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Drug Metabolism and Effects of Carcinogens in Cultured Hepatic Cells

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I. Introduction

A CHARACTERISTIC of all living organisms is the ability to metabolize molecules that are entirely foreign to their internal metabolic machinery; there are relatively few carbon-based molecules that cannot be modified by some living organism. Not only are cells capable of such metabolic transformations, but many cells can also alter their capacity for the metabolic transformation of xenobiotic compounds in response to the presence of the compounds.

The metabolism of drugs and other foreign compounds in the multicellular organism has been monitored by a variety of methods. The entire branch of pharmacology known as pharmacokinetics endeavors to quantify changes in one or more species of xenobiotic molecules as a function of time, dose, route of administration, etc. Such studies, while extremely useful in determining the overall fate of a drug in an organism, do not, under most circumstances, give information concerning the actual mechanisms of the cellular metabolism of the material under study. To determine such mechanisms, biochemical studies on the isolated cellular components involved in such transformations are necessary. In addition, the effects of the extracellular environment on the quantitative capacity of the cell to metabolize xenobiotics are critical in such investigations.

During the last two decades, with the advent of markedly improved techniques for the culture of mammalian cells, many studies have been directed towards the "phar-

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macokinetics" of xenobiotics in cell culture. However, most cells in culture exhibit relatively limited capacities to metabolize foreign compounds. Within the last decade, techniques for the culture of highly differentiated cells possessing a variety of mechanisms for xenobiotic metabolism have been described. Foremost among such tissue culture systems are those that involve the mammalian hepatocyte, a cell type that possesses one of the most extensive repertoires of these mechanisms. This review is designed to acquaint the reader with the techniques for the isolation, characterization, and use of hepatic cells in culture for the study of the metabolism of drugs and related foreign compounds.

II. Initial Studies on the Use of Cultured Cells for the Investigation of Xenobiotic Metabolism

A. *Extrahepatic Tissues*

Although the principal investigations of drug metabolism in cell culture have now centered around the use of hepatic tissues, early studies of drug metabolism and especially the regulation of related enzymes were carried out in both hepatic and nonhepatic tissues in culture. Since nonhepatic, mesenchymal tissues could be cultured with relative ease, these were investigated by several laboratories, most notably those of Nebert and Gelboin (110–112). Alfred and Gelboin (3) demonstrated that the treatment of hamster embryonic cells in culture with 1,2-benzanthracene for periods of 4 to 48 hours induced a 3- to 10-fold increase in the activity of benzpyrene hydroxylase (aryl hydrocarbon hydroxylase). By the use of suitable inhibitors, this increase was shown to reflect an induction of enzyme synthesis. In a more extensive study of this same system, Nebert and Gelboin (111, 112) showed that a number of polycyclic hydrocarbons could also induce aryl hydrocarbon hydroxylase, but that corticosteroids and phenobarbital could not induce the enzyme. With metabolic inhibitors, they also demonstrated the dissociation of translation from transcription beginning several hours after addition of the hydrocarbon.

Later, Ruddon et al. (140) showed a similar effect in mouse 3T3 fibroblasts and made the additional observation that cyclic guanosine 5′-phosphoric acid (GMP) added to the culture in the absence of serum markedly potentiated the induction of aryl hydrocarbon hydroxylase by benzanthracene. At about the same time, Kellermann et al. (74) reported the induction of aryl hydrocarbon hydroxylase in cultured human leukocytes. Their investigations indicated a genetic control of the regulation of aryl hydrocarbon hydroxylase in these blood cells. The more extensive studies by Nebert and his associates, both in cells in culture and in vivo in the mouse, clearly demonstrated a specific genetic locus, the Ah locus, involved in the regulation of aryl hydrocarbon hydroxylase synthesis (167).

Because of the relatively limited drug-metabolizing capacity of these easily cultured mesenchymal tissues, such studies involved only a few types of metabolic reactions, of which aryl hydrocarbon hydroxylation was the most extensively studied. Thus the pharmacologist has continued to seek a cell culture system that exhibits a variety of metabolic reactions of xenobiotics. At present the most promising of these systems in the mammal is the hepatocyte in culture.

B. *Cells Derived from Mammalian Liver*

Since differentiated tissues of the adult are extremely hard to culture and at the same time retain their differentiated characteristics, early investigations of drug metabolism in liver-derived cells employed fetal tissues. Granick (45) demonstrated the induction of the synthesis of δ-aminolevulinic acid synthetase in chick embryo liver cells by a number of chemicals known to induce acute porphyria in vivo. Later studies by Granick and Kappas (47) demonstrated that steroids and their metabolites are also capable of inducing this enzyme in chick embryo hepatocytes in culture. In the same biological system, Ko et al. (77) demonstrated that certain serum factors are required for the development of uridine diphosphate (UDP)-glucuronyltransferase activity. Interestingly, the serum requirements changed with the initial age of the embryo when explantation of the hepatic cells occurred. Somewhat later, Nebert and Gielen (43, 44, 113) reported on the requirements for the induction of aryl hydrocarbon hydroxylase in fetal rat hepatocytes by polycyclic hydrocarbons, phenobarbital, a variety of sera, hormones, and other compounds. They also demonstrated that the requirement for transcription and translation in the induction of this enzyme by either phenobarbital or polycyclic hydrocarbons was essentially identical with that reported earlier in the hamster embryo fibroblast cultures.

Thus, by the early part of the last decade, initial studies on the regulation of several enzymes of drug metabolism and the initial enzyme involved in heme biosynthesis had been reported. However, the most extensive investigations of the regulation of critical components of the drug-metabolizing system, especially those of the cytochrome P-450 system, were in a large measure to await the advances in the technology for the culture of hepatic cells. It is this subject that will be covered more extensively in the remainder of this review.

III. Experimental Systems Employed for the Culture of Cells Derived from Vertebrate Liver

Whereas the culture of nonepithelial cells such as fibroblasts, as well as of primitive epithelium, has been developed to a significant degree, the sustained culture of highly differentiated glandular epithelium from the mammal has not yet achieved a similar degree of refinement. As with most techniques in cell culture, early attempts to culture highly differentiated organs by explantation of fragments of tissue led to a rapid overgrowth by a minority of primitive cells present in all

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tissues. Usually such minority cell types grow out as fibroblasts or as simple epithelium (2). Although some differentiated characteristics of the major epithelial components in vivo may be demonstrated for short periods in culture, little or no replication of such tissues could be shown such as that which can be produced in vivo with appropriate stimuli, e.g., subtotal hepatic resection. Furthermore, such explants are useful for little more than morphologic investigations because the amount of tissue in vitro available for biochemical studies is very restricted.

The culture of adult hepatocytes followed the pattern described above until the development of a reproducible, relatively nondestructive technique for the separation of large numbers of hepatocytes (10^8) into individual cells or small clusters of less than 10 cells. This critical point was established initially by the investigations of Howard et al. (59) and was further refined by Berry and Friend (11) and by Howard and his associates (60). The development of such a technique, which allowed the preparation of isolated hepatocytes in a greater than 75% yield, opened the door for the establishment of primary cultures of adult hepatocytes from several different species. Many of the methods employed for the culture of fetal, neonatal, and adult hepatocytes, as well as cell lines derived from liver, are described below.

A. Culture of Fetal and Neonatal Hepatocytes

Methods for the culture of fetal and neonatal hepatocytes have involved the mincing of the freshly removed tissue and/or enzymatic dissociation of the tissue. In the former case, one of the earliest techniques used was that of Alexander and Grisham (2), who minced livers of 1- to 5-day-old rats and then explanted several of the fragments onto collagenized coverslips maintained in a gas-tight chamber. Morphological, histochemical, and autoradiographic studies could be carried out in this system. Rose et al. (139) had also described an elegant circumfusion system with multiple chambers useful for culturing minced fragments of embryonic mice. In addition, MacDonnell et al. (89) used cut fragments of fetal liver for the study of enzyme release from the explants and of the hormonal regulation of tyrosine aminotransferase. This latter enzyme was also studied in similar cultures by Wicks (179).

The use of enzymatic digestion in the isolation of fetal hepatocytes was employed extensively by Leffert and his associates (e.g., 84, 85). These authors investigated a number of characteristics of short-term cultures of fetal rat hepatocytes that had been isolated by the use of collagenase and then maintained in a medium deficient in arginine. In addition, Buckley and Walton (21) have employed trypsin to isolate hepatocytes from chick embryo liver in order to study the morphology of such cells in culture. More recently, Acosta et al. (1) have used collagenase and hyaluronidase in solution to dissociate cells from the livers of 7- to 10-day-old rats. When these liver cells were maintained in an arginine-deficient medium, they were found to express the L-isozyme of pyruvate kinase, which is characteristic of the adult hepatocyte. Hepatic cell cultures from 5-day-old albino rats were established by Armato et al. (6), by using a "dissociating solution" of trypsin, collagenase, and hyaluronidase buffered with $NaHCO_3$.

Human fetal liver has also been cultured by a number of investigators. Noyes (115) utilized fragments of human fetal liver that had been pressed through a No. 40 mesh stainless steel wire screen. The fragments were cultured on coverslips coated with collagen and also on pieces of Gelfoam sponge. Nau et al. (109) isolated hepatocytes from human fetuses of 9 to 20 weeks gestation by the digestion of slices of the liver with a neutral bacterial protease. These investigators studied the metabolism of several drugs and the effects of phenobarbital and SKF 525-A on the rates of metabolism in slices of human liver cultured for periods up to 100 hours.

The use of fetal cultures has the advantages that one may deal with replicating cells, if this is desirable, and more easily establish short-term primary cultures of the cells. However, in general, the yield of cells of this type is relatively small (10^6 to 10^7), and therefore the amount of material available is limited. The use of adult hepatocytes, as outlined below, affords a far greater yield of nonreplicating cells (10^8 to 10^9), but such cultures are difficult to maintain for extended periods.

B. Culture of Hepatocytes from Adult Animals

Methods similar to those described above for fetal and neonatal tissue have also been used in the preparation of hepatic cells from the adult rodent. In all these cases, enzymatic dissociation with either trypsin or collagenase ± hyaluronidase was the principal technique employed to isolate liver cells. Both normal liver (38, 163) and regenerating liver (57) have been used as the starting tissues, and yields up to 30 million cells from adult rat liver have been reported with this technique (38). Other methods used to separate the cells from liver have included chelating agents (65) and the culture of hepatic fragments, the latter technique being employed with adult human liver (27). In addition, a combination of chelating agents such as tetraphenylborate and ethylenediaminetetraacetic acid (EDTA) together with collagenase and hyaluronidase has been employed to prepare single-cell suspensions from liver as well as from hepatomas (107). Bellemann et al. (10) used this enzymatic combination to digest hepatic slices. Single cells were then obtained by a brief trypsinization of the collagenase-digested slices.

A brief perfusion of the liver followed by enzymatic digestion with mincing, dissection, and/or agitation has also been employed to isolate liver cells of adult animals. In an early investigation by Dickson (28), a special "filter-well" culture was used in a study of the labeling of intermediates with radioactive substrates. Recently, Lef-

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fert et al. (86) used a short (2 to 3 min) perfusion of the liver in situ followed by dissection and agitation in the presence of collagenase. This method was similar to that described for fetal tissue by Leffert and Paul (84), although the use of short-term perfusion was omitted in the latter case.

The principal difficulties with all the methods described above are the very low yield of cells obtained (less than 5% of the total liver cell number) and the question of selection of those cells most able to withstand the rigors of isolation. It was not until hepatic perfusion was established as a reproducible method for obtaining high cell yields that primary liver cell cultures became practical on a large scale.

C. Methods of Primary Culture of Adult Hepatocytes Obtained by the Use of Hepatic Perfusion

1. Liver perfusion methods. As indicated earlier, the basis for the preparation of viable hepatocytes satisfactory for cell culture was the report by Howard et al. (59) that superseded earlier studies in which chelating agents had been used in the perfusion medium in the absence of enzymes (65). The refinements of this new technique by Berry and Friend (11) and again by Howard et al. (60) have been adopted by many laboratories with some variations for the preparation of suspensions of hepatocytes to be explanted directly to cell culture. Variations in this procedure, including a comparison of chelating agents and enzymes used for digestion, have been reported by Fry et al. (35), and the methods have been reviewed by Wagle (174). Such cell preparations have been separated into size classes by using ficoll gradients (29), and the separation of centrolobular and perilobular hepatocytes has also been accomplished after suitable preparation of the animals (175). In addition, Seglen (152, 153) has undertaken studies to determine the optimal concentrations of ions and chelators, as well as optimal characteristics of the enzymatic requirements for obtaining hepatocyte suspensions. Many of the refinements described by Seglen (152, 153) have now been adopted by most workers. Wittman and Jacobus (189) have employed a hydrostatic pressure gauge and a return-flow meter during the perfusion procedure. This modification assures that the cannulae are properly placed in the vessels and that the perfusate is flowing. Although the collagenase perfusion techniques have been applied mainly to the rat, they have also been used to isolate hepatic parenchymal cells from guinea-pig liver (31), mouse liver (133), frog liver (161), and eel liver (55).

Just as brief perfusions were used in conjunction with mincing and enzymatic digestion, prolonged hepatic perfusion has been followed by mincing of the perfused liver in the solution of collagenase and hyaluronidase for both rodent and avian liver (23, 58). Studies have also demonstrated (78) that hepatocytes can be selectively destroyed by treatment of cell suspensions with proteolytic enzymes such as pronase. This treatment results in a cell suspension that contains a significant number of intact sinusoidal cells, but no hepatocytes. This finding may explain the extremely low hepatocyte yields obtained by investigators who employed comparable proteolytic enzymes such as trypsin to digest fragments of liver.

2. Short-term suspension cultures of hepatocytes. The preparation of metabolically active hepatocyte suspensions in good yield has led to the use of such preparations for short-term biochemical investigations. Many of the characteristics of short-term cultures have been reviewed by Jeejeebhoy and Phillips (68). As they pointed out, a variety of different media have been used, varying from a simple salt solution such as Hanks' to the use of tissue culture medium such as Eagle's, as well as media containing a variety of macromolecules such as gelatin or albumin. Unlike the prolonged cell culture systems described below, incubation was carried out in various containers usually immersed in a shaking water bath. Gassing with 95% O_2/5% CO_2 has been used by many investigators who employed the short-term culture systems. In addition, incubation in medium supplemented with rat serum has been shown to improve the response of isolated liver cells to hormones, especially glucagon (156).

One difficulty in using hepatocyte suspensions is the leakage of enzymes from the dispersed liver cells (165). Although Takeda et al. (165) demonstrated that corticosteroids had a preventive effect on the leakage of certain enzymes, this problem remains as a significant one and drastically limits the use of suspensions for any studies other than extremely short-term ones (probably less than 4 hours).

3. Prolonged maintenance of hepatocytes in primary culture. One of the first techniques in which hepatic perfusion was employed for the preparation of rat hepatocytes explanted to a primary culture was that of Bissell et al. (13). These investigators plated cells directly onto plastic Petri dishes and found that albumin synthesis continued for at least 6 days. A similar system in which the induction of tyrosine aminotransferase activity by corticosteroids could be demonstrated was reported by Bonney et al. (16). Comparable systems were then reported by Laishes and Williams (79) and, more recently in the case of mouse liver, by Renton et al. (133).

In most of the studies mentioned above, the hepatocytes survived for approximately one week, and during this period an extensive and continuous loss of hepatocytes from the cultured plate surface was noted. In 1975, Michalopoulos and Pitot (92) used the collagenase perfusion technique described by Bonney et al. (16) to plate isolated adult rat hepatocytes onto collagen gel membranes prepared from rat tail collagen. The advantage of this system was that the viability and function of the cells could be maintained for at least three weeks as evidenced by the corticosteroid induction of tyrosine aminotransferase. Michalopoulos and Pitot contrasted their findings with the plating of hepatic cells on plastic

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dishes or on collagen-coated plates. Other supports for hepatocyte cultures have also been described (146, 154), although without further extension of viability. In comparison, Sirica et al. (158) modified the technique of Michalopoulos and Pitot (92) by plating the hepatocytes on a substratum of nylon mesh coated with a thin collagen gel. In addition to the extended cell longevity, this technique allowed for the removal of the cells from the substratum by gentle treatment with a dilute collagenase solution and provided for their replating. Unfortunately, none of these techniques for culturing adult rat liver cells obtained by perfusion results in any significant degree of hepatocyte replication. The study of Sirica et al. (158) did demonstrate that up to 9% of the cells were in DNA synthesis after one week in culture, but extensive examination did not reveal any significant degree of mitotic activity in such cultures, nor was there any sustained increase in DNA synthesis. Although there had been previous reports of cell replicative activity in adult hepatocyte cultures (86), such studies have not been carefully controlled to determine exactly which cells are replicating, hepatocytes or littoral cells. Thus the mass culture of replicating hepatocytes remains as a goal for future investigations, although the importance of such functions in relation to xenobiotic metabolism is not so clear (see CONCLUSIONS).

D. Culture of Hepatic Cell Strains and Lines

It has not yet been possible to obtain reproducible and substantial levels of mitotic activity of adult hepatocytes in culture, but a number of reports have demonstrated the production of continuous lines or strains of cells from adult liver as well as from hepatomas. Perhaps one of the most noteworthy hepatoma cell lines is the H-4-II-E, first established at the McArdle Laboratory from the Reuber hepatoma (124). Since then, other strains of hepatoma cells have been derived from several transplanted Morris hepatomas (75, 135).

The establishment of cell lines from adult rat liver cells has been reported by several authors (25, 39, 40, 184). In at least one of these cell lines (39), the induction of tyrosine aminotransferase by insulin and corticosteroids was retained, although the karyotype of the cell line was distinctly abnormal. In another study, Bausher and Schaeffer (7) described a diploid rat liver cell line that was relatively resistant to the toxic action of the hepatocarcinogen, aflatoxin B_1, and that exhibited tyrosine aminotransferase activity by a histochemical method, although no evidence for its hormonal regulation was presented. Recently Tokiwa et al. (164) have demonstrated the development of a number of diploid and near-diploid clones from cell lines derived from both adult and newborn rat liver. Such clones exhibited some biochemical characteristics of hepatocytes including the production of albumin and α-fetoprotein. Epithelial cell cultures established from normal rat liver have also been "transformed" in cell culture (18; section VI).

Virtually without exception, the light microscopic and ultrastructural morphology of cell strains derived from liver is significantly different from that of adult liver in vivo or of that reported for primary hepatocytes cultured en masse. Many of the cell lines obtained from normal adult liver resemble endothelium in culture, especially those cell lines that exhibit virtually no hepatic functions. Therefore, with the exception of the hepatoma cell lines, many of which can produce neoplasms when reinoculated in vivo, cell lines derived from normal adult liver may not be parenchymal cells at all. On the other hand, Grisham (50) has recently reviewed the evidence for hepatocyte origin of a number of cell lines and strains derived from rodent liver. He concluded that those cell lines that exhibit some, but not all of the biochemical and morphological characteristics of normal liver may be derived from an intermediate or stem cell type, possibly the cells of the canal of Hering. Such a proposal, if it can be proved, may serve to clarify many of the claims and discrepancies in the reports of differentiated functions of liver cell lines in culture.

IV. Enzymology of Xenobiotic Metabolism in Cell Cultures Derived from Liver

A. Xenobiotic Metabolism in Isolated Hepatocytes from Rats

The advantages of freshly isolated hepatocytes for use in studies of hepatic xenobiotic metabolism have been reviewed previously (12, 19, 56). Several aspects of xenobiotic metabolism have been investigated by using freshly isolated hepatocytes from untreated and adult rats pretreated with various xenobiotics. Freshly isolated hepatocytes from normal rat liver (33, 121) and from rat liver four to six days after partial hepatectomy (52) were shown spectrophotometrically to possess a cytochrome P-450 content that closely resembled that of the parent livers. Spectral studies by Moldéus, Orrenius, and their coworkers (100, 101, 173) have further demonstrated that the major part of cytochrome P-450 in freshly isolated hepatocytes from untreated and phenobarbital-pretreated rats was present in an oxidized, nonsubstrate-bound state. Furthermore, the addition of drugs such as hexobarbital to the medium of these cells resulted in the rapid production of a type 1 spectral change indicative of the formation of a cytochrome P-450/drug substrate complex. As expected, the cellular level of cytochrome P-450, as well as the magnitude of the spectral changes associated with the drug binding and monooxygenation were markedly enhanced in the hepatocytes isolated from the phenobarbital-pretreated animals (100).

In addition to determining cytochrome P-450 content, Guzelian et al. (52) have compared several microsomal monooxygenase activities of freshly isolated hepatocytes from regenerated adult rat liver with those of the caudate lobe of the parent liver. In the freshly isolated hepatocytes, the activities of reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome *c* reduc-

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tase, aminopyrine-N-demethylase, aniline hydroxylase, aryl hydrocarbon hydroxylase, and *p*-nitroanisole O-demethylase were found not to differ significantly from those of the parent liver. Microsomes prepared from freshly isolated hepatocytes from normal adult rat liver were further shown by Fahl et al. (33) to exhibit NADPH cytochrome *c* reductase activity that was not altered from that of microsomes prepared from the intact caudate lobe. Paine et al. (122) also observed no significant difference between the aminopyrine-N-demethylase activity of hepatocytes isolated in the presence of 1.0 mM nicotinamide from normal adult rat liver and that of the intact liver.

Short-term suspensions of rat hepatocytes, generally less than 4 hours, were shown to be active in performing a variety of monooxygenase-mediated metabolisms of xenobiotic substrates. Table 1 is a representative list of xenobiotic substrates found by several laboratories to be metabolized in isolated hepatocyte suspensions prepared from untreated and/or inducer-pretreated adult rats. The metabolic rates of some of these substrates, including α-1-acetylmethadol, *d*-propoxyphene, and N,N-dimethylphenoxyethylamine (12); alprenolol (101); and of the primary oxidative step in benzo(a)pyrene metabolism (171) were shown to be similar in hepatocytes from untreated rats to those observed with liver 9000 × *g* supernatant or microsomes maintained in the presence of NADPH-generating systems. Hepatocytes from the inducer-pretreated animals also exhibited a profile of stimulated xenobiotic-metabolizing activities that was, in general, closely related to that shown by subcellular liver fractions (12, 101, 168, 171). In addition, inhibitors of drug metabolism, such as SKF 525-A or metyrapone, provoked similar inhibitory patterns in both isolated hepatocytes and subcellular liver fractions (12, 19, 101, 168, 171). Despite these similarities, the rates of metabolism of *d*-propoxyphene and α-1-acetylmethadol were not stimulated in the hepatocytes isolated from phenobarbital-pretreated rats, but those activities were increased in the 9000 × *g* supernatant from whole liver by phenobarbital pretreatment (12). However, the results reported for *d*-propoxyphene, as well as for a number of other substrates studied, appeared to resemble more closely the in vivo condition than those obtained with 9000 × *g* supernatant or microsomal fractions from whole liver (12). The rates of metabolism of a number of xenobiotics were also found to be slower in hepatocytes isolated from untreated and/or inducer-pretreated rats than those measured in corresponding subcellular liver fractions (12, 176). It is unlikely that the slower metabolic

TABLE 1

Metabolism of representative xenobiotic substrates in freshly isolated hepatocyte suspensions from untreated and/or inducer-pretreated adult male rats

Substrate	Reaction	Inducer	Effect of Inducer Pretreatment on Reaction Rate	Reference
α-1-Acetylmethadol	N-demethylation	Phenobarbital	No effect	(12)
Aminopyrine	N-demethylation	Phenobarbital	N.D.*	(155, 176)
Antipyrine	N-demethylation	None		(56)
Dansylamide	N-demethylation	None		(56)
Ethylmorphine	N-demethylation	None		(32)
N,N-dimethyl-*p*-chlorophenoxyethylamine	N-demethylation	Phenobarbital	Stimulated	(12)
N,N-dimethylphenoxyethylamide	N-demethylation	Phenobarbital	Stimulated	(12)
d-Propoxyphene	N-demethylation	Phenobarbital	No effect	(12)
Biphenyl	Aromatic hydroxylation	Phenobarbital; 3-methylcholanthrene	Stimulated	(19, 182)
Butamoxane	Aromatic hydroxylation	Phenobarbital	Stimulated	(12)
Quinine sulfate	Aromatic hydroxylation	None		(56)
2-Acetylaminofluorene	Aromatic hydroxylation, N-hydroxylation, deacylation	β-Naphthoflavone	Stimulated aromatic ring hydroxylation	(30)
Benzo(a)pyrene	Aromatic hydroxylation, epoxidation	3-Methylcholanthrene	Stimulated	(22, 171)
8-Methoxybutamoxane	O-demethylation, aromatic hydroxylation	Phenobarbital	Stimulated	(12)
Alprenolol [1-(2-allylphenoxy)-3-isopropylaminopropanol]	Monooxygenation	Phenobarbital	Stimulated	(101)
Hexobarbital	Monooxygenation	Phenobarbital	Stimulated	(72, 100)
Bromobenzene	Epoxidation	Phenobarbital	Stimulated	(168)
Ethinimate	Aliphatic hydroxylation, alcohol oxidation	Phenobarbital	Slightly stimulated	(12)
7-Ethoxycoumarin	O-deethylation	None		(182)
p-Nitroanisole	O-dealkylation	Phenobarbital	Stimulated	(102)

* N.D., not determined.

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rates obtained with the isolated hepatocytes were due to an insufficient cellular concentration of NADPH, since hepatocytes isolated from fed, starved, and/or inducer-pretreated rats were shown to generate more than enough NADPH-reducing equivalents from endogenous hexose monophosphate or tricarboxylic acid substrates to support optimal rates of xenobiotic metabolism (12, 72, 101, 155, 171, 176). In this respect, the cellular NADPH concentration was not rate-limiting unless the hepatocytes were prepared from the livers of fasted untreated or inducer-pretreated rats and incubated in the presence of inhibitors of mitochondrial respiration (101, 171) or of aminotransferases (155, 176). The inclusion of glucose (10 to 25 mM) in the cell suspension medium did not affect the rates of xenobiotic metabolism in hepatocytes isolated from fed or starved rats (12, 101, 171), but stimulated the metabolic activity in hepatocytes prepared from starved, phenobarbital-pretreated rats (101). Although the mechanism of the slower rates of xenobiotic metabolism measured in the isolated hepatocytes is not completely understood, the slower rate obtained with ethinimate was due to a lower V_{max} in the isolated hepatocytes than in liver 9000 × g supernatant (12). On the other hand, whereas the apparent K_m for ethinimate was similar in both preparations, apparent K_m values determined for the N-demethylation of α-1-acetylmethadol (12) and of ethylmorphine (32) were significantly lower in the isolated hepatocytes than in 9000 × g supernatant or microsomes, respectively. In comparison, the apparent K_m for alprenolol metabolism was found to be similar in both isolated hepatocytes and liver microsomes (101). Thus, it is evident that further studies are needed to define more clearly what the rate-limiting effects of membrane transport, monooxygenase levels and their conformation and substrate affinities in intact endoplasmic reticulum, and the possible presence of endogenous activators or inhibitors of drug-metabolizing enyzmes might be on the metabolism of individual xenobiotics in isolated hepatocytes as compared with subcellular liver fractions.

Another important factor governing the overall metabolism of xenobiotics involves the subsequent conjugation of primary metabolites generated by the hepatic mixed-function monooxygenase system with glucuronic acid, sulfate, or glutathione. Several reports have demonstrated an efficient conjugative metabolism in freshly isolated rat hepatocytes in short-term cell suspension. For example, isolated hepatocytes performed conjugations of acetylaminofluorene metabolites with glucuronic acid (30); of p-nitrophenol (12, 102), 7-hydroxycoumarin (182), hydroxylated biphenyl metabolites (19, 182), and phenolic metabolites of p-nitroanisole (102) with glucuronic acid or sulfate; and of the primary metabolites of benzo(a)pyrene with glucuronic acid, sulfate, or glutathione (22, 70). In general, these reactions could be demonstrated in hepatocytes isolated from fed rats in the absence of exogenous cofactors and without the addition of glucose to the medium. However, compounds that lower cellular adenosine triphosphate (ATP) production (menadione, rotenone, 2,4-dinitrophenol) or increase the cytoplasmic reduced nicotinamide adenine dinucleotide (NADH) concentration (ethanol) have been shown to be very effective inhibitors of glucuronidation and/or sulfation in isolated hepatocytes from fed rats, probably as a result of their action in depleting cellular cofactor levels (102, 182). In the case of menadione, rotenone, and 2,4-dinitrophenol, the inhibition of glucuronidation and sulfation appears to be due to a depletion of cellular ATP, which, in turn, is likely to effect a reduction in the cellular levels of uridine diphosphate glucuronic acid (UDPGA) and 3′-phosphoadenosine 5′-phosphosulfate (182). On the other hand, an increased cellular NADH concentration may inhibit the oxidized nicotinamide adenine dinucleotide (NAD^+)-requiring enzyme UDP-glucose dehydrogenase, thereby preventing the synthesis of UDPGA (102). In contrast, incubation of freshly isolated hepatocytes from normal rats with up to 1.0 mM phenobarbital has been found to enhance the synthesis of UDPGA and to increase the cellular UDP-glucose concentration, probably as a result of the inhibition of glycogen synthesis by phenobarbital (114). Not surprisingly, this short-term treatment with phenobarbital did not alter the microsomal protein content nor the UDP-glucuronyltransferase and UDP-glucose dehydrogenase activities of the isolated hepatocytes. Pretreatment of rats with phenobarbital or with 3-methylcholanthrene did not have a major effect on the conjugative activities of isolated rat hepatocytes (19, 171). However, in suspensions of hepatocytes prepared from untreated rats, the glucuronidation of morphine declined at a more rapid rate over a 10-hour period than that of naphthol, which suggests that there is a differential lability of the isozymic forms of glucuronyl transferase in these cells (151).

Viña et al. (172) have shown that the reduced glutathione (GSH) content of freshly isolated rat hepatocytes is, on a micromole/gram of wet weight basis, only about one-half that of the intact liver. This loss could be prevented in the freshly isolated cells by the addition of 0.1 mM ethylene guanosine tetraacetic acid (EGTA) to the perfusion medium. Furthermore, the inclusion of near-physiological concentrations of methionine or homocysteine, but not of cysteine, was found to be necessary to prevent the loss of GSH from hepatocytes isolated in the presence or absence of EGTA and then maintained for 60 min in suspension culture. In contrast, Thor et al. (168) have reported that cysteine was more effective than methionine in increasing the cellular GSH content of hepatocytes isolated from phenobarbital or diethylmaleate-pretreated rats. This discrepancy in the results obtained with cysteine remains unresolved at present.

Other aspects of drug metabolism in short-term cell suspensions of freshly isolated hepatocytes include their ability to form polyglutamate derivatives of methotrexate (41) and to metabolize ethanol and acetaldehyde (160). In the latter case, the alcohol and aldehyde dehydrogen-

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ase activities of the isolated hepatocytes were found to be comparable to those of intact and perfused livers (160).

Although the above studies serve to emphasize the potential value of freshly isolated hepatocytes as a system for assessing hepatic xenobiotic metabolism and its modification by in vivo pretreatments, their use in longer-term studies is obviated by the fact that the hepatocytes tend to degenerate rapidly in cell suspension within a few hours after their isolation. The remainder of this review will be concerned with studies of xenobiotic metabolism in longer-term liver cell culture systems.

B. Monooxygenases of Cultured Liver Cells

Replicating liver cell lines derived from explants of mammalian liver or hepatomas have been uniformly disappointing in terms of their maintenance of measurable levels of cytochrome-P-450-dependent monooxygenase activities comparable to those of intact normal adult liver (13, 19, 95). However, a majority of these cell lines have been shown to exhibit relatively high basal, as well as inducible, levels of the cytochrome P-448-dependent monooxygenase, aryl hydrocarbon hydroxylase (118, 119, 148). Nebert and his associates were the first to employ primary hepatocyte culture to study xenobiotic metabolizing enzyme activity. In an elegant series of papers (42–44, 113, 118), these investigators characterized the P-448-dependent aryl hydrocarbon hydroxylase induction in fetal rat hepatocytes maintained for two to four days in primary culture on a plastic substratum. The enzyme was found to be induced by a variety of structurally diverse compounds, including polycyclic hydrocarbons (42, 43), phenobarbital (42, 43), the insecticide p,p′-DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane) (42, 43), biogenic amines (44), some hormones (44), other endogenous compounds (44, 118), and any one of a large number of hydrophobic compounds added to the culture medium (118). Aryl hydrocarbon hydroxylase induction in culture was further shown by inhibitor studies with actinomycin D and cycloheximide to involve an initial DNA-dependent RNA synthesis and to require continuous protein synthesis (113). Many of the inducing compounds in culture were also typical inducers of the enzyme in vivo. However, phenobarbital was shown by inhibitor and spectral studies to induce only cytochrome P-448 in the cultured fetal hepatocytes, whereas in vivo other cytochrome P-450 species predominate in untreated and phenobarbital-pretreated adult rats (118). Also, unlike the situation in vivo, phenobarbital did not stimulate the proliferation of smooth endoplasmic reticulum in the cultured hepatocytes. The induction of the enzyme in culture by various biogenic amines was also contrasted with their inhibitory or lack of effects on hydroxylase activity in vivo (19, 44). Another interesting feature of the aryl hydrocarbon hydroxylase induction in fetal liver culture was its enhancement by different combinations of polycyclic hydrocarbons, phenobarbital, and certain biogenic amines (44, 118). The most plausible explanation proposed for this additive effect is that a hydrophobic compound may bind to nonspecific sites on the microsomal membranes, or to glass or plastic, thereby allowing for a subsequently greater substrate availability to the enzymatic oxidation sites, thus resulting in an apparently enhanced hydroxylase activity (118). Other possible explanations for the enhancement of the hydroxylase activity in culture include the buildup of hydroxylase metabolites as a result of an inhibition of further metabolism (118) or the presence of several forms of cytochrome P-448 that are inducible in culture by different agents (19). It has been further proposed by Paine (120) that singlet oxygen generation within cells may be a common factor linking the many structurally diverse inducers of cytochrome P-448-dependent aryl hydrocarbon hydroxylase. However, further studies are required to support this attractive hypothesis.

In contrast to the results obtained with fetal rat hepatocytes in primary culture, chick embryo hepatocytes in short-term primary monolayer culture retain a relatively high level of cytochrome P-450 and show a number of P-450-mediated monooxygenase activities that are more comparable to those expressed in ovo, and in adult chicken and mammalian liver in vivo (4, 91, 128, 129). Studies with nonreplicating adult rat hepatocytes in primary culture have also demonstrated a number of differentiated but declining cytochrome P-450-dependent monooxygenase activities. In this respect, Guzelian et al. (52) have shown a differential decrease in the activities of several microsomal monooxygenase activities of adult rat hepatocytes isolated from 4- to 6-day-old regenerated rat livers and maintained in primary culture in plastic culture dishes. In the 24- to 48-hour period immediately after the preparation and culture of the isolated hepatocytes, the activities of aminopyrine-N-demethylase and aniline hydroxylase decreased to less than 20% of their zero time values as determined in freshly isolated hepatocytes. These decreases in enzyme activities closely paralleled the spectrophotometrically measured decline in the concentration of cytochrome P-450 over the same period in cells in culture (see section IV C). On the other hand, the hepatocytes cultured for 24 to 48 hours maintained greater activities of NADPH cytochrome *c* reductase and aryl hydrocarbon hydroxylase that were approximately 64% and 42% respectively of those measured at zero time. However, the activity of *p*-nitroanisole O-demethylase remained almost unchanged from its zero-time value in the cultured hepatocytes, despite spectral and specific inhibitory evidence that this enzyme is cytochrome P-450-dependent. The activities of NADPH cytochrome *c* reductase and epoxide hydratase in microsomes prepared from normal adult rat hepatocytes maintained in primary culture for 10 days on floating collagen membranes were also shown to be reduced, each to approximately 33% of their original levels measured in the intact liver lobe and in freshly isolated hepatocytes,

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while the cytochrome P-450 content of the 10-day-old cultured hepatocytes was found to be only about 10% of its original concentration (33). Michalopoulos et al. (97) have further demonstrated the dissociation of the levels of cytochrome P-450 and NADPH cytochrome *c* reductase in normal adult rat hepatocytes maintained for up to 10 days in primary culture on collagen-coated plates, floating collagen membranes, and confluent human diploid fibroblasts, respectively. The cytochrome P-450 content and the NADPH cytochrome *c* reductase activity of the cultured hepatocytes were both better preserved with time in culture when the cells were maintained on the floating collagen membrane and human diploid fibroblasts than on the collagen-coated plate. Nevertheless, the different rates of decline of cytochrome P-450 and NADPH cytochrome *c* reductase could be demonstrated with each of the three substrata used to support the hepatocytes in culture. In contrast to the findings of Guzelian et al. (52), normal adult rat hepatocytes in 24-hour-old primary culture on collagen gel/nylon mesh substratum exhibited microsomal activities of aminopyrine-N-demethylase and aryl hydrocarbon hydroxylase that were more closely related to those of the freshly isolated hepatocytes. The cytochrome P-450 content of the cultured hepatocytes on the mesh substratum declined to approximately 30% of its original level as measured in the freshly isolated cells (G. Hayao and H.C. Pitot, unpublished data). It is possible that the differences between these results and those of Guzelian et al. could be related to the fact that Guzelian et al. used hepatocytes from partially hepatectomized rats. However, a more plausible explanation is that the functional integrity of the hepatocytes is better preserved when they are maintained on a collagen gel substratum than on plastic. Nevertheless, in hepatocyte cultures, the dissociation of various monooxygenase activities from cytochrome P-450 does not appear to be a function of the substratum. Rather, it may be related to a differential stability of subspecies of cytochrome P-450, as well as to a weak induction of cytochrome P-448 by endogenous inducers in the culture medium (52).

C. Microsomal Cytochromes of Cultured Cells Derived from Liver

One of the features that limits function in adult rodent hepatocytes in primary culture is the rapid decline in their cytochrome P-450. Hepatocytes isolated from normal (91, 93, 97, 121) as well as from regenerated adult rat livers (14, 15, 52) and then maintained under noninducible conditions in primary culture for 24 to 48 hours on either plastic (14, 15, 52, 91, 121), collagen-coated plastic (97, 121), floating collagen membrane (93, 97), collagen gel/nylon mesh (G. Hayao and H. C. Pitot, unpublished data), or human diploid fibroblasts (97) substrata were shown spectrophotometrically to possess a cytochrome P-450 level that was approximately 20% to 33% of that measured in intact liver or in freshly isolated hepatocytes. Michalopoulos et al. (97) have further demonstrated that during the first three days of primary culture, the rate of decline of cytochrome P-450 was similar in normal adult rat hepatocytes maintained on collagen-coated plates, floating collagen membranes, or human diploid fibroblasts. However, after five days in culture, no cytochrome P-450 could be detected in the hepatocytes maintained on the collagen-coated plates, whereas those cultured on the floating collagen membranes and on the human diploid fibroblasts still exhibited a measurable but very low level of cytochrome P-450, which was retained at a slowly declining rate for up to 10 days in culture. Nevertheless, the dramatic early loss of cytochrome P-450 in the cultured adult rat hepatocytes occurred despite the use of various commercial media preparations (14, 15, 52, 91, 93, 97, 121) in the presence or absence of serum, insulin, or antioxidants in the medium (15, 52, 97).

The cytochrome b_5 of normal adult rat hepatocytes in primary culture on floating collagen membranes declined at a slower rate than that for cytochrome P-450 over a 10-day culture period (93). For example, the basal level of cytochrome b_5 measured in hepatocytes after 24 hours in culture was 68% of that of intact liver as compared with a value of 33% for cytochrome P-450. In contrast, adult mouse hepatocytes cultured under very similar conditions on floating collagen membranes were shown to lose about 80% of their cytochrome b_5 and P-450 during the first 24 hours (133). The mouse hepatocytes also lost 90% of their aryl hydrocarbon hydroxylase activity and 97% of their aminopyrine-N-demethylase activity during the same 24-hour culture period, thus indicating a major difference between the differential stability of the xenobiotic-metabolizing components in the mouse as compared with those of the rat. On the other hand, chick embryo hepatocytes in short-term primary culture on plastic have a more stable basal cytochrome P-450 content than do cultured rodent hepatocytes (91). Furthermore, δ-aminolevulinic acid synthetase, the rate-limiting enzyme for heme biosynthesis, is highly inducible in primary cultures of chick embryo hepatocytes by drugs and chemicals that induce cytochrome P-450 in these cells in vivo and in ovo (46, 91, 138), but this enzyme was not increased by drugs in primary cultures of adult rat hepatocytes (91). Paine and Legg (121) have further reported that there is no correlation between the loss of cytochrome P-450 in normal adult rat hepatocytes in primary culture and the stimulation of heme oxygenase activity in these cells. However, these data suggested only that heme oxygenase was not the sole determinant of cytochrome P-450 concentration in the cultured hepatocytes; they did not rule out the possibility of an increased heme synthesis under the culture conditions employed.

Interestingly, cytochrome P-420, the denatured form of cytochrome P-450, was not markedly increased in either adult rat (15, 93, 162) or mouse (133) hepatocytes

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after their first 24 hours in primary culture. This could mean that not all of the cytochrome P-450 is being converted into cytochrome P-420 during this time, or more likely, that cytochrome P-420 also rapidly decreases in culture (133, 162). After the first 24 hours in culture, the cytochrome P-420 level of liver parenchymal cells increased more rapidly than the cytochrome P-450 declined (93, 133). It is probable that the cytochrome P-420 spectrum seen was formed by the combination of albumin or other hepatic proteins with the heme released from cytochrome b_5, as well as from cytochrome P-450 (133).

At present, there have been no published reports that satisfactorily resolve the known multiple forms of cytochrome P-450 (69, 166, 178) in cultured hepatocytes, nor have any direct measurements been made on the effect that culture conditions have on the turnover of the various multicomponents of their mixed-function monooxygenase system. However, it appears that the rate of loss of cytochrome P-450 in rodent hepatocytes, at least, is accelerated when these cells are isolated and placed in primary culture. Whether this loss represents a specific alteration in the rate of degradation of cytochrome P-450 or is due to a more general remodeling of the endoplasmic reticulum of the cultured hepatocytes remains to be determined. However, as will be detailed in section V, the loss of cytochrome P-450 can be temporarily prevented by the addition of various hormonal and chemical factors to the culture medium.

D. Activities of Other Enzymes of Drug Metabolism in Liver Cell Culture

While freshly isolated hepatocytes from adult rats are, in general, more efficient in performing xenobiotic metabolism than those maintained for various times in primary culture, it has been demonstrated that the cultured cells can still exhibit a measurable metabolism of a number of procarcinogens and drugs. For example, hepatocytes from normal or 3-methylcholanthrene-induced adult rats are capable of forming oxidative and conjugative metabolites of benzo(a)pyrene when they are maintained in primary culture on plastic over a 24-hour period (149). Leffert et al. (86) have also reported the limited conversion of 2-acetylaminofluorene to its N-hydroxylated metabolite by adult rat liver cells maintained for five to six days in primary culture according to their culture procedure. Galivan (36) showed that the capacity of adult rat hepatocytes to accumulate methotrexate was lost during their first two to three days in primary culture, but their ability to form polyglutamate derivatives did not appear to be diminished over this same time period. Organ explants of human fetal liver exhibit first-order kinetics in the metabolism of the benzodiazepine drug, prazepam, for up to three days in culture (109). Sirica et al. (158) have demonstrated the expression of and linear increase in the activity of γ-glutamyl transpeptidase, an enzyme involved in the catabolism of glutathione and other γ-glutamyl compounds, in adult rat hepatocytes maintained in primary culture for three to nine days on a collagen gel/nylon mesh substratum. In addition, Leffert et al. (87) observed with their primary rat hepatocyte culture system a measurable amount of glutathione S transferase B (ligandin) whose concentration appeared to be related to the growth cycle of a replicating cell population. However, this replicating liver-derived cell type has not been sufficiently characterized to permit any definite conclusions concerning its exact nature. Nevertheless, it is apparent from these studies that hepatocytes in primary culture are capable of exhibiting a number of specific functions that can influence the metabolism and subsequent fate of drugs, carcinogens, and hepatotoxins.

V. Regulation of Xenobiotic Metabolism in Cultured Liver Cells

A. Maintenance and/or Induction of Microsomal Cytochromes and Monooxygenase Activities in Primary Rat Hepatocyte Culture

Several studies have demonstrated the short-term maintenance as well as induction of microsomal cytochromes and/or monooxygenase activities by various xenobiotics, cofactors, or hormones in primary cultures of hepatocytes prepared from normal or regenerated adult rat liver. The results of these studies are summarized in Table 2. In a number of the studies listed, the media employed were also supplemented with insulin at concentrations of approximately 10^{-7} to 10^{-6} M (26, 33, 93,94, 162) and/or with 5% fetal calf serum (33, 52,93,94, 122) or 1% normal adult rat serum (15, 162). However, it was apparent that supplementation with insulin alone or in combination with serum was ineffective in preventing the rapid decline in cytochrome P-450 (33, 54, 93, 94) or in maintaining several P-450-dependent monooxygenase activities of the culture hepatocytes (33, 97). In addition, estradiol was found to be ineffective in maintaining the cytochrome P-450 content of hepatocytes isolated from regenerating rat liver and cultured for 24 hours on a collagen-coated plastic substratum (54). Decad et al. (26) have demonstrated the maintenance of near in vivo levels of cytochrome P-450 in normal adult rat hepatocytes cultured for 24 hours in Waymouth's medium MB 752/1 supplemented with several peptide and steroid hormones known to increase cytochrome P-450 in vivo, in addition to δ-aminolevulinic acid, linoleic acid, and oleic acid. This medium without estradiol was only 24% less effective in maintaining the cytochrome P-450 of the cultured hepatocytes. On the other hand, Decad et al. (26) have reported that the cytochrome P-450 content of the cultured hepatocytes could be maintained at a level of about 50% that of freshly isolated hepatocytes during the first 24 hours of culture when they were incubated in a defined medium supplemented with 10^{-6} M testosterone. This represented an 18% increase in cellular cytochrome P-450 content over that of the control hepatocytes cultured without testosterone. Both hydrocortisone (93) and dex-

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TABLE 2

Apparent maintenance of "induction" of microsomal cytochromes and monooxygenase activities of adult male rat hepatocytes in primary culture by various xenobiotics, chemicals, cofactors, and hormones

Hepatocyte Source	Age of Culture (days)	Substratum	Agent(s)	Effective Concentration	Exposure Period (days)	Effect	Reference
Normal liver	5–10	Floating collagen membrane	Phenobarbital	2×10^{-3} M	5	3-Fold induction of cytochrome P-450	(33, 94)
Normal liver	5–10	Floating collagen membrane	Phenobarbital	2×10^{-3} M	5	3-Fold stimulation of NADPH cytochrome *c* reductase and epoxide hydratase activities*	(33)
Normal liver	5–7	Floating collagen membrane	3-Methylcholanthrene	1×10^{-5} M	2	4-Fold induction of cytochrome P-448	(94)
Normal liver	0–1	Floating collagen membrane	Hydrocortisone	1×10^{-4} M	1	Partial maintenance of cytochromes P-450 and b_5	(93)
Normal liver†	0–1	Plastic	Nicotinamide	2.5×10^{-2} M	1	Maintenance of cytochrome P-450 and aminopyrine-N-demethylase activity‡	(122)
Normal liver	0–1	Plastic	Isonicotinamide	1×10^{-2} M	1	Maintenance of cytochrome P-450	(123)
Normal liver§	0–7	Plastic	Phenobarbital	1×10^{-4} M	6	5- to 6-Fold stimulation of androstenedione hydroxylase activities	(162)
Normal liver	0–1	Collagen-coated plastic	Medium containing:		1	Maintenance of cytochrome P-450 and metabolism of aflatoxin B_1	(26)
			Estradiol	1×10^{-6} M			
			Glucagon	5×10^{-8} M			
			Hydrocortisone	1×10^{-5} M			
			Testosterone	1×10^{-6} M			
			Thyroxin	1×10^{-5} M			
			δ-Aminolevulinic acid	1×10^{-6} M			
			Linoleic acid	5 mg/l			
			Oleic acid	5 mg/l			
Normal liver	0–7	Collagen-coated glass or plastic	Phenobarbital	2×10^{-3} M	2–3	3- to 4-Fold stimulation of 7-ethoxycoumarin O-deethylase activity; 1.5-fold stimulation of NADPH oxidase activity	(19)
Normal liver	0–7	Collagen-coated glass or plastic	Benzanthracene	13×10^{-6} M	1	2-Fold stimulation of 7-ethoxycoumarin O-deethylase activity‖	(19)
Normal or regenerated liver	0–1	Collagen-coated plastic	Dexamethasone	1×10^{-6} M	1	Partial maintenance of cytochrome P-450	(54)
Regenerated liver	0–1	Plastic	δ-Aminolevulinic acid	8×10^{-5} M	1	Slight production of cytochrome P-450	(51)
Regenerated liver	0–1	Plastic	L-ascorbic acid	4×10^{-4} M	1	Partial maintenance of cytochrome P-450 and b_5	(15)
Regenerated liver	1–2	Plastic	Benzo(a)pyrene	1 μg/ml	1	8-Fold stimulation of aryl hydrocarbon hydroxylase activity; 4-fold stimulation of *p*-nitroanisole O-demethylase activity	(52)
Regenerated liver	2–10	Plastic	Benzo(a)pyrene	1 μg/ml	3	2- to 3-Fold stimulation of *p*-nitroanisole O-demethylase activity	(13, 14)

*Stimulation is used in favor of induction where only enzyme activity was measured.

†Hepatocytes were isolated from normal rat liver in the presence of 1×10^{-3} M nicotinamide.

‡The term maintenance is used to indicate the measurement of near in vivo levels of mixed-function monooxygenase components or activities.

§Androstenedione hydroxylase activities were also significantly stimulated in hepatocytes cultured from normal female rats. Maximum inductive levels of total hydroxylase activities of the female hepatocytes only approximated those of the uninduced male hepatocytes.

‖The stimulation of 7-ethoxycoumarin O-deethylase by phenobarbital and benzanthracene was additive. Enzyme activity stimulated by phenobarbital was cytochrome P-450-dependent; that stimulated by benzanthracene was cytochrome P-448-dependent.

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amethasone (54) prevented in part the decline in cytochrome P-450 content of adult rat hepatocytes during their first 24 hours in primary culture. However, Michalopoulos et al. (93) have found that, whereas 10^{-4} M hydrocortisone was effective in reducing the initial decline in cytochrome P-450, the hormone also stimulated the appearance of cytochrome P-448, but not P-450, as measured spectrophotometrically in normal adult rat hepatocytes cultured for more than five days on floating collagen membranes. In this regard, the cytochrome P-450 content of the hepatocytes cultured for five days in the presence of hydrocortisone had declined to the same low level as that measured in cultures maintained without the hormone. The addition of δ-aminolevulinic acid was also effective in producing a slight increase in the cytochrome P-450 content of adult rat hepatocytes isolated from regenerated rat liver and cultured for 24 hours on a plastic substratum (51).

The mechanisms by which nicotinamide and ascorbate act to maintain the cytochrome P-450 content of the cultured adult rat hepatocytes are unclear (see Table 2). Paine et al. (122, 123) have demonstrated that normal adult rat hepatocytes cultured on plastic for 24 hours in Williams' medium E containing 5% fetal calf serum lose approximately 60% of their initial concentration of total nicotinamide adenine dinucleotides (NAD^+ plus NADH), as well as about 70% of their cytochrome P-450 content. However, the dinucleotide and cytochrome P-450 losses could be largely overcome by supplementation of the medium with 2.5×10^{-2} M nicotinamide. Lower molar concentrations of exogenous nicotinamide were also effective in partially maintaining the cytochrome P-450 content of the hepatocytes during their first 24 hours in primary culture. In addition,the cytochrome P-450 concentration of the hepatocytes cultured for 24 hours in the presence of 2.5×10^{-2} M nicotinamide was increased to the same level as found in intact rat liver when 10^{-3} M nicotinamide was also included in the medium used for cell isolation (122). However, it is unlikely that a causal relationship exists between the NAD concentration of the cultured hepatocytes and the maintenance of their cytochrome P-450 content, since Paine et al. (123) have also found that isonicotinamide, which does not increase the total nicotinamide adenine dinucleotides of normal adult rat hepatocytes in 24-hour primary culture, is twice as effective as nicotinamide on a molar basis in maintaining the initial cytochrome P-450 content of the cultured cells.

Bissell and Guzelian (15) have demonstrated that the cellular concentration of ascorbate is decreased by about 70% during the preparation of isolated hepatocytes from regenerating adult rat liver. However, ascorbate levels remained stable at 35% to 40% of that measured in intact liver when the hepatocytes were cultured for 20 hours in serum-free medium on a plastic substratum. Concomitantly, the cytochrome P-450 content of the cultured hepatocytes decreased progressively to a level of about 20% of that of intact liver during the first 24 hours of culture. With the addition of 10^{-5} M to 10^{-3} M L-ascorbate to the medium at the time of cell plating, there was approximately a 2-fold greater content of cytochrome P-450 in the hepatocytes at 24 hours of culture over that measured in hepatocytes cultured for the same period in unsupplemented medium (15). Ascorbate is also important in the maintenance of the cytochrome P-450 content of mammalian hepatocytes in vivo (15, 116, 192, 193), but its actual role in this process needs to be elucidated. Nevertheless, Bissell and Guzelian (15) have provided indirect evidence that suggests that ascorbate in cultured hepatocytes may be altering the metabolism of the protein or lipid components of cytochrome P-450 rather than that of the heme moiety.

A number of laboratories have now demonstrated the induction of cytochrome P-450 (33, 94) and/or the stimulation of several P-450-dependent monooxygenase activities (19, 33, 162) in primary cultures of adult rat hepatocytes with phenobarbital. Michalopoulos et al. (94) have also shown a marked increase in the smooth endoplasmic reticulum of normal adult rat hepatocytes maintained for 5 to 10 days in primary culture on floating collagen membranes in the presence of 2×10^{-3} M phenobarbital. This concentration of phenobarbital is approximately 40 times the usual pharmacological level in vivo. Furthermore, even though the relative magnitude of inducibility of cytochrome P-450 in culture was similar to that observed in vivo, i.e. approximately a three-fold increase, the actual level of induced cytochrome P-450 in the 5- to 10-day old cultured rat hepatocytes was much lower than that measured in liver after treatment of the animals in vivo with phenobarbital (19), and was also lower than that found in intact liver or in freshly isolated hepatocytes from normal untreated adult rats (33). In addition, Fahl et al. (33) have shown, by substrate-induced difference spectra and by gel electrophoresis, the absence in phenobarbital-induced adult rat hepatocytes cultured for 10 days on floating collagen membranes of most forms of cytochrome P-450, which, by comparison, were present in liver microsomes prepared from phenobarbital-pretreated rats. The properties of the cytochrome P-450 of control and phenobarbital-induced hepatocytes maintained for 10 days on floating collagen membranes were altered from those of the major P-450 cytochromes that are induced by phenobarbital in the intact rat liver. In this respect, the cytochrome P-450 of the control and phenobarbital-treated cultured hepatocytes resembled cytochrome P-448 in terms of its minimal response to type 1 ligands and the product distribution and specific activity shown for the metabolism of benzo(a)pyrene; however, it did exhibit a distinct λ_{max} for the reduced carbon monoxide complex of 450.7 nm. It is possible that this anomalous form of cytochrome P-450 is a normal minor component of adult rat liver microsomes, but that it is dominated in vivo by other forms of cytochrome P-450, which then become selectively de-

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graded after explantation of the hepatocytes into primary culture. On the other hand, the cytochrome P-450 observed in the 10-day-old cultures of adult rat hepatocytes on floating collagen membranes was similar to that reported for neonatal rat liver (63) with respect to its low cellular concentration, its markedly decreased type 1 substrate binding, and the shift in λ_{max} of the reduced carbon monoxide complex. Thus, the altered properties of the cytochrome P-450 of the cultured hepatocytes may reflect the appearance of a new species that becomes expressed as a result of the reversion of the cells to a more undifferentiated state, possibly because of their maintenance in a hormonally deficient medium. This would be consistent with the appearance of fetal-like phenotypic changes demonstrated by Sirica et al. (158) for normal adult rat hepatocytes maintained for a similar period of culture on a collagen gel/nylon mesh substratum. In any case, further studies are required to determine the exact nature of these changes.

Guzelian and Barwick (53) have recently provided evidence to indicate that the degradation of cytochrome P-450 in 24-hour primary cultures of regenerated adult rat hepatocytes is due to a process that is inhibited by cycloheximide and hence may require protein synthesis. They further confirmed that the rapid breakdown of cytochrome P-450 in cultured hepatocytes is not related to their increased heme oxygenase activity (see section IV C). The formation of cytochrome P-450 in 24-hour primary cultures of regenerated adult rat hepatocytes maintained in the presence of δ-aminolevulinic acid was also affected by the addition of cobalt to the medium (51). Since cobalt does not interfere with heme synthesis in these cells, it was concluded that the cation was preventing cytochrome P-450 formation, probably by blocking the association of heme with apocytochrome.

While the above studies have provided useful information concerning the regulation of xenobiotic metabolism in primary cultures of adult rat hepatocytes, they also reflect a number of critical shortcomings. In this respect, most of the studies listed in Table 2 have been limited to cells in the first 24 hours of culture or to a comparable restrictive period and thus do not demonstrate the effects of various chemical or hormonal treatments on the microsomal cytochromes and/or monooxygenase activities over any significant extended time period. In addition, the relative contributions of the various hormonal and chemical components of the medium devised by Decad et al. (26) that are needed to maintain the cytochrome P-450 of cultured hepatocytes have not been adequately determined. Here it is noteworthy to point out that the medium of Decad et al. and the nicotinamide-supplemented medium employed by Paine et al. (122, 123) contain ascorbate. Therefore, it becomes necessary to determine whether the presence of ascorbate is contributing in an additive or permissive manner to the other regulating components of these media in maintaining the cytochrome P-450 of the cultured cells. Bissell and Guzelian (15) have shown that the effects of exogenous δ-aminolevulinic acid and ascorbate are additive in increasing the cellular concentration of cytochrome P-450 in cultured rat hepatocytes. Furthermore, the studies of Fahl et al. (33) have demonstrated the importance of critically characterizing the properties of the control and induced forms of cytochrome P-450 in the cultured hepatocytes. However, since these studies were limited only to hepatocytes in 10-day-old primary culture, it is not known whether the pattern of cytochrome P-450 of earlier cultures is similarly altered. Since phenobarbital primarily affects the centrolobular hepatocytes in vivo, it is also important to determine what effects, if any, hepatocyte heterogeneity might have on the maintenance or induction of their mixed-function monooxygenase components in culture. Finally, a rigorous effort should be made to determine the critical medium conditions required to maintain the differentiated state of the adult rat hepatocyte in primary culture.

B. Effects of Drugs, Chemicals, and Hormones on the Cytochrome P-450-Dependent Monooxygenase Systems of Adult Mouse and Chick Embryo Hepatocytes in Primary Culture

Renton et al. (133) have studied the effects of the interferon-inducing agent, polyriboinosinic acid-polyribocytidylic acid (poly rI.rC), as well as a crude preparation of mouse interferon on the cytochrome P-450 and monooxygenase activities of normal adult mouse hepatocytes maintained for 24 to 48 hours in primary culture on floating collagen membranes. Exposure of the cultures to poly rI.rC at a concentration of 5 μg/ml of medium during the first 24- to 48-hour period after cell plating produced a maximum increase of about 40% in the cytochrome P-450 content of the cultured hepatocytes over that measured in control cultures. Poly rI.rC was also effective in stimulating the aminopyrine-N-demethylase activity of the cultured mouse hepatocytes to a level comparable to that of freshly isolated hepatocytes and of mouse liver microsomes. On the other hand, interferon at a concentration of 1000 units/ml of medium was less potent than poly rI.rC in increasing the cytochrome P-450 content of the cultured hepatocytes, but this glycoprotein was capable of stimulating the aminopyrine-N-demethylase activity of the cells to a level similar to that obtained with poly rI.rC. In addition, both poly rI.rC and the interferon preparation were effective in producing moderate increases in the aryl hydrocarbon hydroxylase activity of the mouse hepatocytes in 24-hour primary culture. However, poly rI.rC was ineffective in inducing the hydroxylase activity of cultured Reuber hepatoma cells (133).

The basis for the induction of cytochrome P-450 and monooxygenase activities by poly rI.rC and crude mouse interferon has not been defined. Poly rI.rC was shown by Renton et al. (133) to induce interferon activity in the mouse hepatocyte cultures, but it is still not clear

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whether a direct causal relationship exists between this response and the induction by poly rI.rC of cytochrome P-450 and monooxygenase activity in the cultured hepatocytes, nor whether the inductive effects of the crude interferon preparation used in this study were the result of interferon or of some as yet undefined impurity. Furthermore, Renton and Mannering (131, 132) have demonstrated that a number of interferon-inducing agents depress hepatic cytochrome P-450-dependent monooxygenase systems when administered to rats and mice in vivo. More recently, these investigators have reported that the interferon-inducing agents poly rI.rC and tilorone suppress the inductive effects of phenobarbital and 3-methylcholanthrene on ascorbic acid synthesis, the level of cytochrome P-450, and the monooxygenase activity in the rat (134). Although the actions of interferon-inducing agents on hepatic P-450 monooxygenase systems in culture and in vivo are paradoxical, such disparate effects may be related to the exceedingly complex nature and the multiplicity of responses to interferon in vivo.

Chick embryo hepatocytes in primary culture have also provided a useful model system for studying the regulation of microsomal mixed-function monooxygenase components. Poland and Kappas (129) first presented preliminary evidence for the induction of cytochrome P-450 and aminopyrine-N-demethylase by phenobarbital in chick embryo hepatocytes in primary culture. Sinclair et al. (157) reported the induction of cytochrome P-450 by isopropylacetamide in short-term primary cultures of chick embryo hepatocytes maintained in a serum-free medium. Isopropylacetamide induced a five-fold increase in cytochrome P-450, approximating the levels induced in ovo, when triiodothyronine and/or dexamethasone were included in the culture medium. Also, as in the case of the rat hepatocytes in primary culture, insulin did not appear to be required for this induction in chick embryo hepatocytes. In an elaborate series of experiments, Althaus, Meyer, and their coworkers (4, 91) have demonstrated the induction of cytochrome P-450 by a variety of xenobiotics in chick embryo hepatocytes maintained in primary culture for up to two days in a chemically defined medium. The induction, which paralleled that observed in ovo, occurred in the absence of freshly added serum or hormones (with the exception of 10^{-7} M insulin) to the medium. In addition, these investigators, by using gel electrophoresis and isotope incorporation studies, demonstrated in this culture system the induction by phenobarbital of the de novo synthesis of a microsomal protein with a molecular weight of 51,000, whereas β-naphthoflavone induced the de novo synthesis of a microsomal protein having a molecular weight of 55,000. While the identity of these proteins as cytochrome P-450 apoproteins is presently only inferred, their induction was shown to be associated with differential increases in aminopyrine-N-demethylase and aryl hydrocarbon hydroxylase activities, respectively.

VI. Effects of Carcinogens, Mutagens, and Toxins on Cultured Cells Derived from Liver

A. Hepatocyte DNA Repair and Mutagenesis Assays for Chemical Carcinogens

It is now evident that the hepatic, mixed-function monooxygenase system plays an important role in converting many chemical carcinogens (procarcinogens) to their highly reactive forms [see Miller and Miller (98, 99) for review]. The ability of cultured hepatocytes to metabolize procarcinogens to forms that react to alter or damage DNA and other macromolecules forms the basis for their potential value as a bioassay for xenobiotic metabolizing capacity, as well as for use as an in vitro screening system for carcinogenic and mutagenic chemicals.

Nonreplicating adult rat hepatocytes in freshly isolated cell suspension and in short-term primary culture have been shown to exhibit DNA repair, measured as unscheduled DNA synthesis, after their exposure to ultraviolet radiation (76, 190), as well as to a number of known direct-acting carcinogens and procarcinogens. Table 3 lists the results from a number of different laboratories of several classes of chemicals (both direct-acting such as methyl methanesulfonate, N-methyl-N′-nitro-N-nitrosoguanidine, and N-acetoxy-2-acetylaminofluorene, and those that require metabolic activation such as 2-acetylaminofluorene, 7,12-dimethylbenz(a)anthracene, 3′-methyl-4-dimethylaminoazobenzene, aflatoxin B_1, and dimethylnitrosamine) tested for their ability to induce unscheduled DNA synthesis in adult rat hepatocyte suspensions and primary cultures and compared with their carcinogenicity in vivo and their mutagenicity in the Ames salmonella/mammalian microsome mutagenicity test (5, 90). Unscheduled DNA synthesis was detected by autoradiography (24, 185, 187, 188) and by the direct measurement of incorporated radioactivity from [^{3}H]-thymidine into hepatocyte DNA isolated on CsCl gradients (20, 96, 159) or by other biochemical procedures (61, 190). As indicated in Table 3, there appears to be, with few exceptions, good correlation between the carcinogenicity and/or mutagenicity of the chemicals tested and their ability to induce unscheduled DNA synthesis in these hepatocyte culture systems. In addition, while there was an indication that the amount of unscheduled DNA synthesis elicited by some of the chemicals, such as aflatoxin B_1 and B_2, was related to their carcinogenic potency (185, 187), it was also evident from these studies that the amount of unscheduled DNA synthesis induced was sometimes greater with weak carcinogens than with potent ones. For example, aflatoxin B_2 was more effective on a molar basis than dimethylnitrosamine in inducing unscheduled DNA synthesis in the cultured hepatocytes (185, 187, 188). Thus, the relative amounts of carcinogen-induced DNA repair shown by these cells may be related to their metabolic capabilities, as well as to the type of DNA damage and repair elicited, rather than to the

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TABLE 3

Carcinogen-induced DNA repair in adult rat hepatocytes maintained in short-term suspension or primary culture

Compound	Culture	Evaluation of DNA Repair	Active Concentration (M)	DNA Repair	Carcinogenicity	Mutagenicity*	Reference
Aromatic Amines							
2-Acetylaminofluorene	S, P†	A, B‡	10^{-6}–10^{-3}	+	+	+	(20, 24, 96, 159, 187, 190)
4-Acetylaminofluorene	P	A		−	−	+	(187)
N-hydroxy-2-acetylaminofluorene	P	A, B	10^{-6}–10^{-5}	+	+	+	(24, 190)
N-acetoxy-2-acetylaminofluorene	P	B	10^{-6}	+	+	+	(190)
Benzidine	S	B	10^{-5}	+	+	+	(20)
2,3-Dimethyl-4-aminobiphenyl	S	B	10^{-5}	+	+	+	(20)
4-Aminobiphenyl	S	B	10^{-4}	+	+	+	(20)
2-Aminobiphenyl	S	B		−	−	+W§	(20)
Polycyclic Aromatics							
3-Methylcholanthrene	S	B	10^{-4}	+	+	+	(20)
Benzo(a)pyrene	S	B		−	+	+	(20)
Benzo(a)pyrene	S, P	B	10^{-6}–10^{-4}	+	+	+	(61, 96)
Anthracene	P	A		−	−	−	(187)
Benz(a)anthracene	S, P	A, B		−	+W	+	(20, 187)
9,10-Dimethyl benz(a)anthracene	S	B	10^{-4}	+	+	+	(20)
7,12-Dimethyl benz(a)anthracene	P	A	10^{-3}	+	+	+	(187)
Azo dyes							
4-Aminobenzene	P	A	10^{-4}	+	+W	+	(187)
3′-Methyl-4-dimethylaminoazobenzene	S, P	A, B	10^{-6}–10^{-4}	+	+	+	(20, 96, 187)
2-Methyl-4-dimethylaminoazobenzene	P	A	10^{-4}	+	+W	+	(187)
p-Phenylazoaniline	S	B	10^{-4}	+	+	+	(20)
3,3′,4,4′-Tetrachloroazobenzene	S	B	10^{-6}–10^{-4}	+	?		(61)
Fungal toxins							
Aflatoxin B_1	S, P	A, B	10^{-7}–10^{-4}	+	+	+	(96, 185, 187)
Aflatoxin B_2	P	A	10^{-5}–10^{-4}	+	+W	+	(185, 187)
Aflatoxin G_1	P	A	10^{-5}	+	+	+	(187)
Aflatoxin G_2	P	A		−	−	−	(187)
Nitrosamines and related compounds							
Dimethylnitrosamine	S, P	A, B	10^{-3}–10^{-1}	+	+	+W	(20, 96, 187, 188)
Diethylnitrosamine	S, P	A, B	10^{-3}–10^{-2}	+	+	+W	(20, (188)
Diphenylnitrosamine	S, P	A, B		−	−	−	(20, 188)
N-nitrosomorpholine	P	A	10^{-3}–10^{-2}	+	+	+W	(188)
N-nitrosopyrrolidine	P	A	10^{-3}–10^{-2}	+	+	+W	(188)
N,N,N-nitrosonornicotine	P	A	10^{-2}	+	+		(188)
4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone	P	A	10^{-3}–10^{-2}	+	+		(188)
Nitrosocarbazole	P	A		−	−		(188)
Dimethylformamide	P	A		−	−		(187, 188)
N-methyl-N′-nitro-N-nitrosoguanidine	S, P	A, B	10^{-5}	+	+	+	(61, 187)
Miscellaneous compounds							
Methyl methanesulfonate	S, P	A, B	10^{-6}–10^{-3}	+	+	+	(61, 159, 185, 187)
Styrene oxide	S	B		−	−	+	(20)
Biphenyl	S	B		−	−	−	(20)

* Data on carcinogenicity and mutagenicity of the compounds tested was taken from Ames et al. (5) and McCann et al. (90), as well as from those listed in the table.

† S, suspensions of freshly isolated hepatocytes; P, short-term primary hepatocyte culture.

‡ A, DNA repair determined by autoradiography; B, DNA repair determined by liquid-scintillation counting.

§ W, weakly positive.

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potency of the carcinogen. With respect to the latter, Regan and Setlow (130) provided evidence for two types of DNA repair in mammalian cells; a "short patch" type of repair as exemplified by ionizing radiation and alkylating agents, and a "long patch" type of repair as elicited by ultraviolet radiation and carcinogens such as N-acetoxy-2-acetylaminofluorene.

These data, although limited, suggest the potential value of the adult rat hepatocyte/DNA repair test systems for use as important supplements to the Ames bacterial mutagenicity test for detecting carcinogenic and/or mutagenic chemicals. In this regard, the hepatocyte suspension and primary culture systems do not require an exogenous microsomal activating preparation in order to be able to metabolize procarcinogens to their ultimate reactive forms. However, it is also obvious from these studies that the testing of additional compounds is required, along with further improvements designed to increase the capacity of the cultured hepatocytes to metabolize procarcinogens, as well as the sensitivity and/or ease of the methods used to quantify DNA repair before hepatocyte DNA repair can be adopted for general use as an in vitro screen for chemical carcinogens and/or mutagens.

Another interesting approach to the detection of potential chemical procarcinogens involves the use of freshly isolated or cultured adult rodent hepatocytes as a feeder system to provide metabolic activation in combination with other mammalian cell types that will respond to the active carcinogenic metabolites produced by exhibiting an increased mutation frequency, growth suppression, or neoplastic transformation in culture. San and Williams (141) employed adult rat hepatocytes in short-term primary culture in order to mediate an increased frequency of 8-azaguanine-resistant mutants of an adult rat liver-derived epithelial cell line by the procarcinogens dimethylnitrosamine, 7,12-dimethylbenz(a)anthracene, and 2-acetylaminofluorene. Similarly, Langenbach et al. (83) demonstrated the induction of ouabain-resistant mutants of V79 Chinese hamster cells by dimethylnitrosamine, diethylnitrosamine, and aflatoxin B_1 when they were cocultivated in primary culture with adult rat hepatocytes. The noncarcinogenic analogues, N-nitrosomethyl-tert-butylamine and aflatoxin G_2, were not mutagenic in this assay system. Wiebkin et al. (181) further showed an inhibition of rat fibroblast growth by cytotoxic chemicals in culture after their short-term incubation with adult rat hepatocytes. A number of different carcinogenic chemicals requiring metabolic activation for full toxicity to be realized were employed. Poiley et al. (127) have used lethally X-irradiated intact adult hamster hepatocytes to metabolize diethylnitrosamine, 2-nitrofluorene, and 4-aminoazobenzene to forms that transformed hamster embryo cells in culture. In addition, Gayda and Pariza (37) demonstrated the increased activation of aflatoxin B_1 for bacterial mutagenesis by primary monolayer cultures of adult rat hepatocytes maintained in a medium similar to that devised by Decad et al. (26) for maintaining the cytochrome P-450 content of these cells. Tong and Williams (169) have also recently defined conditions for detecting genotoxic chemicals, with the use of an adult rat liver epithelial cell line and monitoring mutations at the hypoxanthine-guanine phosphoribosyltransferase locus.

B. Sensitivity and Resistance of Cultured Liver Cells to the Cytotoxic Actions of Chemical Carcinogens and Other Chemicals

Hyperplastic hepatocyte nodules induced in rat liver by 2-acetylaminofluorene or ethionine are resistant to the cytotoxic effects of some hepatocarcinogens, including CCl_4 and dimethylnitrosamine (34). Laishes et al. (81) have demonstrated that cells isolated from 2-acetylaminofluorene-induced rat liver hyperplastic nodules were more resistant in primary culture to the cytotoxic effects of aflatoxin B_1 than were normal adult rat hepatocytes. Schwartz (150) also reported that 7,8-benzoflavone, a competitive inhibitor of polycyclic hydrocarbon hydroxylation, was effective in protecting a cultured rat liver-derived epithelial cell line from the cytotoxicity of aflatoxin B_1, sterigmatocystin, and 7,12-dimethylbenz(a)anthracene. Estrogenic steroids protected rat liver-derived epithelial cells in culture from the cytotoxic effects of aflatoxin B_1 and 7,12-dimethylbenz(a)anthracene (150). In comparison, Bausher and Schaeffer (7, 8) have demonstrated a cloned diploid rat liver-derived epithelial cell line to be more resistant to the cytotoxic effects of aflatoxin B_1 than was a spontaneously transformed fibroblastic cell line derived from fetal rat liver. However, more recent studies by Schaeffer et al. (148) revealed that this liver-derived epithelial cell line exhibited an increased sensitivity, with an increasing level of population doubling in culture, to the cytotoxic effects of aflatoxin B_1 and benzo(a)pyrene. This increased sensitivity was shown to correspond to an enhanced inducibility of aryl hydrocarbon hydroxylase activity by 3-methylcholanthrene as a function of the population-doubling rate. Landolph et al. (82) also found that the toxicity of benzo(a)pyrene to a mouse liver-derived epithelial cell strain in culture increased exponentially with an increase in the number of population doublings. Thus, it is obvious that the sensitivity or resistance of liver cells in culture to the toxic effects of polycyclic hydrocarbon is related in a number of cases to the aryl hydrocarbon hydroxylase activity of the cells. However, Iype et al. (67) have also provided evidence to suggest that the increased resistance of a rat hepatocellular carcinoma line in culture to the cytotoxicity of 7,12-dimethylbenz(a)anthracene compared with that shown by a normal rat liver-derived epithelial cell line was probably due to the carcinoma cells' ability to generate polar metabolites of the polycyclic hydrocarbon more efficiently. This, in turn, resulted in the probable detoxification of its biologically active metabolites.

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Jones et al. (71) examined the enzyme pathways of freshly isolated normal adult rat hepatocytes that are involved in the detoxification of hydrogen peroxide, formaldehyde, and formic acid produced as a consequence of cytochrome P-450-dependent oxidative demethylation. Hydrogen peroxide produced by the cytochrome P-450-dependent reaction was shown to be decomposed primarily by the glutathione peroxidase system when the cellular levels of reduced glutathione were not depleted. Formaldehyde was rapidly oxidized to formic acid by formaldehyde dehydrogenase, an enzyme that requires, but does not consume, reduced glutathione. Formic acid, on the other hand, acted as a substrate for the peroxidatic activity of catalase, but was also found to decrease the catalase concentration by binding to the enzyme as a anionic ligand. In addition, Thor et al. (168) demonstrated the protection of freshly isolated adult rat hepatocytes from bromobenzene toxicity by cysteine and methionine, most likely as a result of their facilitation of cellular glutathione synthesis. Another interesting finding was that of Lowing et al. (88), who described the enhancement of glucose-6-phosphate dehydrogenase, succinate dehydrogenase, NADPH oxidase, and γ-glutamyl transpeptidase activities of adult rat hepatocytes maintained in short-term primary culture by very low doses (i.e., 10 μM) of several chemical carcinogens and some noncarcinogenic isomers. The enzyme activities were decreased at the higher dose levels of the chemicals used, possibly indicating cytotoxicity. Nevertheless, the enhancement in enzyme activities at low doses was probably the result of an adaptive response by the cultured hepatocytes to the cytotoxic effects of the carcinogen and noncarcinogen treatments.

C. Transformation of Cultured Cells Derived from Rat Liver

A number of epithelial-like cell lines and cell strains derived from rat liver can be transformed in culture either under the conditions of nutritional stress (17), "spontaneously" (104, 106, 117, 147, 177), or after exposures to a number of chemical carcinogens, including aflatoxin B_1 (147, 170, 183), dimethylnitrosamine (103–105, 183), 7,12-dimethylbenz(a)anthracene (183), methylnitrosourea (66, 183), N-hydroxy-2-acetylaminofluorene (183), N-methyl-N′-nitro-N-nitrosoguanidine (103–105), methylazoxymethanol acetate (18), and 4-nitroquinoline 1-oxide (73). While, at present, there are no absolute criteria for the malignant transformation of epithelial cells in culture, certain properties of the transformed liver-derived epithelial cells have been determined as a result of these investigations. Some of these properties are presented in Table 4. Of these, Montesano et al. (105) have reported that an increased nuclear:cytoplasmic ratio and growth in soft agar, as well as an increased cytoplasmic basophilia serve as reliable means for determining the malignant potential of the cultured liver-derived cells. While some of the cultured cells exhibited a "piling up" pattern of growth after their transformation (17, 18), this property was not generally observed for many of the transformed liver cell lines and strains studied (64, 103–105, 147, 177, 183, 186). In addition, while an increased agglutinability of transformed liver-derived epithelial cells (17) and fast-growing hepatocellular carcinoma lines (9) by plant lectins has been reported, others have found this not to be the case (73, 186). Williams (186) showed that the agglutinability of several control rat liver-derived epithelial cell lines by concanavalin A was generally higher than that exhibited by several hepatocellular carcinoma lines. Becker (9) also demonstrated that an increased agglutination by plant lectins was not an invarient feature of hepatocarcinogenesis in vivo. The production of extracellular plasminogen activator by rat liver-derived epithelial cells in culture also did not correlate with their tumorigenicity (105). Decreased intracellular communication between transformed rat liver-derived epithelial cells in culture (17) also may not be a suitable criterion for transformation, since normal rat hepatocytes in short-term primary culture have been shown to be deficient in gap junctions (145). A number of transformed rat liver-derived epithelial cell lines (62, 143), some hepatocellular carcinoma lines (136, 143), and hepatocytes cultured from carcinogen-induced "preneoplastic" and neoplastic rat liver (80) exhibit an increased γ-glutamyl transpeptidase activity. However, as previously indicated, this enzyme activity is also rapidly expressed at high levels in normal rat hepatocytes maintained in primary culture (158). Richards and Potter (136) further demonstrated in a number of hepatocellular carcinoma lines a marked variation in their γ-glutamyl transpeptidase activities. Morel-Chany et al. (106) have also provided evidence against the use of isozymic changes as markers of neoplastic transformation in rat liver-derived epithelial cell cultures. Furthermore, transformed liver-derived epithelial cells in culture and hepatocellular carcinoma lines did not exhibit the "criss-cross" cellular pattern (17, 177) characteristic of transformed fibroblasts in culture. It is evident from many of the above studies that the criteria used to judge

TABLE 4

Properties of transformed cultured cells derived from rat liver

Property	References
Cytological pleomorphism and altered nuclear:cytoplasmic ratio	(18, 104, 105, 144, 183, 186)
Aneuploidy	(17, 18, 73, 117, 147)
Antigenic alterations*	(66, 191)
Decreased intercellular adhesiveness	(17, 73, 106)
Increased plating efficiency	(18, 104, 106)
Ability to grow in soft agar	(17, 18, 66, 103, 105, 106, 142, 177, 186)
Ability to grow as carcinoma in vivo following transplantation	(66, 73, 103, 104, 117, 144, 147, 183)

* Antigenic alterations are described for liver-derived cells transformed in culture by chemical carcinogens.

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fibroblast transformation in culture do not adequately pertain to transformation in liver-derived epithelial cell cultures.

The ultimate index of transformation in the rat liver-derived epithelial cells in culture is their ability to give rise to carcinoma when transplanted back into suitable host animals. In this respect, a number of the transformed liver cell lines and strains have developed as solid carcinomas or adenocarcinomas after their inoculation into host animals (103, 104, 117, 183). However, the production of carcinosarcomas (103, 104, 147, 183) and fibrosarcomas (104, 170) was also a common feature in many of these studies. It is likely, at least in some cases, that the development of sarcomas is due to a contamination of the original liver-derived epithelial cell cultures with mesenchymal cells. On the other hand, Schaeffer and Heintz (147) have reported the development of carcinosarcomas, but not carcinomas, in isogenic rats inoculated with aflatoxin B_1-transformed rat liver-derived cells that were originally derived from a single cloned epithelial cell. Explantation of the tumor cells to culture was further shown to give rise to epithelial cells. Thus, it is possible that the mixed morphology of the tumors produced by transplantation is related to a multipotency of the carcinogen-transformed epithelial cells. It is also interesting that at the time of transplantation, the aflatoxin-treated cells reportedly showed no evidence of cytopathology, formation of cell foci, or growth in soft agar.

Since rat liver-derived epithelial cells have been demonstrated to transform "spontaneously" in culture (104, 106, 117, 147, 177), there is some uncertainty as to whether the observed transformation of these types of cultures with chemical carcinogens is due solely to the action of the carcinogen or to other oncogenic factors in the cellular environment as well. Montesano et al. (104) have observed a rather high transformation rate for their untreated rat liver-derived epithelial cell lines. Weinstein et al. (177) have further demonstrated by electron microscopy the presence of type A and C viral particles in a number of rat hepatocellular carcinoma cultures, as well as in a spontaneously transformed rat liver cell line. In contrast, Morel-Chany et al. (106) found no ultrastructural evidence for oncogenic virus or pleuropneumonia-like (PPLO) particles in a spontaneously transformed epithelial cell strain from rat liver. Williams et al. (183) also demonstrated the absence of tumorigenicity in a number of control rat liver-derived cell lines and further showed, in different sublines derived from a single parent line, that transformation by a number of different carcinogens occurred at a variety of ages in culture. Nevertheless, the incidence of "spontaneous" transformation in untreated liver-derived epithelial cells in culture complicates the potential analysis of chemical carcinogenesis in this system.

The relationship between the rat liver-derived epithelial cell and the adult rat differentiated hepatocyte is unclear. Some of the epithelial cell lines and strains have been shown to exhibit low, but measurable, functions specific to the hepatocyte. These include the production of albumin and other serum proteins (17, 18, 66, 147), as well as corticosteroid-mediated induction of tyrosine aminotransferase activity (106, 117). Also, the apparent transformation of several of these rat liver-derived epithelial cell lines or strains by procarcinogens such as aflatoxin B_1, dimethylnitrosamine, and 7,12-dimethylbenz(a)anthracene (183) suggests that at least some of these cultured cells possess the various enzyme systems required for the metabolic conversion of these compounds to active forms. On the other hand, rat liver-derived epithelial cells in culture have been generally shown by morphological and functional studies (49, 66, 147, 177) not to resemble the differentiated adult rat hepatocyte, but rather to be representative of a much less differentiated cell type. It has been hypothesized by Grisham et al. (49, 50) that these liver-derived epithelial cells may be originating from hepatocytic stem cells (see section III), but further studies are needed to establish this interesting possibility.

VII. Conclusions

In this review we have tried to portray a number of examples of the usefulness of cultured hepatic cells in the study of the mechanisms, regulation, and effects of drug and carcinogen metabolism. At the present time there is no single hepatocyte culture system that is ideal for this purpose. However, it is gratifying to note that during the last decade significant advances in the technology needed to maintain fetal, adult, and neoplastic hepatocytes in culture for reasonable periods of time have been achieved.

The studies to date, however, are only a beginning. As already noted, liver tissue explant cultures exhibit a number of features that make them unsuitable for use in controlled studies of hepatic drug metabolism. The liver explant is composed of parenchymal and nonparenchymal cell populations; the hepatocytes are short-lived in these preparations; and explant cultures become rapidly overgrown with outgrowths of mesenchymal cells and simple epithelial cells that lack the complex morphological and functional characteristics of the differentiated hepatocytes. The origin and identity of these less differentiated epithelial cells are still unclear, but their morphology is more comparable to that of endothelium in culture than that of the hepatocyte in vitro. However, as previously indicated, Grisham (49, 50) has hypothesized that at least some of the epithelial cell lines and strains of liver origin may be derived from a hepatic "stem" cell population. The validity of this proposal remains to be determined. Nevertheless, liver-derived epithelial cell lines and strains have been for the most part disappointing in terms of their drug-metabolizing activities and of their expressions of differentiated hepatocytic functions. Also, with successive passage in culture, some liver cell lines may undergo "spontaneous" transformation.

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Although it is possible to culture hepatoma cells as replicating populations for indefinite periods, these cells have lost most of the drug-metabolizing enzymatic apparatus seen in the normal adult rat hepatocyte. Furthermore, hepatoma cells exhibit unstable karyotypes and, therefore, a relative instability of their phenotype in culture for extended periods. A very recent approach employed to exploit the permanence of hepatoma cell lines as a means for "immortalizing" differentiated hepatocyte function has been the hybridization of normal rat hepatocytes with cells of a differentiated hepatoma cell line (180). In this regard, Widman et al. (180) have prepared a hepatocyte/hepatoma cell hybrid that maintained in culture a significant activity of the urea cycle enzyme ornithine carbamoyltransferase. While the somatic cells hybridization of normal hepatocytes with differentiated neoplastic cells of the same type may have potential for obtaining cell lines with sustained hepatocytic drug-metabolizing functions, there are to our knowledge no studies that have systematically examined this possibility.

In comparison with hepatoma and normal liver-derived epithelial cell lines and strains, fetal, embryonic, and early neonatal hepatocytes in culture possess a less variable, and in some species, a better developed enzymatic apparatus for the metabolism of xenobiotics. Studies with chick embryo hepatocytes in short-term primary culture have been more promising than those with fetal or neonatal rodent hepatocytes. However, hepatocytes isolated from a single chick embryo or rodent fetus or neonate can be cultured only in relatively small amounts and with only a limited period of replicative activity in culture. Because the yields of such cells are small, there is a marked limit to the size and number of cultures that can be obtained from a single animal, necessitating in most cases the pooling of hepatocytes from a number of embryonic or fetal livers. Since the techniques utilized for the isolation of these cells always include nonparenchymal cell types in the population, extended culture periods are usually unsuccessful because of an overgrowth of the nonhepatocytic cell elements. However, it has been shown that the overgrowth of fetal and neonatal rat hepatocyte cultures with mesenchymal or fibroblastic cells can be hindered by maintaining the cultures with an arginine-free medium (84). Nevertheless, as has been pointed out, the levels and patterns of drug-metabolizing activities of fetal and early neonatal hepatocytes are altered from those of the adult animal, though it is interesting that chick embryo and fetal and neonatal rat hepatocytes have been shown to acquire some adult cell functions when explanted to primary culture (1, 48, 108). The significance of this phenomenon will be discussed shortly.

The most promising hepatic culture systems are probably those of hepatocytes obtained from animals beyond the neonatal stage. The principal requirement for such cultures is the ability to prepare cell suspensions in high yield from a single liver. On the basis of the age of the animal and the expertise of the investigator, from 2 to 10 $\times 10^8$ cells, of which more than 90% are hepatocytes, may be obtained. Freshly isolated adult rat hepatocytes in short-term suspension culture (1 to 4 hours) offer a number of advantages over the whole animal and other systems such as the perfused liver, liver tissue slices, and liver subcellular fractions used to study xenobiotic metabolism. Unlike the whole animal, the perfused liver, and liver tissue slices, suspensions of freshly isolated hepatocytes are essentially free of other cell populations and their environment can be controlled more rigorously. Hepatocyte suspensions provide more versatility than the perfused liver system in that several variables can be measured simultaneously with cells prepared from a single liver. In addition, freshly isolated hepatocytes in suspension are not subjected to the kinds of substrate and nutrient diffusion problems encountered with liver tissue slices. Freshly isolated hepatocytes also more closely resemble the in vivo situation than do subcellular fractions prepared from whole liver since their structural and functional organization is essentially intact. However, the freshly prepared hepatocyte suspensions are short-lived, thus preventing their use in studies related to the regulation and long-term effects of drug and carcinogen metabolism. Another disadvantage of freshly isolated hepatocytes involves the leakage of enzymes and metabolites from the cell. Furthermore, it is not clear what effects the enzymatic perfusion and subsequent disruption of intercellular membrane relationships might have on the drug transport activities of the isolated hepatocytes.

The primary culture of adult mammalian hepatocytes offers the potential for longer-term studies of xenobiotic metabolism. Such cells can be cultured on suitable substrata for periods up to several weeks. However, in the basal media conditions presently used, the hepatocytes exhibit quantitative and qualitative changes in their metabolic machinery including the enzymes of drug metabolism with time in culture. In most instances the changes are in a negative direction with a resultant loss of function. The rapid decline in cytochrome P-450 of cultured rodent hepatocytes is a particularly dramatic example of this loss. Furthermore, while the morphology of the adult rat hepatocyte in primary culture resembles that seen in vivo, there is observed with time in culture a decrease in endoplasmic reticulum and increases in lipid droplets, microfilaments, and autophagic vacuoles in their cytoplasm (145, 158). The cultured hepatocytes also exhibit fetal gene expression with the appearance of functions normally found during the fetal and neonatal period (87, 158). In addition, hepatocytes derived either from normal or regenerating liver have lost the ability to enter into mitosis, although a significant percentage will, under suitable culture conditions, enter DNA synthesis. However, these cells do not progress through the G_2 phase of the cell cycle (64, 137, 158).

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The inability of adult hepatocytes to divide in culture is a decided disadvantage when considering the aim of obtaining a self-propagating system that may reflect the controls and transitional states of differentiated function shown by hepatocytes in vivo during the course of liver regeneration after partial hepatectomy. The achievement of a proliferating hepatocyte culture system would also provide potential advantages to the study of hepatocarcinogenesis in culture since it is apparent from in vivo studies that the production of liver neoplasms in experimental animals by chemical carcinogens is enhanced when they are administered during states of liver hyperplasia (125). On the other hand, it is also important to note that when adult hepatocytes go into repliction after partial hepatectomy, there is a marked decline in their mixed-function oxidase activity. This is one of the major problems with utilizing hepatocytes from regenerating adult rat liver, as well as from fetal liver, to study xenobiotic metabolism.

Despite these shortcomings, the promise of the greater maintenance of the differentiated state by suitable hormonal and other additions to the medium indicates that conditions will be found that will both preserve and extend the differentiated xenobiotic activities of hepatocytes in primary culture. Already it is possible in short-term primary cultures to maintain substantial drug-metabolizing activity by the inclusion of specific hormones and critical metabolites in the medium. These studies have indicated the usefulness of primary hepatocyte culture as a tool for elucidating factors that regulate the levels of cytochrome P-450 and other components of the mixed-function oxidase system of the hepatic cell in vivo.

The interesting phenomenon of "fetalization" of adult hepatocytes in culture as described by Sirica et al. (158) and Leffert et al. (87) has yet to be explained. In this regard, it is relevant that the properties of cytochrome P-450 of adult hepatocytes in late primary culture are more like those of the neonatal cell in vivo than that of the adult. Pitot and Sirica (126) have suggested that this effect is due to the lack of hormones and other critical regulatory substances in the culture medium, i.e. the hepatocytes revert to their fetal characteristics since the complex in vivo environment of the adult is required to maintain the highly differentiated biochemistry of the hepatic parenchymal cell. It is of interest, as noted earlier, that fetal and early neonatal hepatocytes in culture tend to acquire adult cell characteristics (1, 48, 108). Thus, it is possible that the hepatic cells tend towards some "equilibrium differentiated state" in environments that do not possess all of the hormonal and substrate characteristics of the in vivo situation. Whether this phenomenon can be explained in such simplistic terms or represents some other major mechanisms of differentiation remains to be determined. However, it is evident that many of the difficulties still remaining in the culture of adult hepatocytes and their maintenance in a fully differentiated state comparable to that seen in vivo are among the principal problems facing biology today. Their resolution will greatly increase our understanding of the mechanisms of drug metabolism, as well as other basic biological problems including the control of cell division, the transition from fetal to adult phenotype, and the mechanisms of regulation of genetic expression in highly differentiated mammalian cells. Future studies on the development of new substrata and media that more closely resemble the in vivo environment will likely contribute to the solving of many of these problems.

Finally, adult mammalian hepatocytes in short-term primary culture offer the potential for being a valuable in vitro screening system for chemical carcinogens. In this regard, the results already obtained to induce DNA repair in primary adult rat hepatocyte culture have been quite encouraging and, with further development and testing, it is probable that this system will provide a useful supplement to the Ames salmonella/mammalian microsome mutagenicity test for evaluating potential carcinogens in the environment.

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