PARENCHYMAL CELLS FROM ADULT RAT LIVER IN NONPROLIFERATING MONOLAYER CULTURE

I. Functional Studies

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ABSTRACT

Parenchymal cells from adult rat liver have been established in primary monolayer culture. Donor animals are subjected to a partial hepatectomy and, 4 days later, cells are prepared by collagenase perfusion of the regenerated liver. The hepatic parenchymal cells, separated from nonparenchymal material and suspended in serum-free medium, are placed in plastic tissue culture dishes, where they form a monolayer within 24 h. The monolayer cells exhibit minimal mitotic activity and demonstrate several major metabolic functions characteristic of liver in vivo; these include albumin synthesis and secretion, gluconeogenesis from 3-carbon precursors, responsiveness to insulin and glucagon, glycogen synthesis, and activity of two microsomal enzymes. These functions are present in the monolayer cells for several days at activities similar to those observed in the liver in vivo. The findings indicate that hepatic parenchymal cells in this monolayer system are viable and behave in many respects like normal adult rat liver.

The liver, as a central effector of homeostasis in mammals, plays a complex role in the intact organism. For this reason, methods have been devised which permit study of hepatic physiology in vitro. Four such preparations are in general use: isolated perfused liver, incubated liver slices, suspensions of isolated hepatic parenchymal cells, and "liver" cell lines. Although all have yielded important information, each system has its methodological limitations. Isolated liver perfusion and liver slice incubation invclve unfractionated tissue and consequently fail to discriminate between hepatic parenchymal and sinusoidal cell function (1). More important—and a problem shared by suspensions of isolated hepatic parenchymal cells—is the apparent diminution of metabolic activity after 3 or 4 h in vitro (2, 3). While this period may be sufficient for the study of certain biosynthetic pathways or of rapidly responding metabolic processes, it precludes investigation of many regulatory mechanisms whose response to experimental manipulation is observed only over periods of many hours or days.

In a search for a more stable and homogeneous in vitro preparation, liver cell lines have been developed, derived from explants of liver or from hepatomas (4). Like other tissue culture systems, these hepatic cell lines satisfy the requirements of homogeneity and stability, but they usually fall short of an ideal in vitro system because they fail to retain the multiple differentiated functions of liver cells in vivo. The commercially available Chang liver cells, though derived originally from human liver tissue obtained by biopsy (5), appear to be devoid of differentiated liver function (6). Of the cell lines originating from hepatomas, a few

are capable of conjugating bilirubin (7) or of synthesizing albumin (8, 9) and other serum proteins (9), but none of the systems thus far described exhibits the full range of metabolic functions normally present in the liver in vivo. Indeed, at a recent conference on liver cell culture, the opinion was expressed that the search for a fully differentiated self-propagating liver cell line may be a "will-o'-the-wisp" (10).

One of the reasons for the poor expression of specific metabolic functions in liver cell lines may be their mitotic activity. On the basis of a few well-studied examples, it has been suggested (11) that mitotic activity in culture preempts or deletes the expression of differentiated metabolic function. This tenet has been recently supported by findings in a neuroblastoma cell line whose cells, when exposed to serum, divide at relatively high frequency and demonstrate little specific function. In contrast, when serum is removed and growth suppressed, the cells send out axons and elaborate characteristic enzymes (12).

In the present paper, we describe a method for the study of adult rat hepatocytes in vitro, using an approach which appears to satisfy the requirement of homogeneity, to preserve specific functions and to be stable for several days. The experimental procedure follows general cell culture techniques. Isolated hepatic parenchymal cells are prepared from adult rats, which have undergone a partial hepatectomy 4 days previously, and incubated in a standard defined medium. The preparation differs from cell culture, however, in that conditions are deliberately imposed that minimize cell division. The medium is free of growth-promoting components, such as serum or embryo extract, and high density seeding of cells is carried out, so that cell-to-cell contact is achieved almost immediately. During the first 24 h after plating, the hepatic parenchymal cells, initially round, become cuboidal in shape and form a confluent monolayer which is viable for periods of at least 7 days but static with respect to cell division. Data is presented that documents the presence of differentiated function in the monolayer preparations and its preservation over a period of several days. A detailed description of ultrastructural aspects of these cells in monolayer will be reported in the accompanying paper (13).

MATERIALS AND METHODS

Adult male Sprague-Dawley rats weighing 180–300 g were used. Crude collagenase, 140–200 U/mg, was obtained from Worthington Biochemical Corp., Freehold, N. J. Dialyzed fetal calf serum and Eagle's minimal essential vitamins were from Grand Island Biological Co., Grand Island, N. Y., and Eagle's minimal essential amino acids and Leibovitz' L-15 medium from Microbiological Associates, Inc., Bethesda, Md. Petri dishes were from Falcon Plastics, Oxnard, Calif. or from Lux Scientific Corp., Thousand Oaks, Calif. Firefly lantern extract was from Sigma Chemical Co., St. Louis, Mo., glucagon from Eli Lilly and Co., Indianapolis, Ind., and crystalline insulin from E. R. Squibb & Sons, New York. Benzo-[*a*]pyrene was purchased from Aldrich Chemical Co., San Leandro, Calif., and *p*-nitroanisole from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Other reagents and chemicals were from J. T. Baker Chemical Co., Phillipsburg, N. J., or from Sigma Chemical Co.

Preparation of Isolated Hepatic Parenchymal Cells

Rats were subjected to a two-thirds hepatectomy (median and left lateral lobes) under ether anesthesia and permitted to recover with free access to food and water. 4 days later, the animals were used for the preparation of isolated hepatic parenchymal cells from the regenerated liver. The method of Berry and Friend (14) was employed with the following modifications: hyaluronidase was omitted from the perfusion medium; the perfusion was carried out under room air; and the perfusion medium consisted of 0.8% NaCl, 0.04% KCl, 0.005% Na_2HPO_4, 0.006% KH_2PO_4, 1 mM sodium succinate, and 100 U/ml penicillin, pH 7.5–7.7. The perfusion rate was 30–35 ml/min. Strict sterile technique was observed throughout. Collagenase was prepared fresh each day and sterilized by passage through a washed Millipore filter (0.45 μm pore size). At termination of the perfusion, the liver was placed in a sterile flask, gently dispersed with a blunt tool, and agitated in a metabolic shaker at 37°C for an additional 15 min in 40 ml of the same collagenase-containing medium. The resulting cell suspension was filtered through a double layer of sterile cotton gauze into a centrifuge tube. The parenchymal cells were separated from debris and from sinusoidal cells by four successive centrifugations at 70 *g* for 2 min each, with resuspension of the cells after each spin in monolayer medium (see below) at 27°C. The washed isolated cells finally were resuspended in 10–20 ml of medium and their number estimated in a hemacytometer. The homogeneity of the preparations, with respect to contamination by sinusoidal cells, was determined by methods described previously (15) in which the sinusoidal phagocytes are specifically labeled by injection in vivo of ^{99m}technetium-sulfur colloid, followed by gamma counting.

The perfusion conditions of Berry and Friend (14)

were altered as a result of studies of a microsomal enzyme (*p*-nitroanisole *O*-demethylase) and of a soluble enzyme (lactic dehydrogenase) in the isolated hepatic parenchymal cells. Though cellular lactic dehydrogenase activity, as reported previously (14), was not affected by the cell isolation procedures, the activity of *O*-demethylase was reduced to 20–40% of the activity in the intact parent liver, when the published perfusion conditions were observed (14). By contrast, with the modified perfusion conditions, *O*-demethylase activity was 80–100% of that found in the parent liver.

Preparation of Monolayers

Suspensions of 5–6 × 10^6 cells were placed in 60-mm plastic Petri dishes in 2.5 ml of L-15 medium, which contained 100 U of penicillin/ml and 1 mM succinate. The cell suspensions in the plates were carefully swirled to disperse the cells, and the plates were placed in a humidified incubator at 35°C under air. Visual inspection with an inverted microscope revealed that the rounded cells were essentially in contact in a single layer in the plates. A complete change of medium was carried out after the first 24 h of incubation and every 2 days thereafter. From each rat, a batch of 20–40 monolayers was obtained. Though functional variation among cell preparations was small, an individual batch of monolayers was used for each metabolic study.

Analytical Procedures

SYNTHESIS OF ALBUMIN BY MONOLAYERS: Monolayers were incubated with L-15 medium, in which cold glycine was replaced with 1 μCi/ml of [^{14}C]glycine (sp act 4.92 mCi/mmol). After 2 h of incubation, the incorporation of labeled glycine into albumin in the medium was estimated by solid-phase radioimmunoassay (16). The binding capacity of the anti-rat albumin antibody was estimated from the amount of pure radiolabeled rat serum albumin that was precipitated under standard conditions of incubation (16). For this purpose, rat albumin was labeled with ^{125}I as previously described (16).

p-NITROANISOLE O-DEMETHYLASE: The cell material from two plates was suspended with a polyethylene scraper in a total of 1.0–1.5 ml cold potassium phosphate buffer (0.1 M, pH 7.40) and sonicated. The sonicate was clarified by centrifugation at 18,000 *g* for 10 min. Assay for *O*-demethylase activity was carried out on the supernate with *p*-nitroanisole as substrate (17). The reaction mixture contained approximately 1 mg/ml protein. For comparison, a sample of whole rat liver was homogenized in the same phosphate buffer and *O*-demethylase activity assayed in the 18,000 *g* supernate. Control studies indicated that sonication did not affect the activity of *O*-demethylase under the assay conditions described. For the study of *O*-demethylase induction in monolayers, benzo[*a*]pyrene, dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml, was added to warm L-15 medium in a single-step 1,000-fold dilution, so that the calculated final concentration of benzo[*a*]pyrene in the monolayer medium was 1 μg/ml and that of dimethyl sulfoxide was 0.1%. Control experiments were carried out with dimethyl sulfoxide alone, which had no detectable effect on morphology or function of the monolayers to a concentration of 0.3%.

GLUCONEOGENESIS FROM LACTATE OR PYRUVATE: Studies of gluconeogenesis were carried out in minimal (M) medium that contained the salt mixture of L-15 (18), Eagle's minimum essential vitamins, Eagle's minimal essential amino acids (19), and 100 U penicillin/ml, pH 7.4. Glucose in the monolayer medium was assayed by the glucose oxidase method (Worthington Biochemical Corp.). Pyruvate or lactate, as gluconeogenic substrates, were added to this medium at a concentration of 5 mM, and net glucose production was measured after 2 h of incubation.

ATP CONCENTRATION: ATP was assayed by the luciferin-luciferase method, as described by Holmsen et al. (20). For extraction of ATP from isolated parenchymal cells or from monolayers, 48% ethanol containing 5 mM EDTA and 0.07 M NaCl at 4°C was added directly to the cells, which were then transferred to a centrifuge tube and maintained at 4°C for 15 min for completion of the extraction. The samples were clarified by centrifugation at 20,000 *g* for 10 min. For measurement of ATP, the supernate was diluted in cold 0.07 M NaCl and assayed in an Aminco Photomultiplier microphotometer (American Instrument Co., Inc., Travenol Laboratories Inc., Silver Spring, Md.) connected to a linear recorder. The ATP in individual preparations was quantitated with an internal ATP standard solution. The concentration of ethanol in the assay system never exceeded 3%, which is without effect on the assay (20). For purposes of comparison, ATP was also assayed in whole rat liver. The liver *in situ* was flushed free of blood with cold isotonic saline via the portal vein, immediately excised, and a thin slice rapidly frozen between two blocks of solid CO_2. The time which elapsed between the saline perfusion and the freezing of the liver sample was less than 5 s. The frozen liver slice was powdered in a mortar placed in a dry ice-acetone bath, and the ATP was extracted and assayed as described above.

MISCELLANEOUS PROCEDURES: Standard methods were employed for the measurement of glycogen (21), inorganic phosphorus (22), cyclic adenosine 3′,5′-monophosphate (23), protein (24), glucose-6-phosphatase (25), and lactic dehydrogenase (26).

RESULTS

The isolated hepatic parenchymal cells were morphologically intact and free of debris. It was estimated that with a correction for the recovery of cells from the intact liver (15), the preparation of isolated parenchymal cells contained fewer than 1% sinusoidal cells. From one liver, 100–200 $\times$ 10^6 parenchymal cells were obtained, of which 60–80% were single cells, the remainder being mostly pairs of cells.

Monolayers of Hepatic Parenchymal Cells

Within 6 h of plating, the initially spherical cells adhered to the plastic surface of the Petri dishes, began to flatten and assume a cuboidal shape. After 24–36 h, adaptation of the cells to the in vitro conditions appeared to be complete, in that at least 75% of the plate showed confluent hepatic parenchymal cells with light microscope morphology that resembled normal liver (Fig. 1). Mitotic figures were found in less than 0.1% of the cells. Because the procedure required a high density of cells in the central portion of the Petri dish, plating density at the periphery of the dish tended to be excessive, resulting in piling up of cells, inadequate surface adhesion, and consequently, poor monolayer formation. Thus, a peripheral band of cells usually was lost in the first change of medium after 24 h. This difficulty could be minimized by the use of contoured dishes (Lux Scientific Corp.). In addition, scattered nonviable round cells frequently were observed on the surface of the monolayer, identified by an amorphous appearance and lack of cytologic detail under light microscopy (Fig. 1); these probably represented cells that had been damaged by the preparatory procedures and carried through to the plating stage. Similar nonviable cells also appeared to originate from the monolayer itself as its incubation progressed beyond the 4th day. After about the same duration of incubation, the single confluent sheet of cells began to condense, forming bands and arcs of closely packed cells. Unless indicated otherwise, no functional studies were carried out beyond the 4th day of incubation, although monolayers were observed to exclude trypan blue dye for as long as 2 wk. Growth of fibroblasts or of other mesenchymal cells never occurred in these cell plates, and bacterial contamination was extremely rare.

Viability and Metabolic Functions of Monolayers

Ultrastructural morphology (13) and the exclusion of trypan blue dye (27) provided general

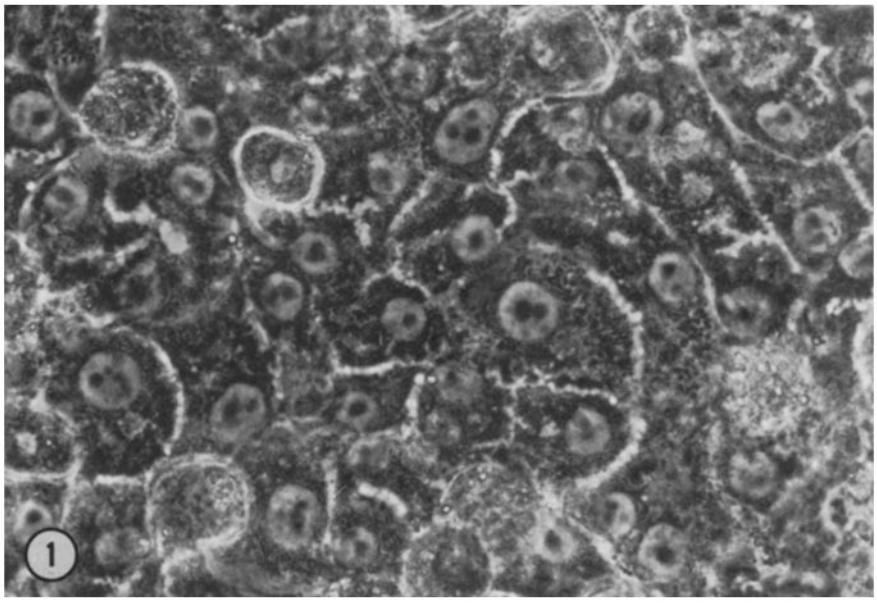

FIGURE 1 Rat hepatic parenchymal cells in monolayer after 4 days of incubation. The amorphous material on the surface of the cell sheet represents nonviable cells. Phase contrast. $\times$ 440.

evidence that the monolayers were viable. In addition, lactic dehydrogenase activity, assayed serially from the 1st to the 5th day of incubation, remained at 80–100% of the activity found in the parent liver. The monolayers were allowed 48 h of adaptation to the in vitro situation before specific metabolic functions were assayed, and, in general, these procedures were carried out between the 2nd and 4th day of incubation. This waiting period allowed for resolution of autophagic vacuoles that may occur during cell isolation and the initial period of monolayer formation (13). Up to the 4th or 5th day of incubation, all of the metabolic functions investigated were measurable at levels similar to those of intact liver. Beyond this time period, the individual functions declined to a variable degree, as indicated below.

ALBUMIN SYNTHESIS: After an initial lag, labeled glycine was incorporated into albumin at a linear rate for at least 2 h (Fig. 2). The lag period presumably represents the time required for transcellular secretion of the newly synthesized albumin. In order to demonstrate that this time lag was not simply due to isotope dilution in a large cellular glycine pool, derived from the high glycine concentration (2.7 mM) in complete L-15 medium, monolayers were preincubated for 1 h in glycine-free medium before addition of the labeled glycine. Under these conditions, the specific activity of the secreted albumin increased, but the lag period was unchanged (Fig. 2). A similar lag period (15–20 min) has been observed between injection of labeled amino acid to animals in vivo and the appearance of labeled albumin in the blood (28, 29). As estimated from the binding capacity of the antialbumin (see Materials and Methods), albumin was synthesized by the monolayers and exported to the medium at a rate of about 1 μg/h per mg cellular protein, which is comparable to the reported rate of albumin synthesis in rat liver in vivo (29). The relative rate of albumin synthesis, as a function of the age of the monolayer, is shown for two different batches of monolayers in Fig. 3. After approximately 4 days of incubation, a decline in albumin synthesis was seen, which was paralleled by reduced activity of other metabolic functions.

GLUCONEOGENESIS FROM LACTATE AND PYRUVATE: Hepatocyte monolayers, incubated in M medium, produced substantial amounts of glucose, which could be almost entirely accounted for by glycogen breakdown (Table I). The presence of Eagle's minimal amino acids in M medium (see Materials and Methods) had no measurable effect on glucose production or glycogen breakdown by monolayers, as compared to data from experiments using amino acid-free M medium. By contrast, the addition of 5 mM lactate or pyruvate to M medium significantly stimulated glucose production while inhibiting glycogenolysis over the same time period. Thus, at least 90% of the glu-

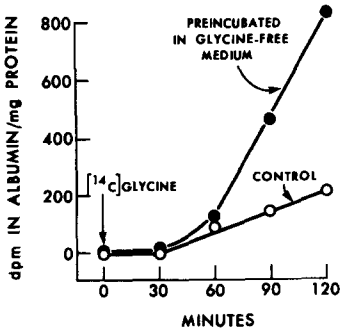

FIGURE 2 Synthesis of albumin by rat hepatocyte monolayers. Monolayers obtained from a single batch of cells were incubated with [^{14}C]glycine, and cumulative incorporation of the label into albumin in the medium over a 2-h period was determined at the times indicated. Results are expressed as disintegrations per minute in immunoprecipitable albumin per milligram total cellular protein. One series of plates (●–●) was preincubated in glycine-free medium for 1 h before addition of the label; control plates (○–○) were maintained in the complete medium.

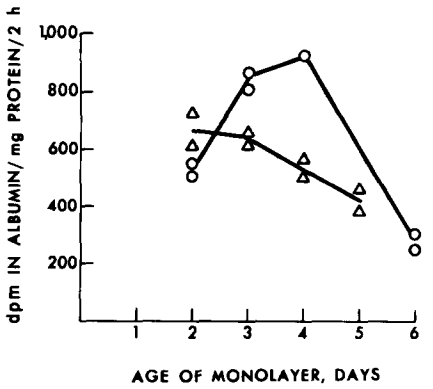

FIGURE 3 Synthesis of albumin by hepatocyte monolayers. On days 2–6 after plating of the cells, individual monolayers were incubated for 2 h with [^{14}C]glycine, and the incorporation of the label into albumin in the medium was determined. Two different batches of cells (○–○ and △–△) were used; each point represents albumin production by a single hepatocyte monolayer.

TABLE I

Gluconeogenesis from Pyruvate and Lactate in Monolayers

Conditions of incubation	Glucose production*	Glycogen consumption*
	μg/mg protein/2 h	μg/mg protein/2 h
M medium	11.2 ± 0.8	9.0 ± 0.5
Added pyruvate, 5 mM	19.5 ± 0.7	1.0 ± 1.5
Added lactate, 5 mM	19.2 ± 0.7	1.5 ± 1.0

* Results are given as the mean of four individual monolayers ± SD, expressed per milligram of cellular protein. Initial glycogen content was estimated from assay of sister monolayers and was 23 ± 2 μg/mg protein (n = 4).

TABLE II

Glycogen Synthesis by Cells in Monolayer

	Conditions of incubation*	Glycogen, μg/mg protein (mean ± SD, n = 4)
1)	Complete medium	14 ± 4
2)	Added glucose, 1 mg/ml	26 ± 3‡
3)	Added glucose, 1 mg/ml, and insulin, 0.4 mU/ml	31 ± 2‡§
4)	Added glucose, 1 mg/ml, and insulin, 1.0 mU/ml	41 ± 8‡§
5)	Added glucose, 1 mg/ml, and insulin, 4.0 mU/ml	51 ± 3‡§

* Monolayers were incubated for 16 h in L-15 medium with the additions indicated.
‡ $P < 0.01$, with respect to (1).
§ $P < 0.05$, with respect to (2).

cose produced in the presence of pyruvate or lactate appears to represent conversion of the individual 3-carbon precursor to glucose (Table I). The rate of gluconeogenesis by monolayers from pyruvate or lactate was comparable to the rates reported for isolated perfused liver (30–32) and for suspensions of isolated hepatic parenchymal cells (14, 30, 33) if allowance is made for the different substrate concentrations employed in the individual studies. (For conversion of milligrams cellular protein to milligrams wet weight of liver, it was assumed that 22% of the total liver mass is protein [34].)

RESPONSE OF MONOLAYERS TO GLUCAGON AND INSULIN: The complete L-15 medium contains no glucose, and the cells in monolayer demonstrate little glycogen, both by chemical analysis and by electron microscopy (13). The cells are, however, capable of glycogen synthesis. Addition of glucose to the complete medium resulted in a significant increase in cellular glycogen. A further dose-related rise in glycogen in the cells was observed with the addition of insulin to the incubation medium (Table II).

The metabolic response of the monolayer to glucagon was studied in terms of glycogenolysis. Addition of 2×10^{-10} M glucagon to the medium produced a rapid release of glucose into the medium with a corresponding loss of cellular glycogen (Fig. 4). The concentration of the hormone used was similar to that employed in studies with isolated perfused liver and is considered to be in the physiological range (32). The time-course of the

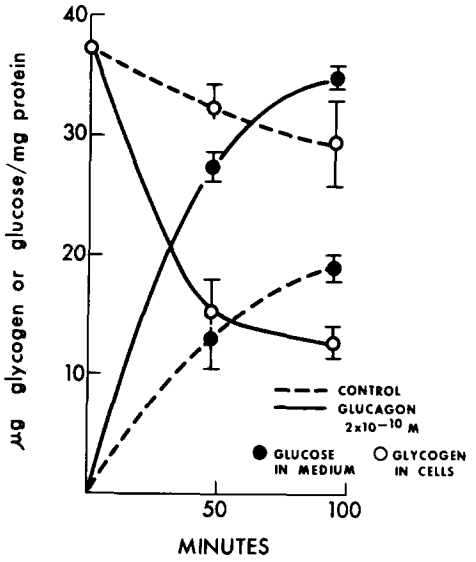

FIGURE 4 Glycogenolysis in monolayers: effect of glucagon. Individual monolayers from the same batch of cells were incubated in M medium in the presence (solid line) or in the absence (dashed line) of glucagon, 2×10^{-10} M. Glucose in the medium (●–●) and glycogen in the cells (○–○) were assayed at the time indicated. Each point represents the mean and SD of four monolayers.

glycogenolytic process indicates that the response to glucagon was essentially completed within 45 min (Fig. 4). In preliminary results, cyclic adenosine 3′,5′-monophosphate increased in response to 2×10^{-8} M glucagon; both the time-course and

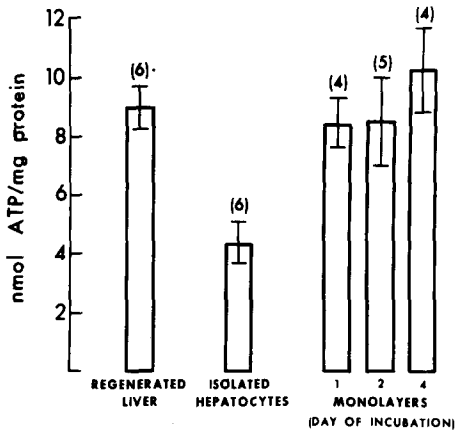

FIGURE 5 ATP concentration in regenerating rat liver, isolated hepatic parenchymal cells and hepatocyte monolayers. ATP concentrations are expressed in nanomoles per milligram cellular protein. Numbers in parentheses indicate the number of experiments, and brackets indicate ±1 SD of the mean.

magnitude of the response were comparable to that reported in isolated perfused liver (35).[1]

ATP CONCENTRATION: ATP concentrations in the liver of the rats used for preparation of hepatocyte monolayers were comparable to those reported in the literature (2, 36). During preparation of the isolated hepatic parenchymal cells, the ATP concentration significantly fell but, after incubation of the cells under monolayer conditions for 24 h, rose to the level found in the intact liver (Fig. 5). These findings suggest that the cells, once in monolayer, may be capable of restoring normal levels of intermediates, such as ATP, lost or degraded during the cell preparation.

MICROSOME ENZYME ACTIVITY: The baseline activities of *O*-demethylase and glucose-6-phosphatase in hepatocyte monolayers differed from one batch of cells to another but generally ranged from 60 to 100% of the respective enzyme activity in rat liver in vivo. Addition of benzo[*a*]pyrene to the incubation medium resulted in a significant increase in *O*-demethylase activity, whereas glucose-6-phosphatase activity was unaffected by the polycyclic hydrocarbon (Fig. 6). The dissimilar response of these two microsomal enzymes to the inducing hydrocarbon mirrors the behavior of these enzymes in the liver in vivo, where glucose-6-phosphatase activity also fails to increase during induction of the drug-metabolizing system (37). In a given batch of cells, *O*-demethylase activity remained constant over the first 6 days of incubation (Fig. 7). The inductive effect of benzo[*a*]pyrene on *O*-demethylase was

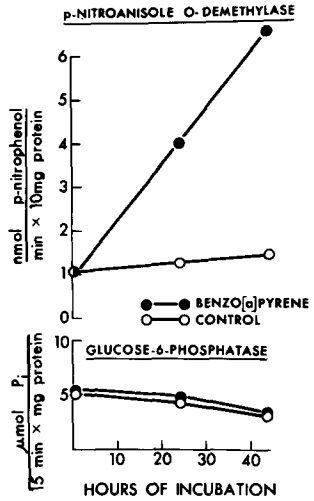

FIGURE 6 Effect of benzo[*a*]pyrene on microsomal enzyme activity in hepatocyte monolayers. Monolayers were incubated in L-15 medium in the presence (●–●) or absence (○–○) of benzo[*a*]pyrene, 1 μg/ml. At the times indicated, two monolayers were assayed for *O*-demethylase activity (average value) and a third monolayer for glucose-6-phosphatase activity. Enzyme activity was measured in the 18,000 *g* supernatant fraction.

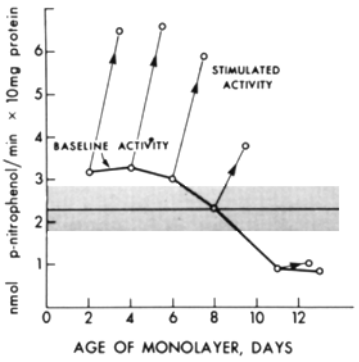

FIGURE 7 Stimulation of *O*-demethylase activity by benzo[*a*]pyrene in hepatocyte monolayers of different age. On days 2–13 after plating, monolayers obtained from a single batch of cells were incubated for 36 h with 1 μg/ml benzo[*a*]pyrene. The activity of *O*-demethylase was compared with that of untreated monolayers of comparable age. Results are expressed as nanomoles *p*-nitrophenol formed per minute per 10 milligram protein in the 18,000 *g* supernatant fraction. The shaded area represents enzyme activity (mean and SD, six animals) of whole regenerating rat liver 4 days after partial hepatectomy.

[1] Cyclic adenosine 3′,5′-monophosphate assays were kindly performed by Dr. H. R. Bourne, University of California, San Francisco, Calif.

reproducibly present at various times during the first 6 days of incubation of the monolayers. Thereafter, the diminishing baseline activity of the enzyme was accompanied by a reduced response to the inducer chemical (Fig. 7).

DISCUSSION

The monolayer system for hepatic parenchymal cells described in this paper has been designed specifically for the study of differentiated hepatic functions in vitro. The presented findings document that various metabolic functions characteristic of hepatocytes are preserved in the incubated cell monolayers over a period of several days. The parameters of viability and function which have been selected for study are typical of liver and at the same time permit a general assessment of cell integrity. Albumin synthesis occurs exclusively in the liver (38) and also serves as a general measure of protein synthesis and secretion. Gluconeogenesis is one of the principal functions of the liver and demonstrates the integrated interaction of the mitochondrial and cytoplasmic compartments of the cell (39). The response to insulin or glucagon serves to verify the presence of membrane receptors for these hormones (40, 41) as well as to demonstrate the integrity of the adenyl cyclase system. The preservation of the smooth endoplasmic reticulum, which is abundantly present in normal liver (42), has been documented by assay of two of its enzymes and their response to inducing chemicals. The operation of these processes at levels comparable to those in the intact organ indicates that the incubated cells maintain a high degree of metabolic integrity. Moreover, within a few hours of plating and incubation, monolayers exhibit repletion of ATP, which was subnormal in the freshly isolated hepatocytes (Fig. 5), and responsiveness to physiological levels of glucagon, which also may be altered in collagenase-prepared cell suspensions (33). This suggests that the conditions under which the monolayers are incubated permit repair of injury that may have occurred during the preparative stages of cell isolation.

Of the existing experimental systems for studying differentiated hepatic function in vitro, the isolated perfused rat liver is perhaps the most widely used, and it served in part as a standard for judging the metabolic performance of the cells in monolayer. In contrast to isolated perfused liver, however, hepatocyte monolayers offer the advantages of cellular homogeneity, stability for days rather than for hours, and the opportunity to conduct multiple experiments with cell material from a single liver. A second frequently used experimental system consists of liver slices incubated in physiologic buffers, which carry out a variety of metabolic functions and which can be prepared in large number from a single liver. The liver slice technique resembles the monolayer system in some functional aspects, but it has the disadvantages of cellular nonhomogeneity and of limited viability as well as the problem of uneven exposure of central areas of the slice to the medium. Moreover, the relative concentrations of glycolytic intermediates appear to undergo marked shifts in excised liver (36). A third experimental system consists of the incubation of suspensions of isolated hepatic parenchymal cells. These are derived usually from nonregenerating (resting) rat liver but are similar in other aspects to the cells used for preparation of the monolayers. The cell suspensions are essentially free of nonparenchymal elements and multiple samples can be prepared from a single liver. However, as with isolated perfused liver, cell suspensions appear to have a limited viability. The ATP concentration in these cell preparations is depressed (Fig. 5), which suggests a deviation from the normal concentration of metabolic intermediates (36), and the relatively high concentrations of glucagon required to stimulate gluconeogenesis (33) may indicate that membrane hormone receptors have been damaged during the preparation of the cells.

Much of the technique for the monolayer system has been borrowed from the methods of cell culture, in that dispersed cells are used as the starting material and a cell monolayer is the result. Hepatocyte monolayers differ from cells in conventional tissue culture primarily in being nonproliferating. Thus, the term "culture" in its strict sense may be inappropriate for the monolayer technique. Most published cell culture methods for liver utilize embryonic material dispersed with proteolytic enzymes and grown in the presence of serum. Chick embryo liver has been used extensively for the study of the heme synthetic pathway (42–45). While important information has been gained by this technique, its relevance to mammalian physiology has been questioned on a number of counts. For example, several compounds that are inactive in mammalian liver have been shown to stimulate heme synthesis in chick embryo liver (42, 46); also, heme catabolism in avian cells apparently proceeds by mechanisms different from those described in various mammalian tissues (47). More-

over, chick embryo liver offers few, if any, technical advantages over the monolayer method, in that the survival of cells in both systems is approximately the same. With the use of rat embryo liver (48), the metabolic peculiarities of avian tissue may be circumvented, but the question of the applicability of observations made with embryonic tissue systems for adult physiology remains unresolved (49). Recently the maintenance of adult rat liver cells in vitro by a "squash" technique (50) or in filter wells (51) has been reported. Differentiated hepatocyte function was not, however, studied under these circumstances.

Organ explants are related to cell culture, in that in both systems cell proliferation is observed under the influence of serum-containing medium. With liver explants, outgrowth of cells occurs reproducibly, which frequently survive for prolonged periods of time (52), and hepatic architecture is at least initially preserved. It is doubtful, however, whether differentiated cell function is long maintained. A "gradient" of cell viability may be detected morphologically, ranging from necrosis and death of centrally located cells to cell proliferation on the surface of the explant (4). Moreover, the growing cell population frequently appears to be mesenchymal in type rather than parenchymal (53, 54), and persistence of differentiated function in liver explants has not yet been demonstrated.

Cell cultures, as well as explants, of liver have permitted isolation of individual cell lines that have survived many passages in vitro. While these cell lines represent an attractive approach to the problem of a stable in vitro cell system, it is often difficult to determine the type of the isolated cell (4). As has been shown in a cloned hepatoma cell line, the phenotype of daughter cells may change markedly from that of the parent cell in the course of relatively few mitotic cycles (55), and with repeated passage of a cell line over a period of months or years, neoplastic changes may appear. Late-passage Chang liver cells were found to be indistinguishable from tumor cells by morphologic and karyotypic criteria (56). Moreover, while particular metabolic features of a given cell line may be retained through many generations in vitro, cultured cells in general appear to undergo alteration to a common enzymatic profile regardless of the origin of the cell (57). A possible exception to these findings is a hepatoma cell line, which exhibits multiple metabolic characteristics of normal liver (9). It is also of interest, however, that while other hepatoma and liver cell lines typically divide every 48 h or less (58–62), this apparently stable, relatively well-differentiated cell line has a generation time of 8–12 days (9).

Thus, it may be one of the important features of hepatocyte monolayers that they exhibit only rare mitotic figures and thereby resemble closely adult rat liver in vivo, in which the parenchymal cells divide rarely, if at all (63, 64). In order to keep the mitotic activity of monolayers at a minimum, the usual techniques of cell culture were modified as follows. A serum-poor medium was used in order to avoid the growth-promoting properties of serum and other biological fluids (65). In addition, since various mammalian cells in culture are known to exhibit density-dependent inhibition of cell division (66–68), plating of cells was carried out at a density such that cell-to-cell contact was achieved almost immediately. Finally, since we found that parenchymal cells from resting adult rat liver required 10% fetal calf serum for monolayer formation, 4-day regenerated liver served as the source of cell material. Bissell and Tilles (69) had previously demonstrated that human fetal liver can be established in monolayers in the absence of serum. We suspected that regenerated liver might be similarly adaptable to serum-free conditions, since such liver is known to exhibit some of the characteristics of fetal liver (70, 71). The combination of these methodological modifications resulted in hepatocyte monolayers which were stable in cell number for several days. An interval of 4 days between partial hepatectomy and preparation of isolated hepatic parenchymal cells was found to be optimal for the reproducible formation of monolayers in serum-free medium. When the period of liver regeneration exceeded 4 days, monolayer formation became progressively less satisfactory unless serum was included in the medium.

Since regenerated liver was used as the cell source for monolayers, the question may arise whether regenerated liver, obtained on the 4th day after partial hepatectomy, may be taken to reflect the metabolic state of resting liver. While this question must be raised anew for each kind of process under study in the monolayer system, the present data suggest that the cells in monolayer are similar to resting liver. After partial hepatectomy, the rate of DNA synthesis reaches a peak after about 24 h, but by 4 days later it has returned almost to normal (74). Other processes, such as albumin synthesis, may be affected

in the opposite direction during the phase of active DNA synthesis but also return to normal after 4 days (28). The activity and inducibility of the microsomal enzymes concerned with drug metabolism, may be markedly altered or temporarily lost during early liver regeneration (72–75). However, 4 days after partial hepatectomy, the activity of these enzymes returns toward normal values, and they respond normally to inducing chemicals. Finally, a variety of hepatic functions are unaffected by partial hepatectomy (74–76). Overall, it appears that potential differences between cells grown in monolayer and normal liver tissue would be unrelated to changes imposed by the regeneration process, more likely being due to nutritional or hormonal deficiencies of the in vitro conditions. For example, hepatic parenchymal cells maintained in a serum-free defined medium for 3 or 4 days might be expected to undergo alteration in adrenal corticosteroid-dependent function; and indeed, we have noted that cells plated and maintained in the presence of 10^{-6} M hydrocortisone exhibit a significantly greater gluconeogenic response to glucagon and epinephrine than do cells from the same preparation established in cortisone-free medium.[2] These findings appear analogous to data from studies of isolated perfused livers from adrenalectomized animals (77). Thus, the monolayer method offers the possibility for study of the various hormonal and nutritional modulators of normal hepatocyte metabolism.

Critical to the success of the described method is the density of the initial inoculum. Optimally, the plate should contain a single layer of contiguous round cells. Excessively large inocula, leading to piling up and overlap of cells, prevent proper cell attachment, while excessively small inocula, which prevent cell-to-cell contact, result in spotty confluence in the plate and consequently short-lived preparations. Also important is the depth of the medium used. Whereas 2.5–3.0 ml of medium per 60-mm plate yield optimal results, the 5 ml volume frequently used with plates of this size prevents monolayer formation. This effect may be related to diminished gas exchange through the greater depth of fluid (78).

The proposed in vitro system, allowing study of differentiated hepatic function in stable isolated cells, has countless applications for metabolic investigations. It presently is being used for the study of several aspects of the synthesis and degradation of components of rat liver membranes, including the regulation of heme and hemoprotein synthesis, lipoprotein formation, and cholesterol metabolism.

[2] D. M. Bissell and L. E. Hammaker. Unpublished observations.

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REFERENCES

1. Bissell, D. M., L. Hammaker, and R. Schmid. 1972. Hemoglobin and erythrocyte catabolism in rat liver: the separate roles of parenchymal and sinusoidal cells. *Blood J. Hematol.* **40**:812.
2. Ross, B. D., R. Hems, and H. A. Krebs. 1967. The rate of gluconeogenesis from various precursors in the perfused rat liver. *Biochem. J.* **102**:942.
3. Howard, R. B., and L. A. Pesch. 1968. Respiratory activity of intact isolated parenchymal cells from rat liver. *J. Biol. Chem.* **243**:3105.
4. Watanabe, H. 1970. Recent progress in the study of liver culture. *Prog. Liver Dis.* **3**:49.
5. Chang, R. S. 1954. Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc. Soc. Exp. Biol. Med.* **87**:440.
6. Perske, W. F., R. E. Parks, Jr., and D. L. Walker. 1957. Metabolic differences between hepatic parenchymal cells and a cultured cell line from liver. *Science (Wash. D. C.).* **125**:1290.
7. Rugstad, H. E., S. H. Robinson, C. Yannoni, and A. H. Tashjian, Jr. 1970. Metabolism of bilirubin by a clonal strain of rat hepatoma cells. *J. Cell Biol.* **47**:703.
8. Ohanian, S. H., S. B. Taubman, and G. J. Thorbecke. 1969. Rates of albumin and transferrin synthesis in vitro in rat hepatoma-derived H 411-EC cells. *J. Natl. Cancer Inst.* **43**:397.
9. Tashjian, A. H., Jr., F. C. Bancroft, U. I. Richardson, M. B. Goldlust, F. A. Rommel, and P. Ofner. 1970. Multiple differentiated

functions in an unusual clonal strain of hepatoma cells. *In Vitro.* **6**:32.

10. POTTER, V. R. 1972. Workshop on liver cell culture. *Cancer Res.* **32**:1998.
11. MALAMUD, D. 1971. Differentiation and cell cycle. *In* The Cell Cycle and Cancer. R. Baserga, editor. Marcel Dekker, Inc., New York. 137.
12. BLUME, A., F. GILBERT, S. WILSON, J. FARBER, R. ROSENBERG, and M. NIRENBERG. 1970. Regulation of acetylcholinesterase in neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **67**:786.
13. CHAPMAN, G. S., A. L. JONES, U. A. MEYER, and D. M. BISSELL. 1973. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. II. Ultrastructural Studies. *J. Cell Biol.* **59**:735.
14. BERRY, M. N., and D. S. FRIEND. 1969. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43**:506.
15. BISSELL, D. M., L. HAMMAKER, and R. SCHMID. 1972. Liver sinusoidal cells. Identification of a subpopulation for erythrocyte catabolism. *J. Cell Biol.* **54**:107.
16. BISSELL, D. M., and J. G. TILLES. 1972. Radioimmunoassay of serum albumin produced by human embryonic liver in monolayer culture. *J. Cell Biol.* **53**:819.
17. NETTER, K. J., and G. SEIDEL. 1964. An adaptively stimulated *O*-demethylating system in rat liver microsomes and its kinetic properties. *J. Pharmacol. Exp. Ther.* **146**:61.
18. LEIBOVITZ, A. 1963. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Hyg.* **78**:173.
19. EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.).* **130**:432.
20. HOLMSEN, H., I. HOLMSEN, and A. BERNHARDSEN. 1966. Microdetermination of adenosine diphosphate and adenosine triphosphate in plasma with the firefly luciferase system. *Anal. Biochem.* **17**:456.
21. HASSID, W. Z., and S. ABRAHAM. 1957. Chemical procedures for analysis of polysaccharides. *Methods Enzymol.* **3**:37.
22. FISKE, C. H., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375.
23. GILMAN, A. G. 1970. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**:305.
24. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
25. SWANSON, M. A. 1955. Glucose-6-phosphatase from liver. *Methods Enzymol.* **2**:541.
26. BEUTLER, E. 1971. Red cell metabolism. A Manual of Biochemical Methods. Grune & Stratton, Inc., New York. 58.
27. GIRARDI, A. J., H. MCMICHAEL, JR., and W. HENLE. 1956. The use of HeLa cells in suspension for the quantitative study of virus propagation. *Virology.* **2**:532.
28. SCHREIBER, G., J. URBAN, J. ZÄHRINGER, W. REUTTER, and U. FROSCH. 1971. The secretion of serum protein and the synthesis of albumin and total protein in regenerating rat liver. *J. Biol. Chem.* **246**:4531.
29. PETERS, T., JR., and J. C. PETERS. 1972. The biosynthesis of rat serum albumin. VI. Intracellular transport of albumin and rates of albumin and liver protein synthesis *in vivo* under various physiological conditions. *J. Biol. Chem.* **247**:3858.
30. INGEBRETSON, W. R., and S. R. WAGLE. 1972. A rapid method for the isolation of large quantities of rat liver parenchymal cells with high anabolic rates. *Biochem. Biophys. Res. Commun.* **47**:403.
31. HEMS, R., B. D. ROSS, M. N. BERRY, and H. A. KREBS. 1966. Gluconeogenesis in the perfused rat liver. *Biochem. J.* **101**:284.
32. EXTON, J. H., and C. R. PARK. 1968. Control of gluconeogenesis in liver. II. Effects of glucagon, catecholamines, and adenosine 3′,5′-monophosphate on gluconeogenesis in the perfused rat liver. *J. Biol. Chem.* **243**:4189.
33. JOHNSON, M. E. M., N. M. DAS, F. R. BUTCHER, and J. N. FAIN. 1972. The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate, and fatty acids. *J. Biol. Chem.* **247**:3229.
34. GEAR, A. R. L. 1970. Inner- and outer-membrane enzymes of mitochondria during liver regeneration. *Biochem. J.* **120**:577.
35. EXTON, J. H., S. B. LEWIS, R. J. HO, G. A. ROBISON, and C. R. PARK. 1971. The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. *Ann. N. Y. Acad. Sci.* **185**:85.
36. FAUPEL, R. P., H. J. SEITZ, W. TARNOWSKI, V. THIEMANN, and C. WEISS. 1972. The problem of tissue sampling from experimental animals with respect to freezing technique, anoxia, stress, and narcosis. A new method for sampling rat liver tissue and the physiological values of glycolytic intermediates and related compounds. *Arch. Biochem. Biophys.* **148**:509.
37. ORRENIUS, S., and J. L. E. ERICSSON. 1966. On the relationship of liver glucose-6-phosphatase to the proliferation of endoplasmic reticulum in phenobarbital induction. *J. Cell Biol.* **31**:243.
38. MILLER, L. L., C. G. BLY, M. L. WATSON, and W. F. BALE. 1951. The dominant role of the

liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-ε-C^{14}. *J. Exp. Med.* **94**:431.

39. LARDY, H. A., V. PAETKAU, and P. WALTER. 1965. Paths of carbon in gluconeogenesis and lipogenesis: the role of mitochondria in supplying precursors of phosphoenolpyruvate. *Proc. Natl. Acad. Sci. U. S. A.* **53**:1410.
40. RODBELL, M., H. M. J. KRANS, S. L. POHL, and L. BIRNBAUMER. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. III. Binding of glucagon: method of assay and specificity. *J. Biol. Chem.* **246**: 1861.
41. CUATRECASAS, P. 1972. Properties of the insulin receptor isolated from liver and fat cell membranes. *J. Biol. Chem.* **247**:1980.
42. MARVER, H. S., and R. SCHMID. 1972. The porphyrias. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 1087.
43. GRANICK, S. 1966. The induction *in vitro* of the synthesis of δ-aminolevulinic acid synthetase in chemical porphyria: A response to certain drugs, sex hormones, and foreign chemicals. *J. Biol. Chem.* **241**:1359.
44. SASSA, S., and S. GRANICK. 1970. Induction of δ-aminolevulinic acid synthetase in chick embryo liver cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **67**:517.
45. STRAND, L. J., J. MANNING, and H. S. MARVER. 1972. The induction of δ-aminolevulinic acid synthetase in cultured liver cells. The effects of end product and inhibitors of heme synthesis. *J. Biol. Chem.* **247**:2820.
46. KAPPAS, A., C. S. SONG, R. D. LEVERE, R. A. SACHSON, and S. GRANICK. 1968. The induction of δ-aminolevulinic acid synthetase in vivo in chick embryo liver by natural steroids. *Proc. Natl. Acad. Sci. U. S. A.* **61**:509.
47. NICHOL, A. W. 1970. The formation of biliverdin by chick macrophages in tissue culture. Observations on the effect of inhibitors. *Biochim. Biophys. Acta.* **222**:28.
48. GIELEN, J. E., and D. W. NEBERT. 1971. Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. I. Stimulation of enzyme activity in nonhepatic cells and in hepatic cells by phenobarbital, polycyclic hydrocarbons, and 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane. *J. Biol. Chem.* **246**: 5189.
49. GREENGARD, O., M. FEDERMAN, and W. E. KNOX. 1972. Cytomorphometry of developing rat liver and its application to enzymic differentiation. *J. Cell Biol.* **52**:261.
50. ALWEN, J., and J. J. GALLHAI-ATCHARD. A method of maintaining parenchymal cells from adult rat liver *in vitro*. *J. Cell Sci.* **11**:249.
51. DICKSON, J. A. 1971. Metabolism of adult rat liver cells in filter-well culture. *Exp. Cell Res.* **64**:17.
52. HILLIS, W. D., and F. B. BANG. 1962. The cultivation of human embryonic liver cells. *Exp. Cell Res.* **26**:9.
53. WATANABE, H. 1966. A fine structural study of liver culture. *Exp. Cell Res.* **42**:685.
54. DEMOISE, C. F., J. T. GALAMBOS, and A. FALEK. 1971. Tissue culture of adult human liver. *Gastroenterology.* **60**:390.
55. AVIV, D., and E. B. THOMPSON. 1972. Variation in tyrosine amino-transferase induction in HTC cell clones. *Science (Wash. D. C.).* **177**: 1201.
56. MOORE, A. E., C. M. SOUTHAM, and S. S. STERNBERG. 1956. Neoplastic changes developing in epithelial cell lines derived from normal persons. *Science (Wash. D. C.).* **124**:127.
57. DAVIDSON, E. H. 1964. Differentiation in monolayer tissue culture cells. *Adv. Genet.* **12**:143.
58. THOMPSON, E. B., G. M. TOMKINS, and J. F. CURRAN. 1966. Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proc. Natl. Acad. Sci. U. S. A.* **56**:296.
59. KAIGHN, M. E., and A. M. PRINCE. 1971. Production of albumin and other serum proteins by clonal cultures of normal human liver. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2396.
60. GERSCHENSON, L. E., M. ANDERSSON, J. MOLSON, and T. OKIGAKI. 1970. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. *Science (Wash. D. C.).* **170**:859.
61. COON, H. G. 1968. Clonal culture of differentiated rat liver cells. *J. Cell Biol.* **39**(2, Pt. 2): 292. (Abstr.).
62. BOREK, C. 1972. Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. *Proc. Natl. Acad. Sci. U. S. A.* **69**:956.
63. MACDONALD, R. A. 1961. "Lifespan" of liver cells. Autoradiographic study using tritiated thymidine in normal, cirrhotic, and partially hepactectomized rats. *Arch. Intern. Med.* **107**: 335.
64. POST, J., and J. HOFFMAN. 1964. Changes in the replication times and patterns of the liver cell during the life of the rat. *Exp. Cell Res.* **36**:111.
65. TODARO, G., Y. MATSUYA, S. BLOOM, A. ROBBINS, and H. GREEN. 1967. Stimulation of RNA synthesis and cell division in resting cells by a factor present in serum. Growth Regulating Substances for Animal Cells in Culture. *Wistar Inst. Symp. Monogr. No.* 7. 87.

66. EARLE, W. R., K. K. SANFORD, V. J. EVANS, H. K. WALTZ, and J. E. SHANNON, JR. 1951. The influence of inoculum size on proliferation in tissue cultures. *J. Natl. Cancer Inst.* **12:** 133.

67. GURNEY, T. 1969. Local stimulation of growth in primary cultures of chick embryo fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **62:**906.

68. VASILIEV, J. M., I. M. GELFAND, L. V. DOMNINA, and R. I. RAPPOPORT. 1969. Wound healing processes in cell culture. *Exp. Cell Res.* **54:**83.

69. BISSELL, D. M., and J. G. TILLES. 1971. Morphology and function of cells of human embryonic liver in monolayer culture. *J. Cell Biol.* **50:**222.

70. CHURCH, R. B., and B. J. MCCARTHY. 1967. Ribonucleic acid synthesis in regenerating and embryonic liver. II. The synthesis of RNA during embryonic liver development and its relationship to regenerating liver. *J. Mol. Biol.* **23:**477.

71. STANISKLAWSKI-BIRENCWAJG, M., J. URIEL, and P. GRABAR. 1967. Association of embryonic antigens with experimentally induced hepatic lesions in the rat. *Cancer Res.* **27:**1990.

72. HILTON, J., and A. C. SORTORELLI. 1970. Induction by phenobarbital of microsomal mixed oxidase enzymes in regenerating rat liver. *J. Biol. Chem.* **245:**4187.

73. GRAM, T. E., A. M. GUARINO, F. E. GREENE, P. L. GIGON, and J. R. GILLETTE. 1968. Effect of partial hepatectomy on the responsiveness of microsomal enzymes and cytochrome P-450 to phenobarbital or 3-methylcholanthrene. *Biochem. Pharmacol.* **17:**1769.

74. HENDERSON, P. T., and K. J. KERSTEN. 1970. Metabolism of drugs during rat liver regeneration. *Biochem. Pharmacol.* **19:**2343.

75. VON DER DECKEN, A., and T. HULTIN. 1960. The enzymatic composition of rat liver microsomes during liver regeneration. *Exp. Cell Res.* **19:**591.

76. BECKER, F. F., and M. W. BITENSKY. 1969. Glucagon and epinephrine responsive adenyl cyclase activity of regenerating rat liver. *Proc. Soc. Exp. Biol. Med.* **130:**983.

77. EXTON, J. H., and C. R. PARK. 1965. Control of gluconeogenesis in the perfused liver of normal and adrenalectomized rats. *J. Biol. Chem.* **240:** 955.

78. MCLIMANS, W. F. 1972. The gaseous environment of the mammalian cell in culture. *In* Growth, Nutrition and Metabolism of Cells in Culture. G. H. Rothblat and V. J. Cristofalo, editors. Academic Press, Inc., New York. **I:**137.