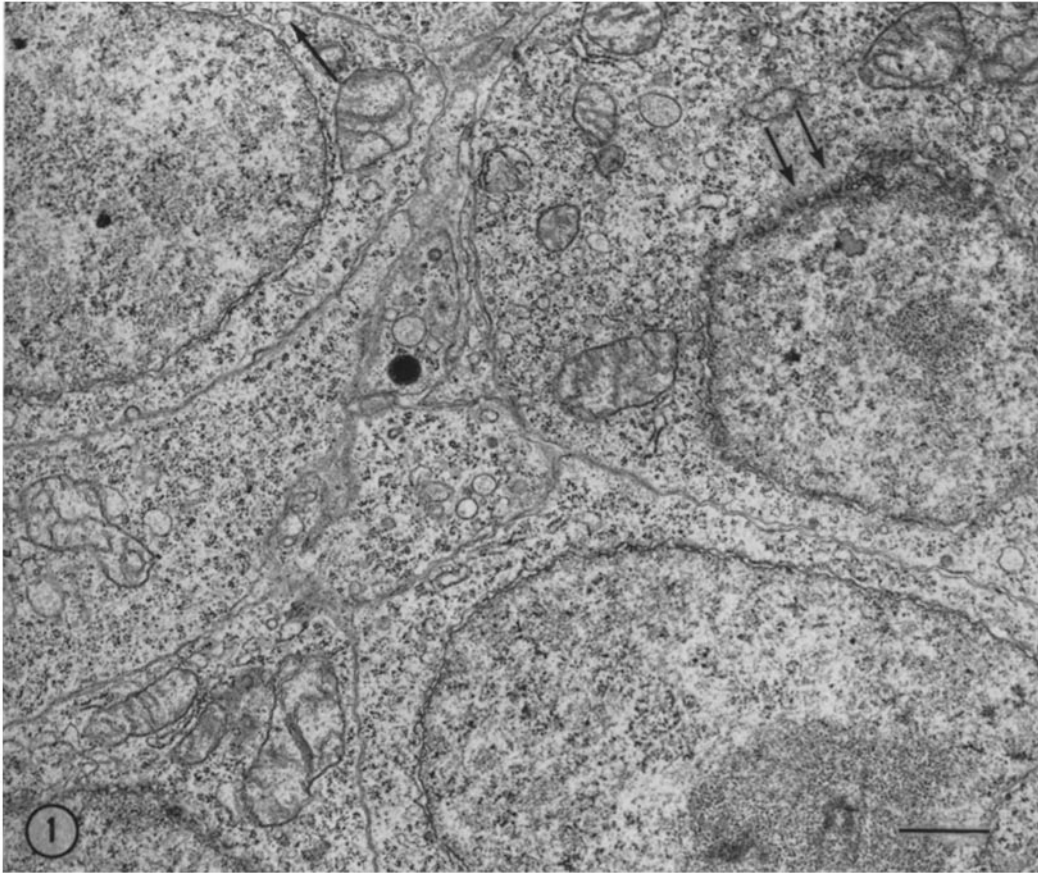
RESULTS

Development In Ovo

GROWTH: The liver of the chick appears at the end of the 2nd day of embryonic life as a diverticulum of the anterior wall of the intestinal umbilicus. The organ weighs approximately 1 mg on day 5, 50–80 mg on day 11, and 1 g at hatching on day 21. Almost all the increase in size is the result of parenchymal cell proliferation (6). Connective tissue, sinusoidal, and hematopoietic cells make up only a very small proportion of the total number of cells in chick embryo liver.

ULTRASTRUCTURE: Major ultrastructural changes take place in the chick liver cell during

the early embryonic period. The following changes occur between days 5 and 11 (Figs. 1–6): (a) *Ribosomes and rough endoplasmic reticulum:* The free ribosome concentration decreases moderately between days 5 and 8, markedly between days 8 and 11 (compare Figs. 1, 3, and 5 illustrating 5-, 8-, and 11-day cells). There is a large increase in the concentration of rough endoplasmic reticulum between days 5 and 8, followed by some decrease to day 11 with a change in morphology from mainly vesicles to flattened cisternae which partly envelop mitochondria or are aligned parallel to plasma membrane. These observations have led us to conclude that rough endoplasmic reticulum in chick embryo liver matures in two phases: rapid membrane proliferation between days 5 and 8, followed by extensive binding of free ribosomes to membrane between days 8 and 11. The chemical measurements of Pollak and Ward (7) also indicate a marked increase in membrane up to day 8 followed by some decrease. Rough endoplasmic reticulum and outer nuclear membrane are more densely covered with ribosomes on day 11 (Figs. 5 and 6) than on day 8 (Figs. 3 and 4) when membrane concentration has reached a maximum but before extensive ribosome binding has occurred. (b) *Nucleus:* The inner and outer nuclear membranes lose their ruffled appearance between days 5 (Fig. 1) and 11 (Fig. 5) and become smooth and parallel. The inner membrane is already smooth on day 8, a time at which the outer membrane is still ruffled (Fig. 4). Between days 5 and 8 when outer nuclear membrane is ruffled, there is a large increase in rough endoplasmic reticulum, particularly the vesicular form. Occasionally, a vesicle can be seen attached to outer nuclear membrane by an isthmus (Figs. 1 and 4, arrows). It is possible that small vesicles of rough endoplasmic reticulum are formed by a budding process from outer nuclear membrane and that these vesicles fuse to form larger sacs (Figs. 3 and 4) and finally cisternae. A high rate of such budding with subsequent fusion has been suggested by Rebhun (8) as a possible mechanism of annulate lamellae formation. Between days 5 and 11, there does not appear to be any change in the size of the nucleus or of nuclear pores, or in the distance between pores (Figs. 1 and 3). Between days 5 and 11, a prominent granular nucleolus is present, probably reflecting a high rate of ribo-



FIGURES 1 and 2 Liver from 5 day chick embryo.

FIGURE 1 Cells show many free ribosomes (most in small aggregates), a small amount of rough ER, no smooth ER, and no glycogen. Mitochondria have ruffled membranes, electron-translucent matrices, and no granules. Nuclear membranes are ruffled and have pores (arrows at right). Note vesicle attached by isthmus to outer nuclear membrane (arrow at left). Scale line on all figures indicates 1 μ . $\times 12,000$

FIGURE 2 Note Golgi complex (at left) with electron-translucent cisternae and vesicles, also (at right) bile canaliculus bounded by cells with tight junction but no microvilli. $\times 18,900$.

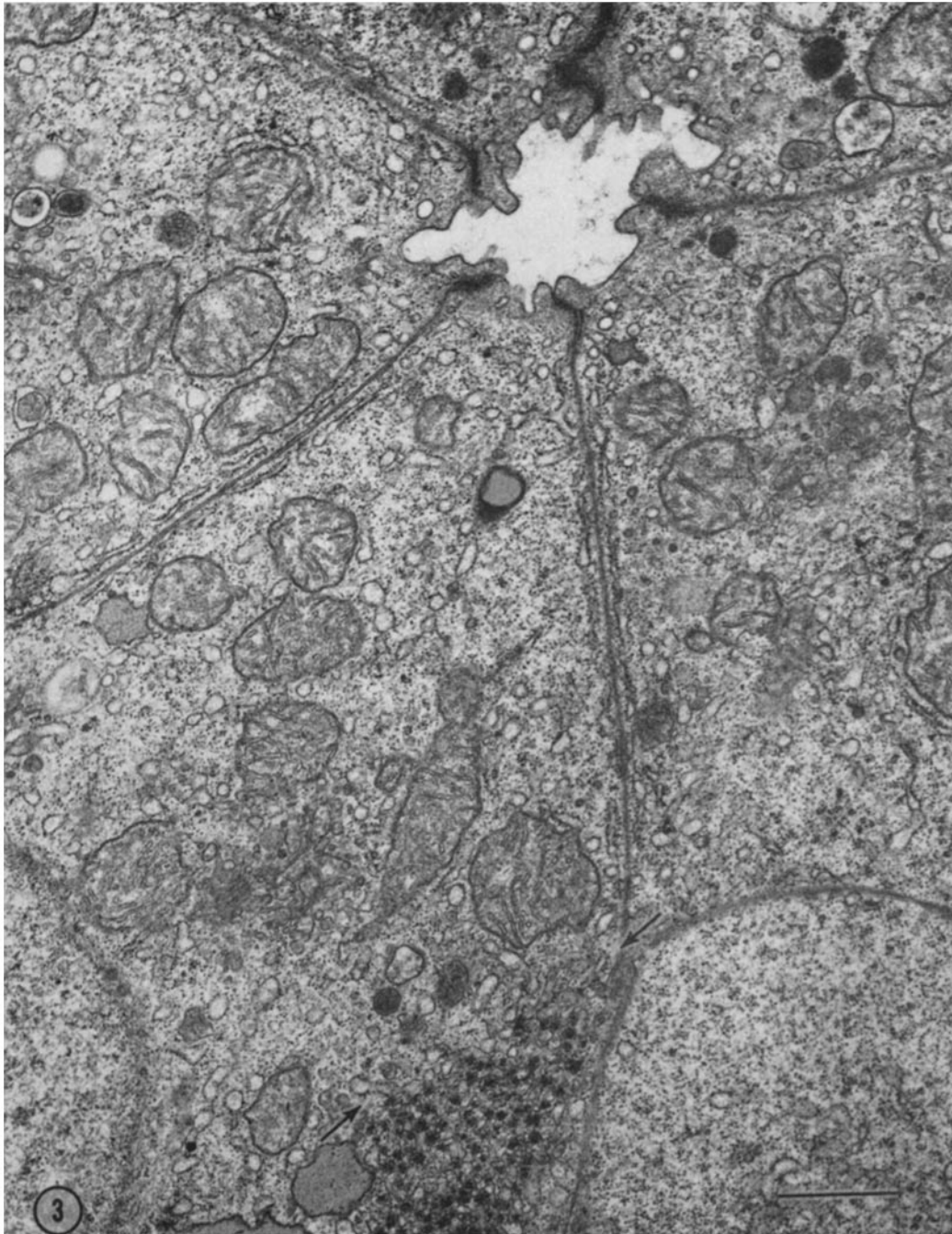


FIGURE 3 Liver from 8 day chick embryo. Cells show many rough ER vesicles and rough ER cisternae, the latter near mitochondria and plasma membranes. Many free ribosomes are present. A bile canaliculus (top) is bordered by cells with rudimentary projections. Glycogen particles (bottom) are embedded in a lattice of smooth ER. Note connections between smooth and rough ER at margins of glycogen area (arrows); see also Fig. 4, *inset*. $\times 17,200$.

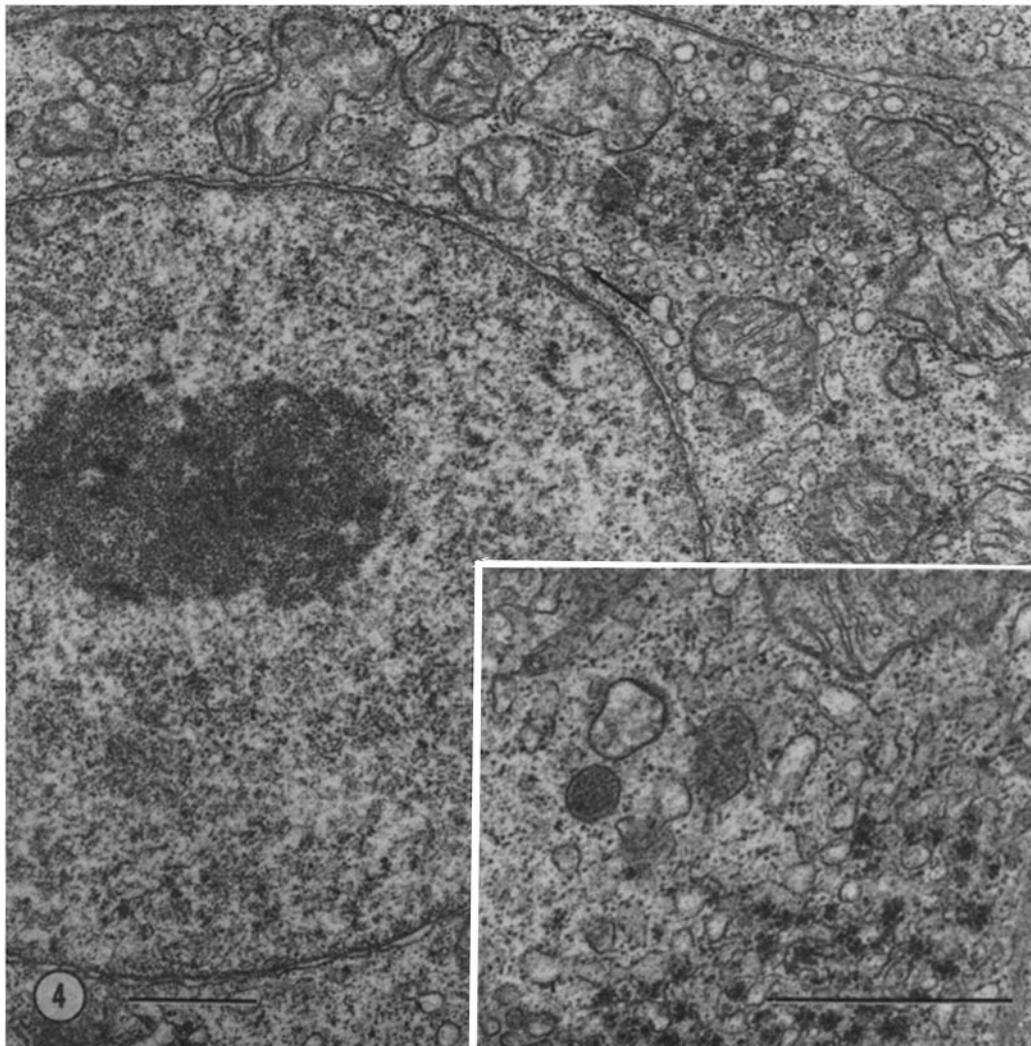
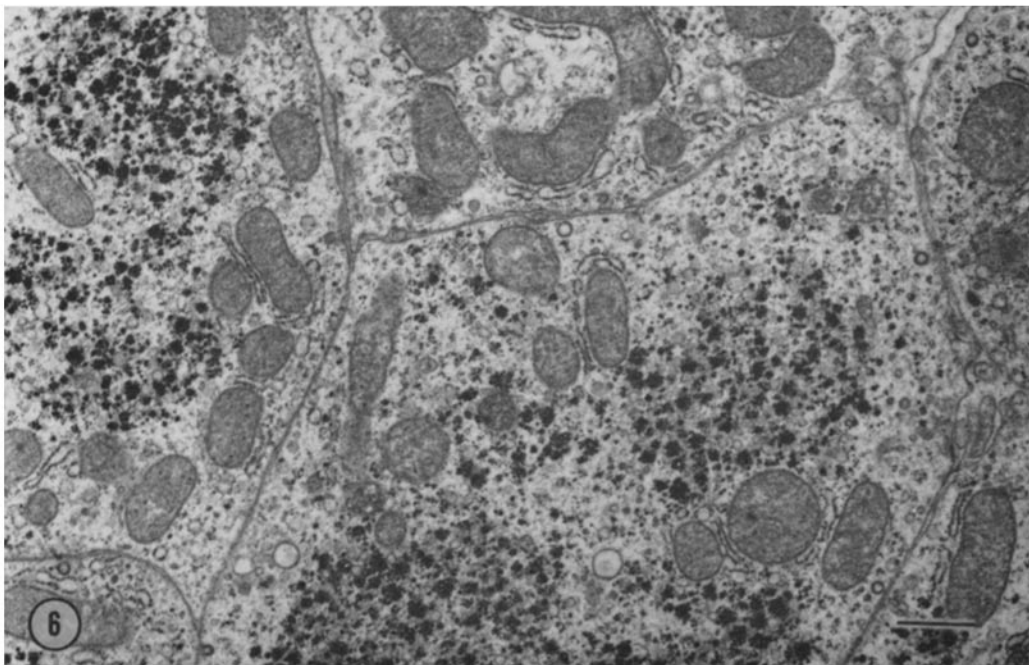
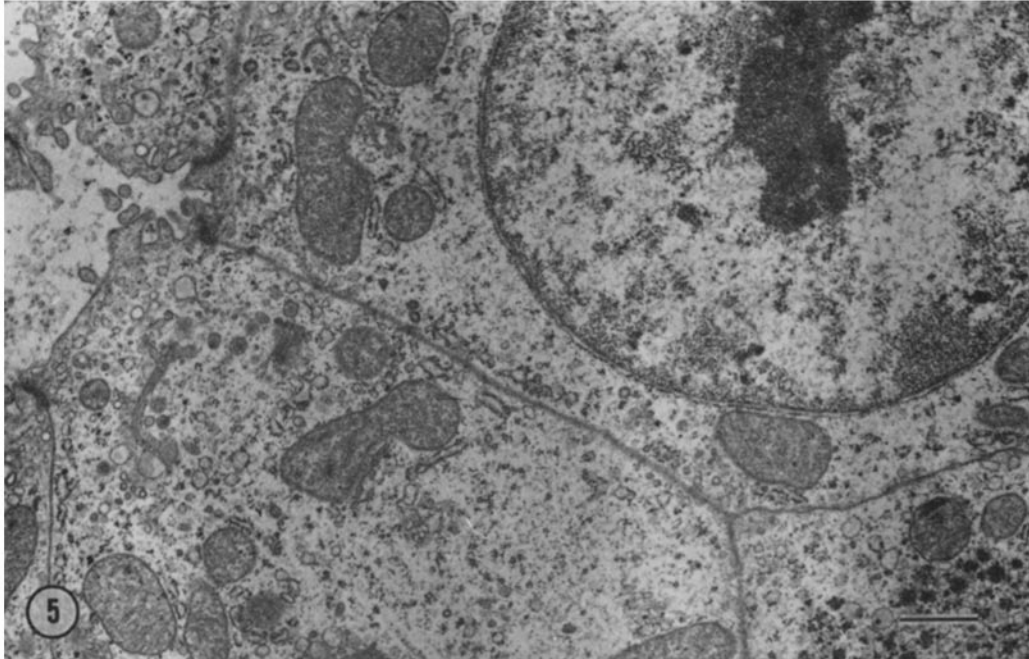


FIGURE 4 Liver from 8 day chick embryo. Note the smooth inner nuclear membrane and ruffled outer nuclear membrane with attached vesicle (arrow); note also small area of smooth ER with glycogen. $\times 17,200$.

Inset, Note connections between smooth and rough endoplasmic reticulum at margins of glycogen area (lower left and upper right). $\times 32,250$.

some synthesis. The concentration of total ribosomes (free and membrane-bound) in chick embryo liver remains constant (7) through this period; a high rate of ribosome synthesis would be necessary to maintain this concentration in the presence of a high rate of cell proliferation and growth (6). (c) *Glycogen and smooth endoplasmic reticulum*: Glycogen particles embedded in tubular lattices of smooth endoplasmic reticulum appear sometime after day 5 (Figs. 3 and 4) and are a

prominent feature of at least half the cells by day 11 (Fig. 6). The smooth endoplasmic reticulum appears to be continuous with rough reticulum on the margins of the lattice work (see Fig. 3 and Fig. 4, *inset*). These connections and the sequence of appearance of first rough and then smooth endoplasmic reticulum suggest that smooth reticulum arises from the rough. (d) *Mitochondria*: Mitochondria change in several ways between days 5 and 11. Both inner and outer membranes



FIGURES 5 and 6 Liver from 11 day chick embryo.

FIGURE 5 Cells show few free ribosomes. There is more cisternal than vesicular rough ER. The rough ER and outer nuclear membranes are densely covered with ribosomes. The mitochondria have smooth contours, moderately dense matrices, and very dense granules. Nuclear membranes are smooth. A bile canaliculus (upper left) is bordered by cells with microvilli. A compact Golgi complex with moderately dense material in cisternae and in some of the vesicles is oriented towards the bile canaliculus. $\times 10,500$.

FIGURE 6 Note several prominent areas of smooth ER with glycogen. $\times 10,500$.

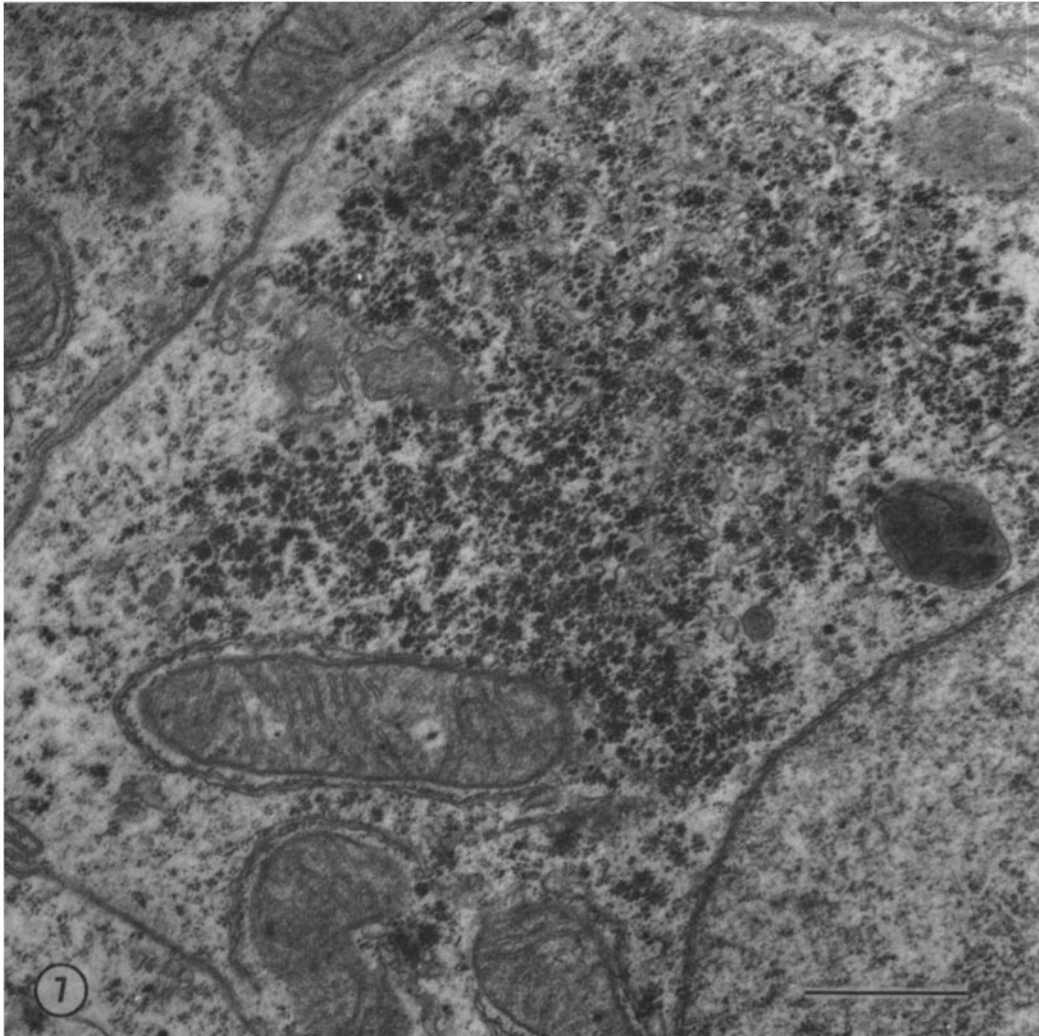


FIGURE 7 Liver from 11 day chick embryo after 4 days' organ culture. Micrograph shows very large, flattened cisternae of rough ER encompassing mitochondria. Note area of smooth ER with glycogen. $\times 21,600$.

lose their ruffled appearance (compare Figs. 1, 5, and 6). There is an increase in mitochondrial size, in the number of cristae, and in the density of matrix. Small, very dense granules appear in the matrix. (e) *Golgi complexes and bile canaliculi*: After day 5, Golgi complexes become more numerous and more compact (vesicles grouped closer to the cisternae), and in many instances they can be seen oriented toward the canalicular pole of the cell (compare Figs. 2 and 5). A moderately dense material accumulates in some of the vesicles and in the cisternal components (Fig. 5).

This may indicate the onset of secretory activity. Bile canaliculi develop microvilli (compare Figs. 2 and 5). Tight junctions, presumably sealing off canicular channels from extracellular spaces, are already present on day 5 (Fig. 2).

To summarize, the embryo liver cell by the 11th day of egg incubation is comparable to the adult cell in electron microscope appearance. Some of these observations have been reported by Stephens and Bils (9), by Pollak and Shorey (10), and by Karrer (11).

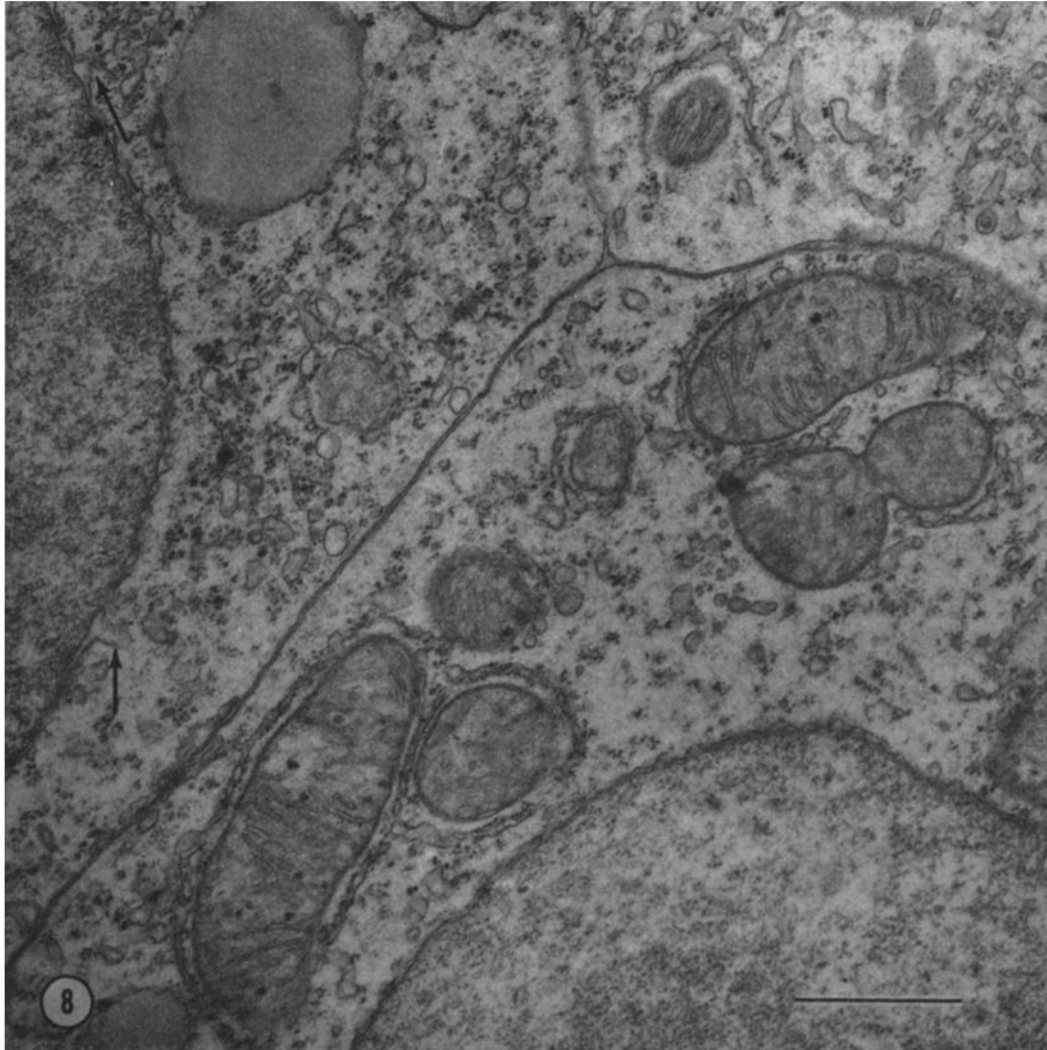


FIGURE 8 Liver from 11 day chick embryo after 4 days' organ culture. Micrograph shows areas of smooth ER devoid of glycogen. Note ruffled nuclear membranes with blebs (arrows). $\times 21,600$.

Development during Organ Culture

GROWTH: Chick embryo liver tissue (see Methods section) cultured on rafts over Eagle's medium becomes flattened and spread out. Changes in liver cell proliferation and protein have been described previously (6); there are large decreases in cell division and a 20–50% fall in protein over 6 days of culture. Necrosis is sometimes observed in the center of the tissue after several days of culture.

ULTRASTRUCTURE: (a) *11 day embryo liver.*

The 11 day embryo liver cell is not much changed during 4 days' organ culture though it appears to form additional rough and smooth endoplasmic reticulum (Figs 7 and 8). The cisternae of rough endoplasmic reticulum become markedly enlarged and flattened (compare Figs. 6 and 7 illustrating 11 day liver before and after culture). New smooth endoplasmic reticulum is observed in the form of tubular lattices devoid of glycogen (Fig. 8). Nuclear membranes become ruffled, and large blebs suggest the formation of cytoplas-

mic membrane (Fig. 8, arrows). There is "no large loss" of glycogen during culture as judged from the electron micrographs; cells appear to retain existing lattice works of smooth endoplasmic reticulum with enmeshed glycogen particles (Fig. 7). The stability of chick embryo liver glycogen during aerobic culture has been noted previously (12).

(b) *5 day embryo liver.* The 5 day embryo liver cell during organ culture on rafts over Eagle's medium develops all the ultrastructural features of the 11 day cell except for glycogen and smooth endoplasmic reticulum. The process of maturation is already evident after 2 days of culture (Fig. 9), but is more complete after 6 days (Figs. 12 and 13). The rate of ultrastructural development appears to be approximately the same during culture and *in ovo*. At 2 days of culture (Fig. 9), one observes decrease in free ribosomes, increase in cisternal rough endoplasmic reticulum, changes in mitochondria, and smoothening of inner nuclear membrane (Fig. 10). At 6 days (Fig. 12), one notes very large and flattened rough cisternae, large mitochondria, Golgi complexes with electron-opaque material, and biliary microvilli (Fig. 13).

Annulate lamellae, structures normally never seen in fresh chick liver at any age, are present in virtually all cells after 2 days of culture and are still evident in some cells after 6 days of culture. These structures are frequently close to the nucleus (Fig. 10). Sometimes they lie alongside the outer nuclear membrane, the pores of the nucleus and lamellae apparently being held in alignment by traversing cylinders with electron-translucent cores (Fig. 11). The lamellae at their margins are frequently continuous with rough endoplasmic reticulum (Fig. 10, arrows).

The association between annulate lamellae and outer nuclear membrane suggests that the lamellae are derived from nuclear membrane in some way (13). The lamellae may indicate an unusually high rate of formation of cytoplasmic membrane from outer nuclear membrane (8). They appear to generate new rough endoplasmic reticulum, for in many places annulate lamellae are continuous with rough endoplasmic reticulum; after 6 days of culture, there are far fewer and smaller lamellar structures than at 2 days but much more rough reticulum. The origin and function of the annulate lamellae remain, however, a matter of speculation.

Liver cells from the 11 day embryo do not form annulate lamellae during culture. There also appears to be less new rough endoplasmic reticulum formed in the 11 day than in the 5 day embryo liver cell during culture. This may account (see Discussion) for the smaller development of UDP-glucuronyltransferase activity in 11-day cells than in the 5-day cells reported previously (6).

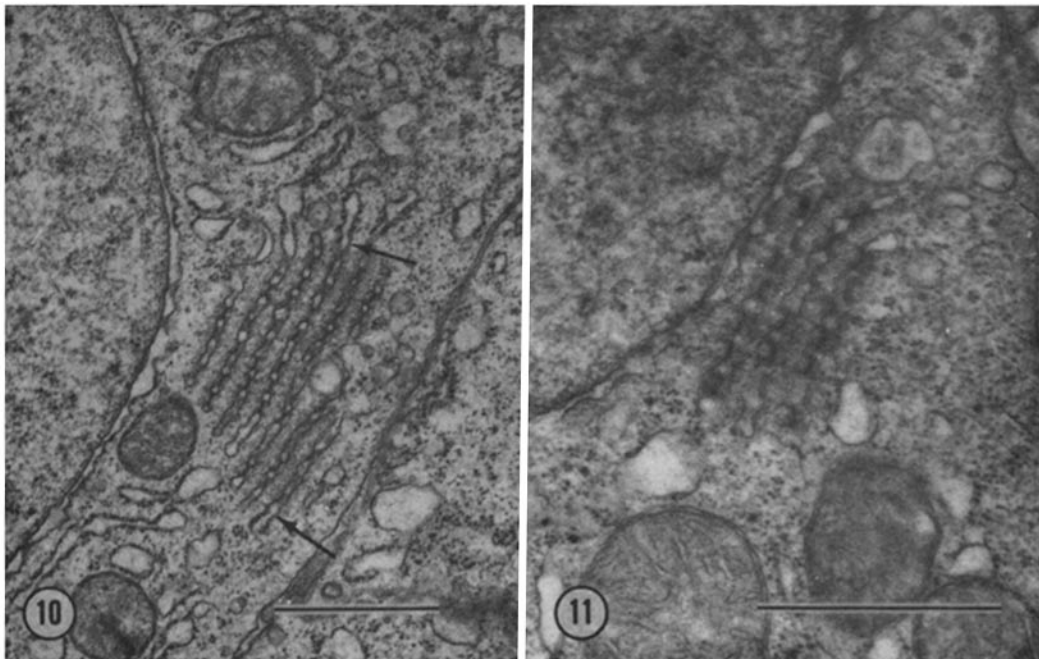
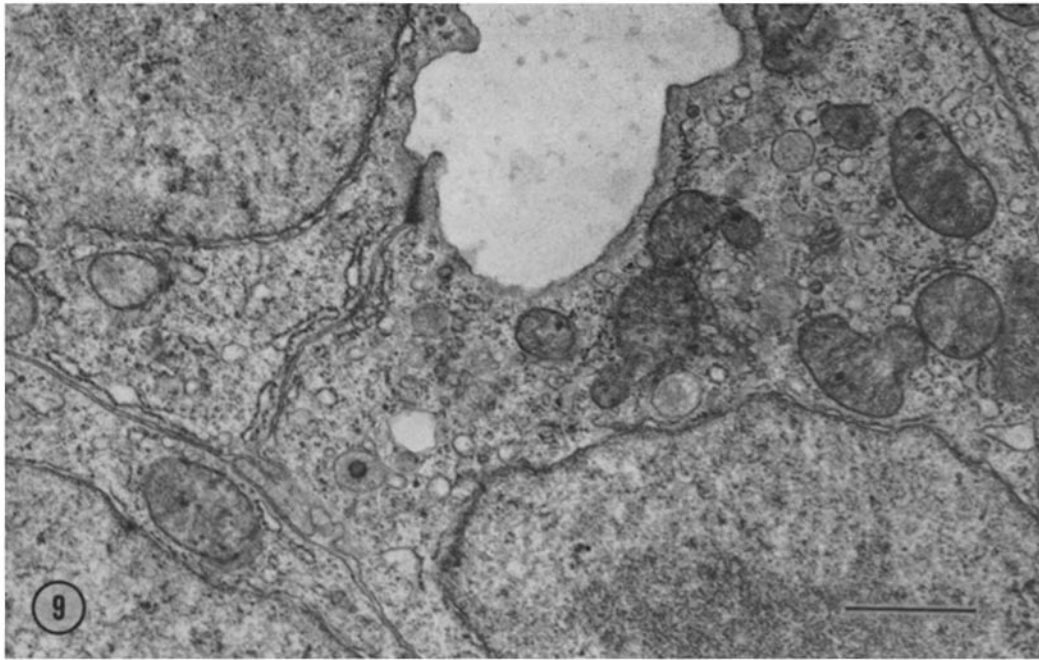
Development on Chorioallantoic Membrane

Livers from 5-day chick embryos were grafted to the chorioallantoic membrane of 12-day embryos and examined after 6 days. The grafts become vascularized and approximately doubled in weight. Presumably, there was some cell division, but much less than occurs during normal growth (6). The cells developed all the ultrastructural features of the 11 day embryo liver cell, including smooth endoplasmic reticulum and glycogen. There was little or no development of UDP-glucuronyltransferase activity (6).

DISCUSSION

The morphological sequences just described may profitably be compared with concurrent enzyme changes. Correlation of these two aspects of maturation appears to throw some light on control of functional differentiation in the developing chick-embryo liver cell.

UDP-glucuronyltransferase activity towards *o*-aminophenol is a feature of adult liver which develops to high levels on hatching in the chick, and at birth in mammals (4). This enzyme is one of a large group of microsomal enzymes concerned with detoxifying endogenous and exogenous substances (14). Its activity is present in the rough and smooth endoplasmic reticulum of the adult rabbit liver cell at a ratio of approximately two to one (15). It has been demonstrated in the microsomal fraction of adult chick liver (4), and of embryonic chick liver after culture (5, 6), although its distribution there between rough and smooth endoplasmic reticulum is not known. The presence (and increase) of only rough, not smooth, endoplasmic reticulum in the 5 day chick embryo liver cell during culture, when UDP-glucuronyltransferase activity is increasing to adult levels (6), suggests that the enzyme during normal development (4) is localized initially in rough reticulum. Rough

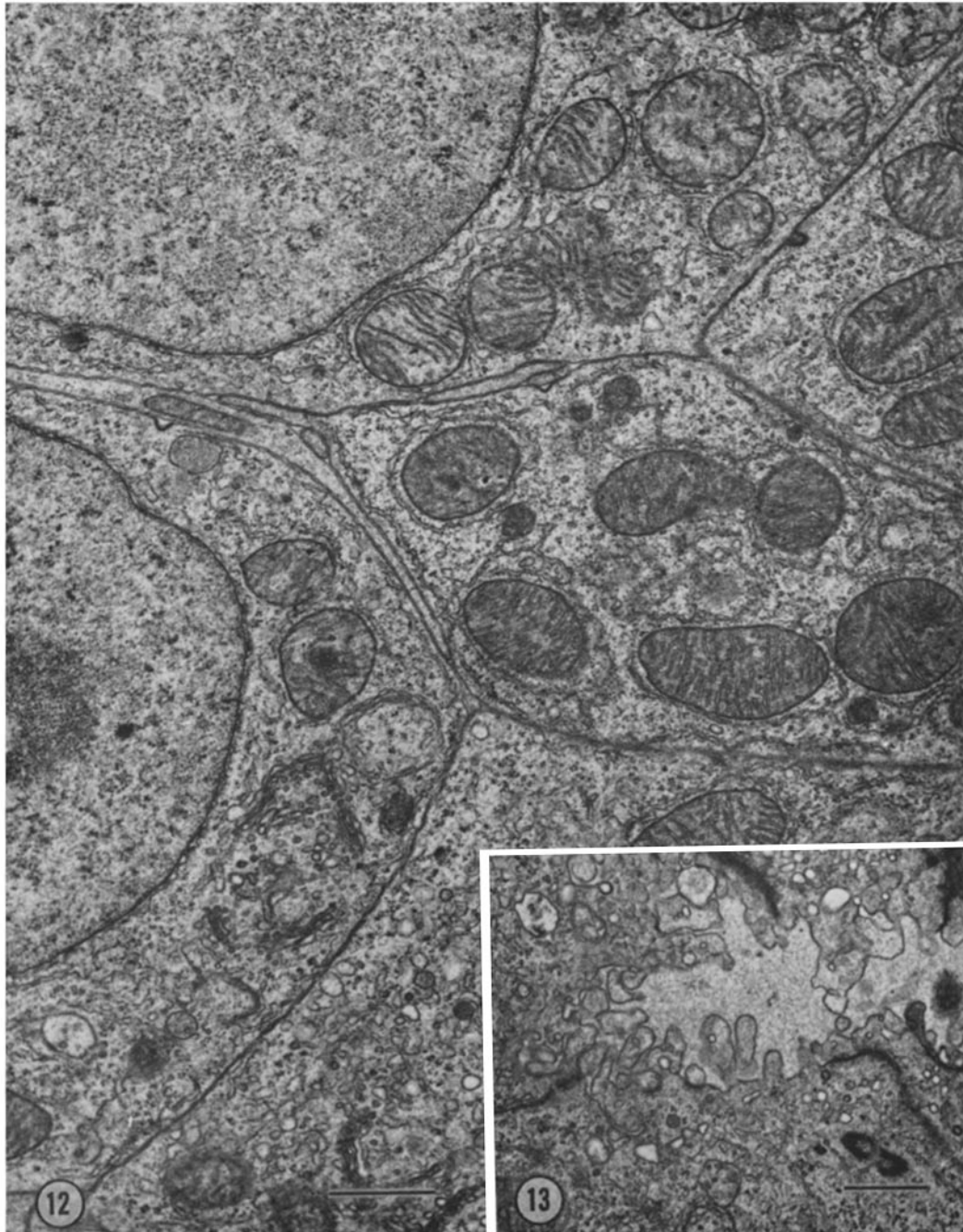


FIGURES 9-11 5 day liver after 2 days' organ culture.

FIGURE 9 Micrograph shows mitochondria with smooth membranes, granules, and increased matrix density. Note rough ER cisternae and decrease in free ribosomes. Bile canaliculus (top) is bounded by cells with tight junctions but no microvilli (apparently unchanged during 2 days of culture). $\times 17,200$.

FIGURE 10 Note annulate lamellae and connections to rough ER (arrows). The inner nuclear membrane has become smooth; the outer nuclear membrane remains ruffled as in 8 day embryo liver cell (see Fig. 4). $\times 21,400$.

FIGURE 11 Note annulate lamellae with cylindrical densities traversing the pores of the lamellae and extending to nuclear pores. $\times 32,400$.



FIGURES 12 and 13 5 day liver after 6 days' organ culture.

FIGURE 12 The micrograph shows large mitochondria with smooth membranes, many parallel cristae, dense matrices, and granules in matrix. There are very large, flattened rough ER cisternae (examine cell in center). Golgi complex is compact (examine cells at lower left corner). Note absence of smooth ER and glycogen. $\times 15,000$.

FIGURE 13 Bile canaliculus is bounded by cells with irregular microvilli. Note centriole at lower right corner. $\times 12,000$.

reticulum containing enzyme might then be converted to smooth reticulum. Such a sequence has been suggested (16, 17) for glucose-6-phosphatase activity when it appears perinatally in mammalian liver (18, 19).

UDP-glucuronyltransferase activity and rough endoplasmic reticulum increase in both 5-day and 11-day chick embryo liver cells during organ culture. Development of both enzyme and rough reticulum is greater in the 5 day than in 11 day cultured liver (6). Is development of this enzyme activity, then, always associated with proliferation of rough endoplasmic reticulum? Evidence indicates that the association is not always present. Rough endoplasmic reticulum will rapidly proliferate in 5-day embryo liver cells whether these cells are left *in ovo*, grafted to chorioallantoic membrane, or cultured on rafts; however, UDP-glucuronyltransferase activity increases only during culture (4-6). Apparently, development of this enzyme activity, but not development of rough endoplasmic reticulum, is inhibited by the embryonic environment (6). Thus, induction mechanisms for enzyme and membrane synthesis are probably different.

UDP-glucuronyltransferase activity can develop in culture systems in which the endoplasmic reticulum does not visibly proliferate, but breaks up. Segments of 8-11 day embryo liver immersed in a serum-Tyrode's medium develop adult levels of UDP-glucuronyltransferase activity within 6-8 days and maintain them for 2-3 wk (5). Under these conditions, liver tissue becomes encapsulated by proliferating spindle-shaped cells, probably derived from sinusoidal epithelium (20). Rapidly (within 2 days, and well before UDP-glucuronyltransferase activity is maximal), nearly all liver cells show extensive chromatin clumping, ballooning-out of outer nuclear membrane, swelling and rupture of mitochondria, disappearance of glycogen, and fragmentation of endoplasmic reticulum and loss of ribosomes.¹ Dissection proved that UDP-glucuronyltransferase activity develops in the degenerating hepatic cells, not in the rapidly proliferating capsule (5). Radioautography with leucine-³H indicated that proteins are synthesized at comparable rates in liver and capsule cells.¹ As UDP-glucuronyltransferase activity is associated with the "microsomal fraction" of these deranged hepatic cells (5), the relevant part of the endo-

plasmic reticulum presumably survives there; the extensive damage naturally renders interpretation of electron micrographs difficult, but no proliferation of the endoplasmic reticulum was recognized.¹

Smooth endoplasmic reticulum and glycogen (unlike the other features of adult liver) were not formed in 5-day embryo livers during culture in Eagle's medium; these structures, however, appeared in 5-day embryo livers grafted to the chorioallantoic membrane of 12-day old chick embryos. Also, once present, as in the 11 day liver, glycogen and smooth endoplasmic reticulum are stable during culture. Therefore, perhaps some factor in the circulating blood of the advanced embryo is required to initiate their synthesis. There is evidence indicating that synthesis of glycogen and smooth endoplasmic reticulum requires insulin. The maturation of the pancreatic islets has been correlated with the onset of glycogen storage in chick embryo liver (21-23). Work now in progress (24)² has shown that insulin (but not other hormones) will induce the formation of glycogen, glycogen synthetase activity, and smooth endoplasmic reticulum in 5-day chick embryo livers maintained on rafts over Eagle's medium. Chicken serum or chick embryo extract, however, can also induce glycogen storage (25). Insulin could be the inducing principle in the serum and embryo extracts. It is possible that enrichment of Eagle's medium with additional glucose, amino acids, and vitamins might permit development of smooth endoplasmic reticulum and glycogen. This would suggest that the insulin effect is not specific. Thus far, however, enrichment of this kind has not aided development except to diminish protein loss.

To summarize, the chick embryo liver cell appears in most aspects (rough endoplasmic reticulum, mitochondria, biliary microvilli, and Golgi complex) to develop independently of extrinsic factors other than nutrients; but the embryonic environment may exert some control by inhibitors, as in the case of UDP-glucuronyltransferase activity (5, 6) and by hormonal inducers, as in the case of smooth endoplasmic reticulum, glycogen synthetase, and glycogen. It must be recognized, however, that cultured cells may exhibit requirements not ordinarily limiting a process *in vivo*, or may even become freed from controls which *are* limiting in the organism.

¹ C. A. Benzo, A. M. Nemeth, and G. J. Dutton. Unpublished observations.

² C. A. Benzo and G. de la Haba. Manuscript in preparation.

This work is part of a continuing study of liver development carried out in collaboration with Dr. Geoffrey J. Dutton, Biochemistry Department, The University, Dundee, Scotland.

This study was supported by a National Institutes of Health Grant (HD00375) to Dr. Nemeth, and by a Medical Research Council Grant to Dr. G. J. Dutton.

Received for publication 26 March 1970, and in revised form 28 September 1970.

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