# STUDIES ON PRIMARY CULTURES

# OF DIFFERENTIATED FETAL LIVER CELLS

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## ABSTRACT

A method for culturing non- or slowly growing, differentiated fetal rat liver cells is described. It involves the use of collagenase as a digesting agent and of a selective medium deficient in arginine which suppresses the growth of nonparenchymal liver cells. Evidence is presented that surviving cells (a) retain liver-specific urea cycle functions measured by their capacity to transform ornithine into arginine, (b) synthesize DNA in glucose-deficient medium, and (c) synthesize and secrete albumin. This primary cell culture responds to partially hepatectomized rat serum and may be an appropriate assay system for the study of mechanisms which regulate liver regeneration.

## INTRODUCTION

The establishment of cultures of liver cells has been one of the more elusive problems of tissue culture. In 1952, undifferentiated cells derived from mouse liver were cloned and grown in culture (1). Liver explant cultures were also used in early attempts to grow liver cells for short time periods in vitro: outgrowth consisted mainly of undifferentiated granular cells with little organized formation (2). The survival of cultured, adult liver–derived explants was extended for several months by using a collagen substratum (3). A modification of this method was described recently (4), but the outgrowing cells were reported to retain only "some" differentiated functions. Recently, methods to culture hepatocytes have been reported, and cloned cell lines of rat liver origin have been established (5–8). However, these propagated cell lines and also cells in monolayer derived from cultured liver explants (4) have not retained *all* functional characteristics of hepatocytes, e.g., ability to synthesize arginine. Cells in tissue culture derived from liver

which do not exhibit one (or more) hepatic function(s) can not be defined as normal hepatocytes. In this light, culture of normal hepatocytes has not yet been achieved.

Liver regeneration has been used as a model for the study of the regulation of mammalian cell division, but lack of controlled experimental conditions in vivo has limited the understanding of the processes involved (9–11). Little is known about the growth control mechanisms by which hepatocytes are maintained in the liver as a resting population, capable of rapid synchronous proliferation after partial hepatectomy (9–11). In vitro liver cell culture systems might, therefore, be useful for studying mechanisms regulating the growth of hepatocytes.

We wish to describe a selection method for culturing rat and human fetal liver cells, which retain prominent liver-specific metabolic functions. A nonselective method (arginine-containing medium) for culturing human fetal liver cells has also

been described (12). In a second report, to be published elsewhere,[1] we describe preliminary studies with this cell culture system demonstrating enhanced stimulation of DNA and protein synthesis by serum from partially hepatectomized rats in comparison to normal rat serum.

## MATERIALS AND METHODS

### Materials

Fetal calf and calf serum were purchased from Grand Island Biological Co., Grand Island, N.Y. Collagenase was obtained from Sigma (Cat. No. C-1030) (Sigma Chemical Co., St. Louis, Mo.). Thymidine-methyl-$^3$H, L-ornithine-3-$^3$H, and L-leucine-4,5-$^3$H were obtained from New England Nuclear Corp., Boston, Mass. Rat albumin antisera were purchased from Cappel Laboratories, Downington, Pa. Guinea pig complement, sheep hemolysin, and erythrocytes were obtained from Hyland Laboratories, Los Angeles, Calif.

### Animals

200–250 g, Fisher 344, timed, pregnant rats were supplied from Simonsen Laboratories, Gilroy, California.

### Growth Media

The cells were cultured in Dulbecco and Vogt's modification of Eagle's medium deficient in arginine and/or glucose and supplemented with heat-inactivated, dialyzed fetal calf serum (usually 10%), 1650 units per ml penicillin and 33.3 μg per ml streptomycin sulfate. The cells were plated in 55 mm Nunc (Roskilde, Denmark) plastic dishes.

### Fibroblast Cultures

3T3 and SV40 virus-transformed 3T3 cells (SV3T3) were obtained from Drs. M. Vogt and R. Dulbecco. Routinely, the cells were grown in complete medium containing 10% calf serum. They were transferred, using 0.05% trypsin in Ca$^{++}$- and Mg$^{++}$-free Tris/saline solution, pH 7.4. After centrifugation, the cells were washed twice and plated at the appropriate density in 55 mm Nunc plastic dishes.

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[1] D. Paul, H. Leffert, G. Sato, and R. W. Holley. 1972. Stimulation of DNA and protein synthesis in differentiated fetal rat liver cells in culture by serum from partially hepatectomized rats. *Proc. Nat. Acad. Sci. U. S. A.* In press.

### Liver Cell Culture

Pregnant rats at 19–21 days of gestation were anesthetized with ether, and fetuses were obtained under sterile conditions. The fetuses were decapitated immediately, and the livers were removed and placed in arginine-deficient medium containing 10% dialyzed fetal calf serum (37°C). The pooled livers were cut into four pieces with scissors, and the medium was removed. The tissue was incubated in a solution of collagenase (3 mg/ml, 2 ml/liver) for 8 min at 37°C in a 125 ml Erlenmeyer flask and stirred at moderate speed with a magnetic stirrer. The supernatant was removed carefully with a Pasteur pipette and placed in a bottle containing 5 ml of arginine-deficient medium supplemented with 10% dialyzed fetal calf serum at 0°C. Four or five such enzymatic digestions were performed and the supernatants were pooled. One volume of medium was added to the single-cell suspension in collagenase and the suspension was centrifuged for 7 sec at the maximum speed of a clinical centrifuge (International Equipment Co., Needham Heights, Mass., Model HN-S) in 40 ml conical centrifuge tubes. The supernatant was removed and the pellet was resuspended in 10 ml of medium. Cell aggregation was minimized by a single vigorous pipettation. The various cell suspensions were pooled and the cell number was determined (hemocytometer, Coulter Counter [Coulter Electronics, Inc., Hialeah, Fla.]). Usually, $10^6$ cells were plated in plastic dishes (5 ml medium) and incubated at 37°C in an humidified 10% $CO_2$–90% air incubator. The time from excising the tissue to plating the cells was 2 hr. The medium was changed 24 and 48 hr after plating.

### Assay for Thymidine-$^3$H and Ornithine-$^3$H Uptake

The method of determining the incorporation of thymidine-$^3$H into trichloroacetic acid (TCA)-precipitable material in 3T3 cells is described elsewhere (13). The same method was used to determine the uptake of ornithine-$^3$H into TCA-precipitable material.

After incubation of liver cells with ornithine-$^3$H or thymidine-$^3$H, the cultures were washed and trypsinized, and portions of the cell suspension were used for the determination of cell number and radioactivity of the TCA-precipitable material retained on Millipore filters (Millipore Corporation, Bedford, Mass.). A modification of the assay for the incorporation of thymidine-$^3$H into TCA-precipitable material has been described in detail elsewhere.[1]

Radioautography was performed with washed and formalin-fixed cultures which were treated with 5% TCA. Kodak AR-10 stripping film was used.

## Transformation of Ornithine into Arginine

A simple assay was devised in order to test for the presence of ornithine-carbamoyltransferase (2. 1. 3. 3), argininosuccinate synthetase (6. 3. 4. 5), and argininosuccinate lyase (4. 3. 2. 1).

Cells to be tested were incubated in arginine-deficient medium containing 50 $\mu$Ci ornithine-$^3$H (SA, 23.6 Ci/mmole) for 24 hr at 37°C. The medium was removed, the cells were washed, and 1-2 ml of 6 N HCL was added. After some hours, the solution was incubated at 120°C for 24 hr in a closed tube. After hydrolysis, the sample was dried by evaporation in a desiccator and redissolved in 0.35 M citrate buffer, pH 5.28. Amino acid analysis was performed with a Beckman automatic amino acid analyzer (Model 120C) (Beckman Instruments, Inc., Fullerton, Calif.). For routine assay only, the basic amino acids were separated on a 0.9 × 13 cm column packed with Beckman PA-35 ion exchange resin. After chromatography, 2 ml fractions were collected and the radioactivity was determined in a liquid scintillation counter. Radioactive arginine is detected only when the three enzymes are present in the cells under study.

## Determination of Rat Albumin by Complement Fixation

5 × 10$^6$ liver cells were plated in arginine-deficient medium containing 10% dialyzed fetal calf serum in 55 mm dishes. 11 days after plating, the media from two dishes were pooled. The cells were scraped off the dishes, added to 4 ml of water, and subjected to freezing and thawing (four times). The debris was re-

moved by centrifugation. Both culture medium and cell extract were used for the determination of albumin by complement fixation following the procedure described by Wasserman and Levine (14). Heat-inactivated rat serum and arginine-deficient medium containing dialyzed fetal calf serum (10%) were used as controls. All samples were dialyzed extensively against water. Rabbit anti-rat albumin was heated at 60°C for 20 min to inactivate complement and was stored at −20°C.

## RESULTS

Initial attempts to culture fetal rat liver cells began by digesting minced pieces of fetal liver tissue with collagenase for 20 min. The resulting cultures contained single cells and small pieces of tissue. These cultures were incubated in arginine-deficient medium in order to suppress (over)growth of nonparenchymal liver cells (15). A few days after plating, cells migrated out of the tissue pieces and formed a monolayer culture. The appearance of these cultures did not change during the following 3-4 wk.

When fetal liver tissue was digested stepwise with collagenase as described in Materials and Methods, the tissue was dissociated into single cells. The "recovery efficiency" (i.e., the number of attached cells per dish 14 hr after plating divided by the number of cells plated) of such cells is shown in Fig. 1. It may be seen that the recovery efficiency at high cell density (> 10$^5$ cells per cm$^2$) is about 10%. Established cell lines (3T3, SV3T3) plate with an efficiency of 70-100%.
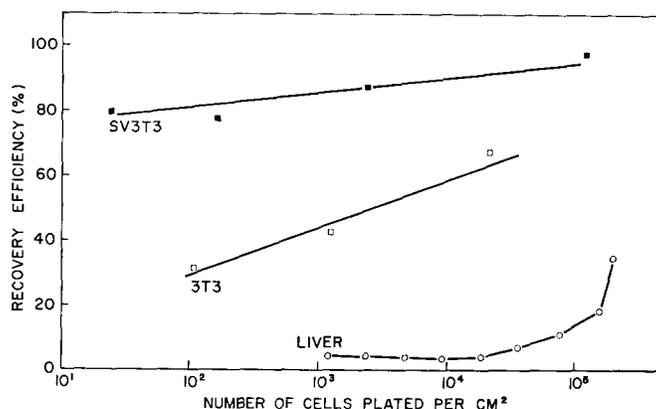


FIGURE 1  Recovery efficiency of cells as a function of cell density. Varying numbers of liver, 3T3, and SV3T3 cells were plated in 55 mm plastic tissue culture dishes in 5 ml of arginine-deficient medium supplemented with heat-inactivated, dialyzed fetal calf serum (10%). Cells were allowed to attach to the dish overnight, and 14 hr after plating, the cells were trypsinized and counted in a Coulter Counter. Abscissa: number of plated cells per cm$^2$. Ordinate: recovery efficiency in per cent.
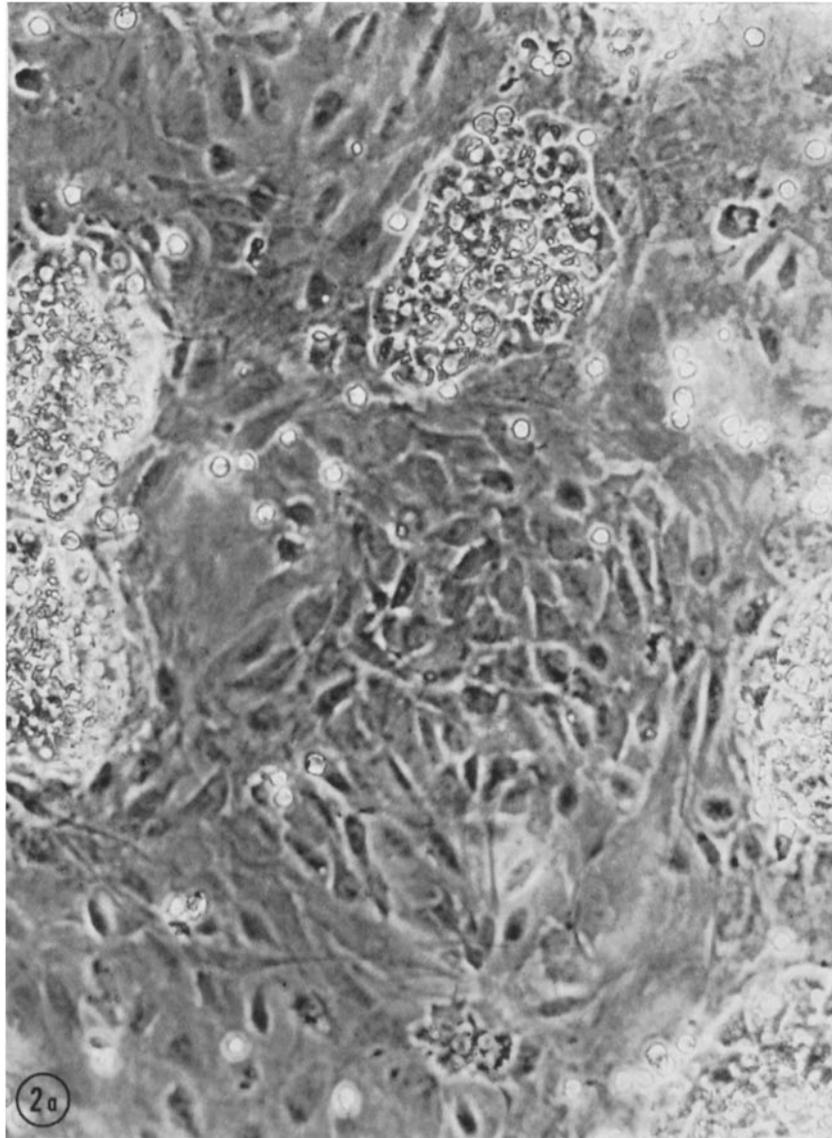
FIGURE 2   Micrographs of fetal rat liver cells in culture. Cells were plated as described in Materials and Methods. The cultures were incubated in arginine-deficient medium supplemented with dialyzed fetal calf serum (10%). The cultures shown are 10 days old. Approximately 5% of the cells are binucleated; approximately 1% are tri- or tetranucleated cells. Cells forming aggregates may be distinguished from less densely spread, single cells. (a) Phase-contrast microscopy. × 100. (b) Radioautograph showing nuclear uptake of thymidine-$^3$H. × 100.

The method of plating fetal rat liver cells described in this paper has been used successfully to plate human fetal liver cells derived from hysterotomy tissue.

Photomicrographs of fetal rat liver cultures are shown in Figs. 2 a and 2 b. It may be seen that two different types of cellular formations are present: tightly packed cell aggregates in monolayer and less densely spread single cells. Aggregation is a frequent occurrence with cell suspensions derived

(2b)
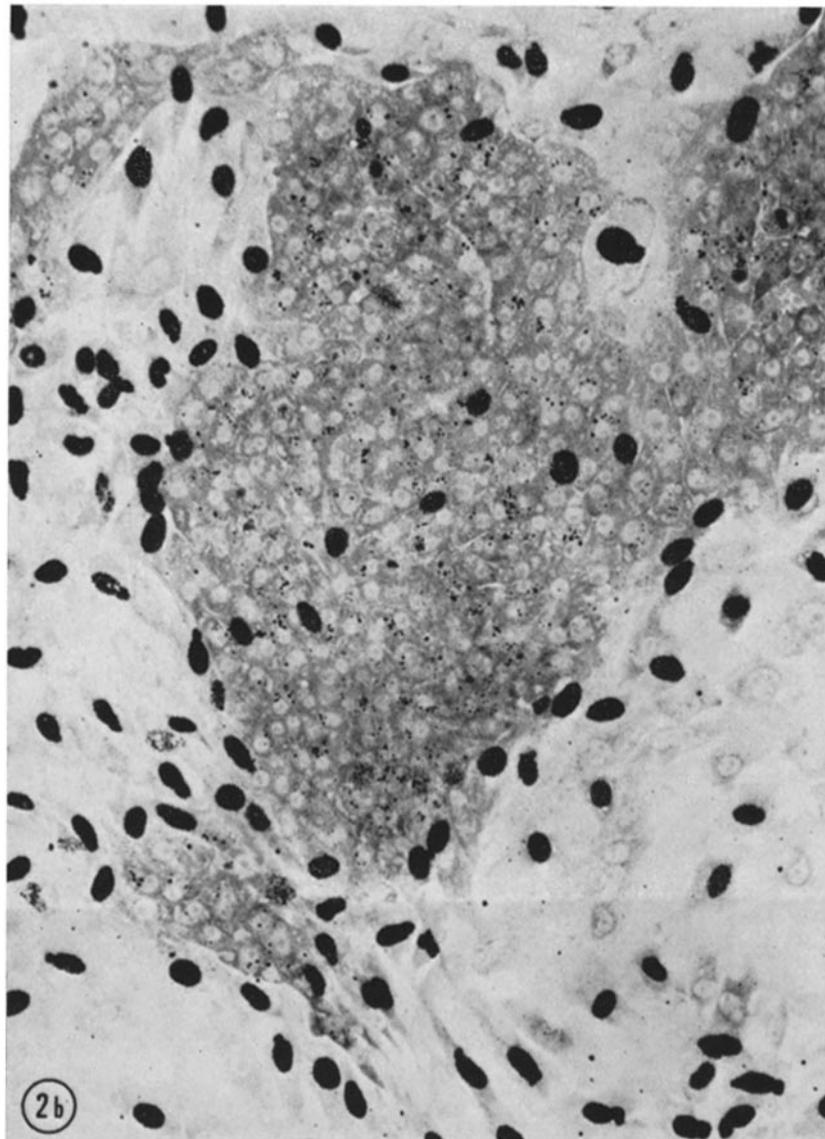
from fetal tissues. Although single-cell suspensions were plated, this aggregation was observed shortly after plating. It is not clear whether the formation of aggregates occurred in suspension before cellular attachment or afterwards by cellular migration on the surface of the dish.

In order to characterize the cells derived from fetal rat liver, the following liver functions were tested: transformation of ornithine into arginine, synthesis of DNA in glucose-deficient medium, and albumin synthesis and secretion.

## Enzymes of the Urea Cycle

Arginase is an enzyme widely distributed in animal tissues and not restricted to liver. Therefore, the ability of the cells to produce urea was not measured but, instead, their capacity to synthesize arginine from ornithine was studied because the three enzymes involved in this transformation are unique to liver. The method is described in Materials and Methods. The results of such experiments are shown in Fig. 3. It can be seen that 3T3
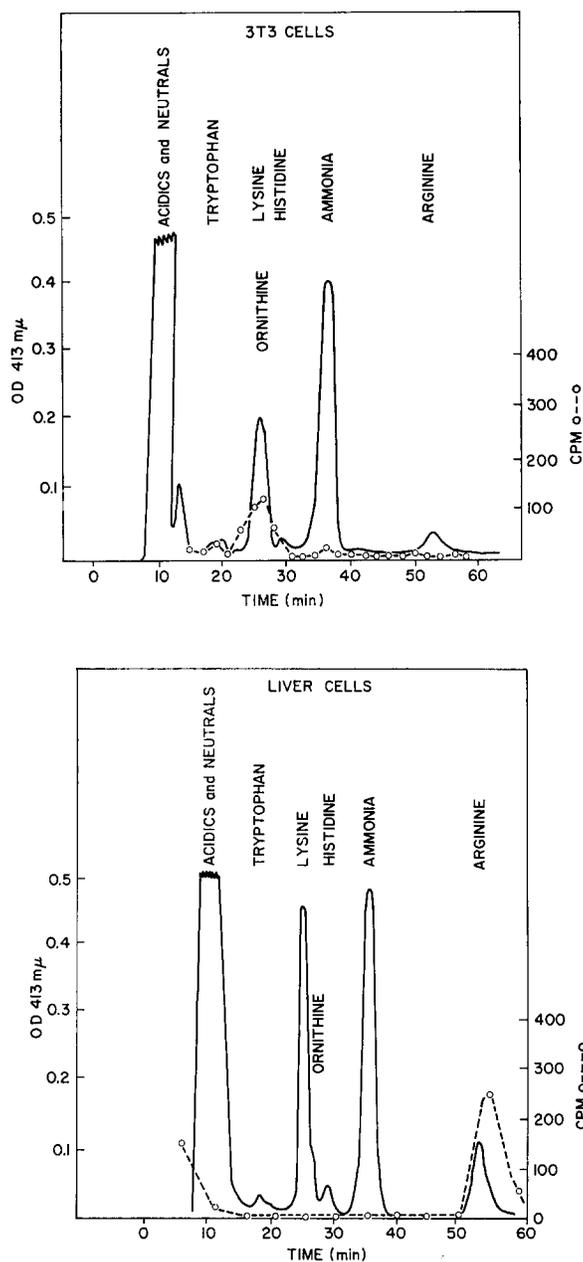
FIGURE 3  Assay for the transformation of ornithine-$^3$H into arginine-$^3$H. Cells to be tested were incubated in the presence of ornithine-$^3$H in arginine-deficient medium for 24 hr. The cells were washed, dissolved in 6 N HCl, and hydrolyzed. The dried sample was dissolved in the appropriate buffer, and amino acid analysis was performed as described in Materials and Methods. The elution volume was collected in 2 ml fractions, and the radioactivity was determined in a liquid scintillation counter. (a) 3T3 cells; (b) fetal rat liver cells. Abscissa: time after starting the fractionation, in minutes (fraction number [elution volume]). Ordinates: (left) OD413 m$\mu$; (right) counts per minute.
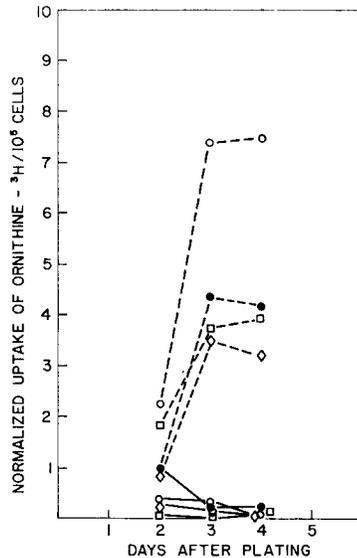
FIGURE 4 Stimulation of ornithine-$^3$H uptake in deficient medium. $10^5$ 3T3 and $10^6$ liver cells were plated in different media supplemented with dialyzed fetal calf serum. At different times after plating, the cultures were pulsed for 4 hr with $4 \times 10^{-4}$ M ornithine-$^3$H (10 $\mu$Ci/ml). The medium was removed, the cultures were washed, and the radioactivity per culture was determined as described in Materials and Methods. Solid lines: 3T3 cells; broken lines: liver cultures. ●——●, complete medium; ○——○, medium deficient in arginine; ◇——◇, medium deficient in glucose; □——□, medium deficient in arginine and glucose. Abscissa: days after plating. Ordinate: normalized specific ornithine-$^3$H uptake.

fibroblasts do not have the capacity to synthesize arginine (Fig. 3 $a$). Furthermore, it has been established that two rat liver-derived cell lines (5, 6), mouse L cells, human embryonic fibroblast $\times$ mouse L cell hybrid cells, and C6 rat glial tumor cells do not transform labeled ornithine into arginine. In contrast, the cells derived from fetal rat liver convert the bulk of labeled ornithine into arginine (Fig. 3 $b$). In addition, the incorporation of ornithine-$^3$H–metabolites into TCA-precipitable material of fetal rat liver cells is stimulated in arginine-deficient medium as compared with cultures incubated in complete medium (Fig. 4). This effect could not be observed in 3T3 cells.

Human fetal liver cell cultures plated as described here also have the capacity to synthesize arginine from ornithine.

The synthesis of arginine and its incorporation into TCA-precipitable material by fetal liver cell cultures suggested the possibility of using ornithine-$^3$H as a label to identify arginine-synthesizing cells in radioautography experiments. Radioautograms of 3T3 fibroblasts and liver cultures were made on day 6 after a 24 hr incubation with 50 $\mu$Ci ornithine-$^3$H. 3T3 fibroblasts showed a low number of cytoplasmic grains (approximately 2–3% of that of liver cells), as expected from their inability to synthesize arginine (Fig. 3 $a$). In the liver cultures, the cells within monolayer aggregates and also some outgrowing single cells showed dense cytoplasmic labeling. In separate experiments with both fibroblast and liver cultures obtained by collagenase digestion of minced pieces of liver (see above), cytoplasmic incorporation of leucine-$^3$H occurred in all cells, indicating that cells which did not show cytoplasmic grains after incubation with ornithine-$^3$H were still viable. When liver cultures were incubated with thymidine-$^3$H, the precursor was actively incorporated into the nuclei; and the larger fraction of DNA-synthesizing cells was present in the less densely spread single cells surrounding the monolayer aggregates (Fig. 2 $b$). When liver cultures were simultaneously labeled with thymidine-$^3$H and ornithine-$^3$H, few cells were observed which showed both nuclear and cytoplasmic labeling: nuclear labeling was predominant in cells no longer in contact with aggregates and cytoplasmic labeling was mainly localized in aggregates.

## Synthesis of DNA and Cell Division in Deficient Media

When growing 3T3 cells were incubated in medium deficient in glucose, DNA synthesis ceased, no further cell division occurred, and few fibroblasts survived after the 6th day (Fig. 5 $a$, $b$). When fetal liver cells were incubated in the same medium, DNA synthesis continued and the number of attached cells per dish increased slightly (Fig. 5 $a$, $b$). These experiments suggested that fetal liver cells—but not 3T3 fibroblasts—produce glucose. Similar results were obtained when the medium was deficient in glucose and/or arginine.

If ornithine was added to arginine-deficient medium, no increase in cell division was observed with liver or 3T3 cultures. However, when no medium changes were made (see Materials and Methods), considerable cell division in liver cultures was observed in the absence of ornithine and arginine. This would suggest that conditioning
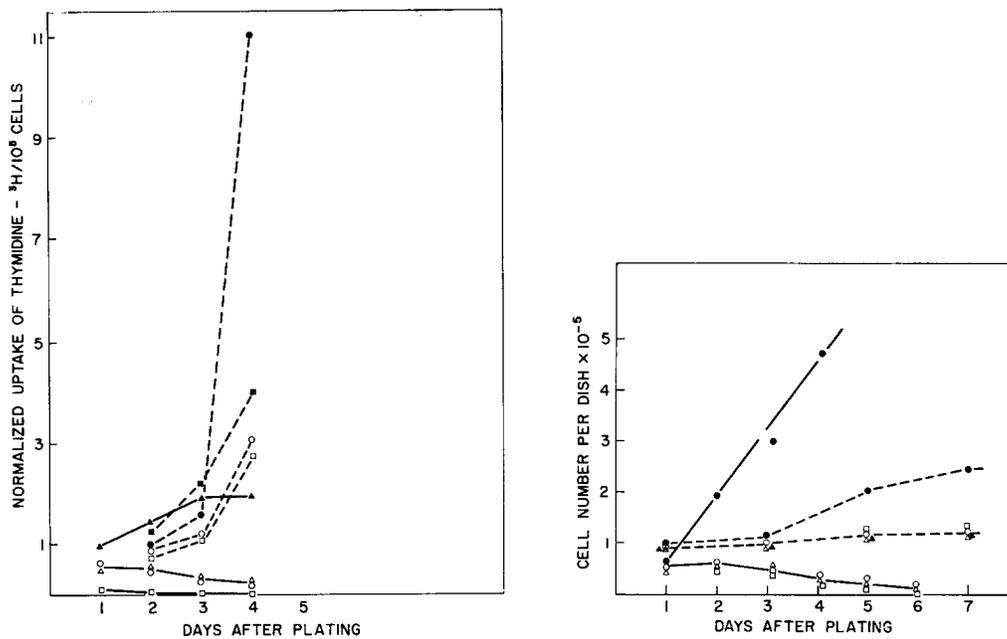
FIGURE 5  DNA synthesis and growth in fetal liver and 3T3 cultures in different media. $10^5$ 3T3 cells and $10^6$ liver cells were plated in different media supplemented with dialyzed fetal calf serum. (a) At different times after plating, the cultures were pulsed with thymidine-$^3$H (1 $\mu$Ci/ml, 3 $\times$ $10^{-6}$ M thymidine) for 1 hr (3T3 cells) and 18 hr (liver cells). The medium was removed, the cultures were washed, and the radioactivity per culture was determined as described in Materials and Methods. Solid lines: 3T3 cultures; broken lines: liver cultures. ●——● and ▲——▲, complete medium; ■——■ and △——△, medium deficient in arginine; ○——○, medium deficient in glucose; □——□, medium deficient in glucose and arginine. (b) In parallel experiments the number of attached cells at different times after plating was determined. Solid lines: 3T3 cultures; broken lines: liver cultures. ●——●, complete medium; ○ ——○, medium deficient in arginine; △——△, medium deficient in glucose; □——□, medium deficient in argninei and glucose; ▲——▲, medium deficient in arginine and supplemented with 4 $\times$ $10^{-4}$ M ornithine. Abscissa: days after plating. Ordinate: number of cells per dish.

rather than nutritional factors are required for liver cell division. These observations will be published in detail elsewhere.[2] In 3T3 cultures, ornithine cannot be used as precursor for arginine synthesis (Fig. 3).

In complete medium, the number of cells per dish increased in both liver cells and 3T3 fibroblast cultures (Fig. 5). In liver cultures it is likely that dividing cells growing in complete medium included many cells not synthesizing arginine; these cells may be an undifferentiated population. Evidence supporting this hypothesis was presented some years ago (15), indicating that the growing population of cells in primary liver cultures incubated in complete medium consisted of fibro-

blast-like cells (present in the heterogeneous plating) and not of differentiated parenchymal liver cells.

### Synthesis and Secretion of Albumin

The complement fixation assay for rat serum albumin in liver cell culture medium and liver cell extracts is described in Materials and Methods. The results are shown in Fig. 6. To show that specific complexes had formed with material secreted from liver cells, the assays were also done in the presence of antigen excess (e.g., by addition of rat serum). As seen in Fig. 6, complement fixation was reduced. The results suggest that albumin may be synthesized and secreted into the medium.
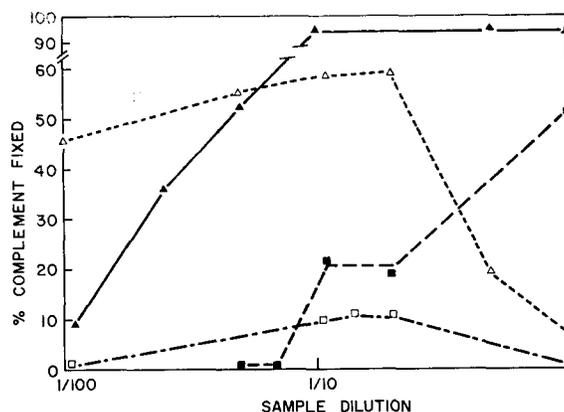
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[2] H. Leffert and D. Paul. Manuscript in preparation.

FIGURE 6  Determination of albumin in liver cell extracts and in culture medium by complement fixation. Liver cells ($5 \times 10^6$) were plated in arginine-deficient medium supplemented with dialyzed fetal calf serum. Cell extracts and culture medium were prepared as described in Materials and Methods. The determination of albumin was performed after the procedure published by Wasserman and Levine (14). Abscissa: dilution of the dialyzed sample. Ordinate: per cent of complement fixed. △——△, fresh medium (unexposed to cells), containing 10% dialyzed fetal calf serum (control); ▲——▲, liver cell extract; ■——■, medium from cultured liver cells (day 11 after plating); □——□, excess rat serum added to dilutions of medium from cultured liver cells.

## Cell Survival in Serum-Free Medium

3T3 and SV3T3 cells die approximately 3–4 days after the culture medium is changed to serum-free medium. When cells derived from fetal rat liver are changed from arginine-deficient, fetal calf serum-containing medium to serum-free medium, they survive for at least 7 wk and at this time still have the capacity to synthesize arginine from ornithine.

### DISCUSSION

The development of a differentiated liver culture might be potentially useful for studying the regulation of DNA synthesis and cell division of liver cells by serum factors. The problem of culturing hepatocytes has been approached by using a selective medium (deficient in arginine) which suppresses growth of, and eventually kills, nonarginine-synthesizing cells (e.g., 3T3 fibroblasts, Fig. 5 b). Cell division has been observed in arginine-deficient liver cultures whose medium has not been changed.[2] This would imply that arginine-synthesizing cells (hepatocytes) have been selected for by arginine deficiency. These observations along with evidence which eliminates the possibility of cross-feeding in primary cultures (i.e., arginine synthesized by hepatocytes, allowing for the survival and growth of nonarginine-synthesizing cells) will be discussed elsewhere.[2]

The observation that many differentiated functions may be lost or altered if cells are repeatedly subcultured (15) makes it unlikely that these liver cell cultures will be useful for establishing differentiated cell lines. However, liver-derived cell lines with some differentiated functions have been described (1). Nevertheless, when plated in arginine-deficient medium at high cell densities, these primary cultures may be held for at least 7 wk without loss of differentiated functions.

The recovery efficiency of established lines such as 3T3 and SV3T3 cells is a linear function of the number of cells plated per dish. As can be seen in Fig. 1, this is not the case for liver cells. Instead, at high cell densities the recovery efficiency increases markedly, which suggests that a cooperative effect is important for survival of primary liver cells in culture. This is a common but poorly understood observation for primary cells.

The fetal rat liver cells obtained by the method described in the present communication have been characterized. They are capable of transforming ornithine into arginine (Fig. 3). The synthesis of arginine in the urea cycle involves three steps, catalyzed by three enzymes (see Materials and Methods) which are liver specific. The conversion of ornithine into arginine in liver cells allows the use of ornithine-³H as a label for arginine-synthesizing cells in radioautographic experiments (Figs.

3 b and 4). Albumin is known to be synthesized by liver cells; the results from complement fixation studies suggest that albumin is present in cell extracts and in the culture medium (Fig. 6). Further studies are necessary to firmly establish these findings. The observation that liver cells synthesize DNA in the absence of glucose in the medium can be interpreted in two ways: either the cells break down glycogen or they synthesize glucose via the glyconeogenesis pathways. Further work is necessary to identify the pathway by which glucose is supplied to the cells.

Cultured liver cells, prepared as described here, respond to serum of partially hepatectomized rats by an increased incorporation of thymidine-$^3$H and leucine-$^3$H into TCA-precipitable material compared with control cultures incubated with normal rat serum.[1]

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