Liver Cell Models in \textit{in Vitro} Toxicology

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\textit{In vitro} liver preparations are increasingly used for the study of hepatotoxicity of chemicals. In recent years their actual advantages and limitations have been better defined. The cell models, slices, and mainly primary hepatocyte cultures, appear to be the most powerful \textit{in vitro} systems, as liver-specific functions and responsiveness to inducers are retained either for a few days or several weeks depending on culture conditions. Maintenance of phase I and phase II xenobiotic metabolizing enzyme activities allows various chemical investigations to be performed, including determination of kinetic parameters, metabolic profile, interspecies comparison, inhibition and induction effects, and drug–drug interactions. \textit{In vitro} liver cell models also have various applications in toxicology: screening of cytotoxic and genotoxic compounds, evaluation of chemoprotective agents, and determination of characteristic liver lesions and associated biochemical mechanisms induced by toxic compounds. Extrapolation of the results to the in vivo situation remains a matter of debate. Presently, the most convincing applications of liver cell models are the studies on different aspects of metabolism and mechanisms of toxicity. For the future, there is a need for better culture conditions and differentiated hepatocyte cell lines to overcome the limited availability of human liver tissues. In addition, strategies for \textit{in vitro} analysis of potentially toxic chemicals must be better defined. — \textit{Environ Health Perspect 106}(Suppl 2):511–532 (1998). \url{http://ehpnet1.niehs.nih.gov/docs/1998/Suppl 2/511-532guillouzo/abstract.html}

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\section*{Introduction}

Because of its interposition between the digestive tract and the rest of the body, the liver occupies a central role in major functions of the organism. It receives large amounts of nutrients and noxious compounds entering the body through the digestive tract and portal vein. Among the major hepatic functions are uptake of amino acids, lipids, carbohydrates, and vitamins, and their subsequent storage, metabolic conversion, and release into the blood and bile. The liver is also the principal organ involved in the biotransformation of exogenous substances, with its capacity to convert hydrophobic substances into water-soluble products that can be secreted readily from the body. However, more than a thousand xenobiotics are potentially hepatotoxic. The capacity of a chemical to produce liver damage \textit{in vivo} often results from the interaction of a series of complex cellular processes that are involved in the uptake, biotransformation, and elimination of these potentially toxic compounds. Hepatotoxins may react with the basic cellular constituents—proteins, lipids, RNA, and DNA—and induce almost all types of lesions of the liver, most frequently steatosis. Whatever the agent responsible for injury, the reaction of the liver involves a common sequence of events that can be analyzed at the tissue, cellular, and molecular levels. However, it is difficult \textit{in vivo} to distinguish the primary effects of a compound from those induced secondarily because liver functions are under the influence of various endogenous and exogenous factors that result in complex interactions with other organs. Moreover, most of our understanding of liver injury induced by chemicals remains confined to animal models, and data obtained in animals cannot be extrapolated with certainty to the human situation. Because of the drawbacks of \textit{in vivo} studies of drug- and chemical-induced hepatotoxicity, \textit{in vitro} liver systems represent a better experimental approach to screen potential hepatotoxic compounds and investigate mechanism(s) by which chemicals induce liver lesions. The most frequently used isolated liver preparations include isolated perfused organ, tissue slices, subcellular fractions, and isolated and cultured hepatocytes. The latter model is the most popular system and is widely used for toxicity studies. In this review the advantages and limitations of \textit{in vitro} models in toxicity studies will be discussed, with special reference to the isolated hepatocyte model and future developmental needs.

\section*{In Vitro Liver Models}

Among the \textit{in vitro} liver preparations recognized as useful experimental models in toxicology, intact cellular systems, i.e., the isolated perfused organ, tissue slices, and the isolated hepatocyte, offer various advantages over other systems such as liver cell lines, subcellular fractions, and genetically engineered cells (Table 1). However, the isolated hepatocyte is the most widely used model.

\section*{Different \textit{in Vitro} Liver Preparations}

\textit{The Isolated Perfused Organ}. The isolated perfused liver represents the closest \textit{in vitro} model of the \textit{in vivo} situation. The major advantages are that the three-dimensional architecture is preserved and that the bile flow can be collected and analyzed separately. It is also the only \textit{in vitro} model that allows consideration of issues related to hemodynamics (1). This model has been used for investigating drug- and chemical-induced hepatotoxicity (2) and represents an interesting tool in studies of physiologically based toxicokinetic modeling. However, the isolated perfused liver model is difficult to handle and its functional integrity is not maintained beyond a few hours. Although more than one test compound was added to the perfused medium in some studies, and concentration–response relationships have
been established in a single organ (3), this model is not appropriate for the analysis of various experimental conditions from a single organ. In addition, relative to other in vitro models, it does not significantly reduce the number of animals used and human organs are not available.

Liver Slices. The tissue slice model also retains tissue organization and cell-cell and cell-matrix interactions. However, bile flow cannot be analyzed separately. For some time, tissue slices have had major limitations, primarily because of poor diffusion of oxygen and nutrients from the incubation medium. The development of a newly designed tissue slice resulted in renewed interest for this model. Liver slices of nearly identical dimensions (approximately 250 \( \mu \)m thick) can be prepared with minimal tissue trauma (4,5). This system appears appropriate for short-term studies on human liver, as tissue slices can be obtained from both liver fragments and needle biopsies. Histologic examination can be conducted. Recent studies have shown that tissue slices can remain metabolically active for 2 to 3 days (6,7). However, discrepancies exist in cell viability and functional capacity of tissue slices, probably related to the different experimental conditions. Moreover, some authors claim that slices can be cryopreserved. However, to our knowledge, viability of parenchymal cells after thawing has not been demonstrated.

Isolated Liver Cells. Isolation and Culture of Hepatocytes. Preparation of rat hepatocytes. A considerable step forward in obtaining intact adult rat hepatocytes was made by the introduction of collagenase and hyaluronidase as dissociating agents (8). The liver is quickly perfused with cold Ca\(^2+\)-free Hanks buffer containing the two enzymes, removed, cut in small pieces, and incubated with the enzymatic solution in a shaking waterbath. Berry and Friend (9) further improved the technique by perfusing the liver in situ with a Ca\(^2+\)-free Hanks buffer containing collagenase and hyaluronidase and obtained high cell yields and viability. This technique was modified and simplified by Seglen (10), who developed the two-step collagenase perfusion method now widely used. The first step involves the perfusion of a calcium-free buffer. The second step is circulation of a calcium-supplemented buffer containing collagenase. Hepes is the most frequently used buffer. The initial perfusion facilitates desmosomal cleavage and further dispersion of liver cells. The addition of Ca\(^{2+}\) to the enzyme solution ensures adequate collagenase activity.

The two-step collagenase perfusion method yields large numbers of viable parenchymal cells. About 40 to 60 \( \times \) \( 10^6 \) hepatocytes/g of tissue are usually obtained from a young rat liver; cell viability ranges between 85 and 95% as determined by the trypan blue exclusion test; the preparation contains less than 5% contaminating nonparenchymal cells. Organs can be hypothermically preserved for several hours before their disaggregation without marked loss of the viability of isolated cells (11).

Hepatocyte subpopulations can also be prepared. Perivenous or periportal hepatocytes can be obtained by selective destruction of the perportal or perivenous part of the microcirculation by digitonin perfusion before applying the basic collagenase method (12,13). Two whole livers must be used. Hepatocyte subpopulations are prepared from whole cell populations by counterflow centrifugation according to their degree of ploidy (14). Enriched hepatocyte couplet populations can also be obtained by slight modifications of the classical

| Model                     | Advantages                                                                 | Limitations                                                                 |
|---------------------------|-----------------------------------------------------------------------------|----------------------------------------------------------------------------|
| Isolated perfused liver   | Functions close to those of the \( \textit{in vivo} \) organ (all enzyme     | Short-term viability (2-3 hr)                                              |
|                           | equipment preserved)                                                        | Study of one or a few compounds only                                       |
|                           | Lobular structure preserved                                                  | Bile excretion decreased after 1 to 3 hr                                  |
|                           | Functional bile canaliculi                                                  | No study on human liver                                                   |
|                           | Collection of bile possible                                                 | Suitable only for liver of small animals                                  |
|                           | Short-term kinetic studies (extraction)                                     | No significant reduction in the number of animals used                    |
| Liver slices              | Lobular structure preserved (all enzyme equipment preserved)                | Viability: 6 hr to 2 days                                                 |
|                           | Selective intralobular effects detectable                                   | No collection of bile possible                                            |
|                           | Studies on human liver possible                                             | Not all the cells preserved similarly (interassay variability)            |
| Isolated hepatocytes*     | Functions close to those of \( \textit{in vivo} \) hepatocytes              | Short-term viability (2-4 hr)                                             |
|                           | Studies on several compounds at different concentrations                   | No bile canaliculus                                                      |
| Primary hepatocyte cultures* | Functions expressed for several days in certain conditions               | Early phenotypic changes                                                  |
|                           | Induction/inhibition of drug metabolizing enzymes                          | Altered bile canaliculi                                                  |
| Liver cell lines          | Unlited cell number                                                         | Various drug enzyme activities lost or decreased                          |
|                           | Some functions preserved                                                   | Genotype instability                                                      |
| Subcellular fractions     | Drug enzyme activities preserved                                           | Short-term studies                                                        |
| 38000 \( \times \) g fraction | Drug enzyme activities preserved                                      | Short-term studies                                                        |
| Microsomes                | Production of metabolites for structural analysis                          | No cytosolic phase II enzyme reactions                                    |
| Genetically engineered    | One or more human enzymes expressed                                         | Cofactors required for activity                                           |
| cells                     | Available mainly for CYPs                                                   |                                                                           |
|                           | Unlimited cell number                                                      | Use for specific purposes only                                           |

*Other cell types, e.g., Kupffer, endothelial, stellate, and bile duct cells, can also be isolated, cultured, and even cocultured with hepatocytes.
two-step collagenase perfusion, such as addition of trypsin inhibitor in the collagenase solution (15). The cell preparation contains approximately 30% couples; after counterflow elutriation a fraction containing 85% couples has been obtained (16). All these methods have been applied only to rodent hepatocytes.

The use of a crude collagenase preparation that also contains other proteolytic enzymes reportedly alters surface properties of parenchymal cells. To overcome this drawback some investigators have attempted to develop dissociation techniques without the use of collagenase. The enzyme is replaced by a chelating agent. Addition of 2 mM ethylenediaminetetraacetate (EDTA) to the collagenase-free perfusion medium allows viable hepatocytes to be obtained (17,18); however, the yield of hepatocytes by this method is lower and the generation of substantial numbers of damaged cells is unavoidable, making their removal by the use of a percoll gradient necessary (18). This method improves maintenance of functional capacities in cultured hepatocytes compared to the standard collagenase method (19), but conflicting data have been reported (20).

Preparation of hepatocytes from other species. The two-step collagenase perfusion protocol, first designed for rat liver, has been applied to the liver of various species, including human (21–23). Viable hepatocytes have been obtained from whole livers, lobe portions, or wedge biopsies. Isolated hepatocytes are usually prepared from whole livers when small animals are used, e.g., fish, frogs, birds, hamsters, and rabbits. Wedge biopsies are frequently used to obtain parenchymal cells from larger mammals such as dogs, monkeys, ruminants, and humans. However, even for the human liver, the dissociation of the whole organ has been described (24). If the basic collagenase protocol is used, appropriate modifications must be made with respect to cannula diameter, perfusate volumes, and flow rates. Comparative conditions for dissociation of various human samples have been recently summarized (25).

Characteristics of isolated hepatocytes. Freshly isolated hepatocytes retain responsiveness to signals to which they reacted prior to liver dissociation. However, some phenotypic changes have been observed just after tissue disruption. Freshly isolated rat hepatocytes reportedly express c-fos (26) and exhibit changes in abundances of various cytochrome P450 (CYP) transcripts (27). Moreover, conspicuous individual variations in cellular functions are frequently observed in human hepatocytes (28) (Table 2). In addition to age, sex, liver diseases, and genetic polymorphisms, premedication and the duration and conditions of liver preservation before cell disruption may greatly influence the metabolic activities of freshly isolated human hepatocytes (29,30). The conditions of organ preservation before dissociation are also critical. Vons et al. (31) reported that human livers stored for 7 ± 2 hr in cold University of Wisconsin solution (Dupont, Paris) gave lower cell yields than surgical liver biopsies when expressed per gram of tissue. Moreover, most of these cell populations attached poorly to plastic. Critical parameters are probably the length of hypothermic preservation and extent of washing with buffer before liver dissociation.

Survival of isolated hepatocytes in suspension is short, not exceeding a few hours, and the functional capacity is dependent on the composition of the incubation medium (32). However, when entrapped in alginate beads, isolated hepatocytes survive much longer. We have shown that under these conditions, rat hepatocytes incubated in a culture medium were capable of conjugating bilirubin for 24 hr or more (33).

Freshly isolated hepatocytes can also be transiently preserved by hypothermia or cryopreserved for months or years. When stored at 4°C either in the Leibovitz medium (Gibco Laboratories, Cergy-Pontoise, France) or in the University of Wisconsin solution (Dupont) (the most effective solution for cold organ preservation), rat hepatocytes survived for 1 or 2 days (34–36). However, survival and metabolic competence are better maintained in the Leibovitz medium. After 2 days of storage only the cells preserved in this medium are able to correctly attach to plastic and survive in culture (36).

Various procedures for cryopreservation of hepatocytes have been described; the importance of the nature and concentration of cryoprotectant, cooling rate, and conditions of thawing have been stressed (37–44). Parenchymal cells from various species can be frozen (40). However, cell viability is reduced and functions including drug metabolizing enzyme activities are more or less impaired (40,44). About 50% of hepatocytes that are able to attach and survive in culture when plated just after isolation still possess these properties after freezing in liquid nitrogen and thawing. The length of preservation is not a critical parameter. Whether cryopreserved for a few days or for 4 years, human hepatocytes were equally viable after thawing (40).

Among the species studied, dog hepatocytes appear to be the most sensitive cells to the freeze/thaw process (40,44). Among the most sensitive functions to the freeze/thaw process are cytosolic phase II enzyme activities and protein neosynthesis, which are not restored in thawed hepatocytes in pure culture. However, when thawed parenchymal cells are set up in coculture with rat primitive biliary cells, they recover levels of functional activities close to those found in fresh hepatocyte cocultures after a few days (45). A recent study has shown that when entrapped in alginate beads, hepatocytes exhibit only limited reduced cell viability and retain their functions at high levels (46).

The results with human hepatocytes deserve further comments. Indeed, although data are reproducible and similar to those

| Table 2. Drug metabolizing enzyme activities in human hepatocytes. |
|---------------------------------------------------------------|
| **Substrate (reaction)**                  | **Enzyme activity** | **Range** | **Mean** | **n** |
| Ethoxyresorufin-O-deethyl (cytochrome P450) | 0.2–8             | 3.0       | 19       |
| Phenacetin (cytochrome P450)               | 0.1–25            | 4.7       | 25       |
| Pentoxifylline (cytochrome P450)           | 0.1–5             | 0.7       | 19       |
| Methenylxanthine (cytochrome P450)        | 0.1–2             | 0.7       | 6        |
| Dextromethorphan (cytochrome P450)        | 0.1–2             | 0.5       | 6        |
| Nifedipine (cytochrome P450)              | 0.5–13            | 5.3       | 8        |
| Lactic acid (cytochrome P450)             | 0.3–2             | 0.8       | 7        |
| Paracetamol (cytochrome P450)             | 0.0–16            | 4.1       | 26       |
| Glucuron conjugation                      | 0.1–14            | 3.6       | 27       |
| Sulfoconjugation                          | 0.05–0.5          | 0.2       | 14       |
| 1-Chloro-2,4-dinitrobenzeno (cytochrome P450) | 0.1–7             | 1.1       | 32       |

Abbreviations: GSH, glutathione; n, number of tested cell populations from different donors. *Activities were measured 16 to 48 hr after hepatocyte seeding. †Micromoles metabolites formed per minute per milligram cellular protein. ‡Nanomoles metabolites formed per hour per milligram cellular protein. §Unit per milligram cellular protein. Data from Guillouzo and Chesnè (28).
obtained with animal hepatocytes when the cells are prepared from fresh, histologically normal liver pieces, they are much more variable with parenchymal cells isolated from pathologic and/or hypothermically preserved liver samples. Further studies are required to better define the critical conditions for correct cryopreservation of such cell populations. Some biochemical parameters probably should be measured and short-term incubation or culture of the cells before freezing will improve survival and function after thawing.

**Culture of isolated hepatocytes.** To survive for prolonged periods, liver parenchymal cells must be put in culture. However, major problems have been encountered with primary liver cell cultures; they are related to limited survival (not exceeding a few days) and the loss of liver-specific functions such as drug metabolizing enzymes (47,48). CYP concentration decreases approximately 50% during the first 24 to 48 hr in rodent hepatocytes (49–52), but the sum of the amounts of immunoreactive protein declines more slowly. Steward et al. (53) reported that the total spectral CYP measured in rat hepatocytes declined 68% during the first 3 days of culture in a serum-free medium, whereas the sum of the amounts of immunoreactive protein for seven CYP enzymes declined only 24% during the same period. These findings suggest that loss of heme rather than protein accounted for much of the observed CYP loss in cultured rat hepatocytes. CYP mRNAs also drop dramatically during the first days of culture (54,55). Several immunoblotting and Northern blotting analyses have shown that CYP enzymes are differently affected (55–58). Induction of specific CYPs by phenobarbital is difficult to achieve (59); several other CYPs, although decreased, remain capable of responding to inducers in vitro (53,56–58,60,61). However, the response can be different from that observed in vivo (62). Differential changes are also observed for uridine diphosphogluconate (UDP)-glucuronyltransferase forms (63) and glutathione S-transferase (GST) subunits (64–67). UDP-glucuronyltransferase activity is more stable than that of sulfotransferases (66). The loss of specific functions is associated with an early drop of transcription rates of liver-specific genes, e.g., albumin and transferrin, that represent only 1 to 10% of those found in the liver after 24 hr of culture (68,69).

Phenotypic changes with time in culture also include overexpression in adult rat hepatocytes of P-glycoprotein, the product of multidrug resistance gene(s), after 2 to 3 days of culture, making the cells much less sensitive to anticancer drugs. Indeed, this adenine triphosphate (ATP)-dependent drug efflux pump is functional, as shown by a parallel decrease in intracellular accumulation of doxorubicin and the marked effect of known blockers such as verapamil and cyclosporin (70).

Most *in vitro* studies on determination of drug metabolizing enzyme activities have been performed on rodent hepatocytes. However, major species differences have been observed, particularly when comparisons involve human hepatocytes. Indeed, many investigators have clearly demonstrated that functional alterations occur later and are more progressive in human hepatocytes than in their rodent counterparts (29,71–73). About 50% of the initial CYP content is still found after 1 week of culture (29,73), and all major CYPs (i.e., CYP1A, CYP2C, CYP2E, and CYP3A) remain well expressed and responsive to specific inducers (72). Specific inductions were demonstrated with rifampicin (72,74), 3-methylcholanthrene (72), omeprazole (75), and dihydralazine (76). As found in *vivo*, rifampicin controls expression of CYP3A at the transcriptional level (77). Phase II enzymes are also preserved in cultured human hepatocytes (73,78,79). However, an early decrease has been observed for GST activity, which was not followed by an increase as found in rat hepatocytes (79). Nevertheless, GSTs remain responsive to inducers. A specific induction of GST alpha-class transcripts by phenobarbital, 3-methylcholanthrene, and dithiolethiones has been reported in primary human hepatocyte cultures (80). P-Glycoprotein is only slightly overexpressed (81).

An additional particular feature of human hepatocytes is the frequent biphasic expression of some mRNAs. Some transcripts, particularly CYP mRNAs, show a conspicuous drop followed by an increase after 2 or 3 days of culture (72,82). This behavior could have some marked implications in metabolic capacity of the cells and supports the view that human hepatocytes can be used for various studies even after 2 or 3 days in primary culture.

Various functions are cell density dependent. Thus albumin secretion rate per cell is much higher when rat hepatocytes are plated at low density (83). In conventional culture conditions cell divisions, if any, are rare, and the cells do not survive for more than 1 to 2 weeks.

Hepatocyte subpopulations have also been investigated in primary culture. Tetraploid hepatocytes express higher levels of liver-specific functions than diploid cells, suggesting that all genes are functional (14). Periporal and perivenous hepatocytes retain their functional differences (84,85).

The reasons for the loss of liver-specific functions have been described and remain a matter of debate. The loss of a surface factor during collagenase preparation of isolated hepatocytes, as well as disruption of the extracellular matrix and cell–cell contacts have been proposed to be responsible for the early and conspicuous phenotypic changes. Recently Wright and Paine (20) evaluated the levels of CYP in both rat hepatocyte cultures prepared with the chelating agent EDTA to avoid proteolysis and in liver slices where both the extracellular matrix and cell–cell interactions are preserved. These authors found a loss of CYP in both systems, suggesting that there is no direct relationship between such functional alterations and the use of collagenase, the loss of cell–cell contacts, and/or the absence of an extracellular matrix (20).

Obviously, different events occur during the isolation process, cell seeding, and attachment that can all be considered critical steps. Overexpression of transcripts of immediate or early genes such as c-jun and c-fos and activation of nuclear factor kappa B are associated with cell isolation. Rapid degradation of particular mRNAs and transcriptional silencing of certain genes occurs rapidly after cell isolation. Both liver-specific (e.g., some CYPs) and housekeeping (e.g., copper–zinc superoxide dismutase) functions are involved. Other changes are related to cell attachment, which results in a redistribution of membrane receptors. A better knowledge of mechanisms involved in all these events is needed to improve *in vitro* survival and function of hepatocytes.

**Factors influencing hepatocyte survival and function.** A number of studies dealing with the definition of more suitable culture conditions have led to the conclusion that three groups of factors can affect cellular functions *in vitro*: soluble factors, extracellular matrix components, and cell–cell interactions (86,87).

**Exogenous soluble factors.** The composition of the culture medium has been widely explored (88–95) (Table 3). A variety of soluble factors, including hormones, growth factors, and trace elements, improve hepatocyte survival and function *in vitro* (87,95,96). In addition a number of nonphysiologic soluble factors are also effective; they include ligands such as metyrapone and ionicotinamide (90),...
Table 3. Soluble factors affecting survival and liver-specific functions of hepatocytes in primary cultures.

| Factors                  | Survival | Functions                                      | Reference                       |
|--------------------------|----------|------------------------------------------------|----------------------------------|
| Corticosteroids          | +        | Some functions are improved^b                  | Guguen-Guillouzo et al. (89)     |
| Insulin                  | +/-      | Less effective than with corticosteroids     | Laishes and Williams (89)        |
| Isonicotinamide          | -        | Maintenance of CYPs improved                  | Paine (90), Houe et al. (91)    |
| Metyrapone (acts as a ligand) | -       | Maintenance of CYPs improved                  | Paine (90)                      |
| Dimethylsulfoxide        | +++      | Many functions improved                       | Isom et al. (82)                |
| Nafenopin                | ++       | Production of plasma proteins improved (inhibition of apoptosis) | Bayly et al. (93)               |
| Pyruvate                 | ±        | Some functions improved                        | Tomita et al. (94)              |
| Iron-containing diferric transferrin | ±       | Functions improved^b                          | Block et al. (95)               |

^Increased survival: -, no; +, a few days; ++ to +++, a week to several weeks. *Only the most stable functions in vitro; e.g., albumin production, are improved.

drug inducers such as phenobarbital (97), and solvents such as dimethylsulfoxide (92), which is the most effective. However, dimethyl sulfoxide must be used at a concentration as high as 2% to give maximum effects (92).

Some complex serum-free hormonally defined media have been proposed for culturing hepatocytes (98), but they do not allow satisfactory maintenance of specific gene transcription and expression of differentiated functions beyond a short period. Only transitory improvement of plasma protein production rates (99) and drug metabolizing enzyme levels and response to inducers (100) has been reported in rat hepatocyte cultures.

In standard culture conditions i.e., in the absence of mitogenic factors, rat hepatocytes are blocked in the mid to late G1 phase at the restriction point (101). A number of studies have involved the identification of soluble factors that may stimulate DNA synthesis in hepatocytes plated at low densities (102,103). Two groups of humoral growth-promoting agents can be distinguished: the primary mitogenic factors and the comitogenic factors. Thus, epidermal growth factor requires insulin or angiotensin II as maximal stimulation of DNA synthesis. Serum factors such as hepatocytin B and the hepatocyte growth factor also stimulate hepatocyte proliferation. In addition various substances and culture conditions can modulate the response of hepatocytes to growth stimuli in vitro. They include energetic components of the basal medium, e.g., pyruvate (102), lactate (102), glucose (95), proline (104), and iron-containing diferric transferrin (104). When rat hepatocytes are grown in serum-free medium containing low Ca²⁺ concentration (0.4 mM) and epidermal growth factor, a high percentage go through one cycle of division and a few replicate twice (105). Nicotinamide appears to be very effective (91,106); the mechanism mediating these effects remains unclear (95). About 15% of rat hepatocytes undergo three rounds of cell division when maintained in a serum-free medium containing 10 mmol/liter nicotinamide and 10 ng/ml epidermal growth factor (106).

Several extracellular matrix substrates can also promote cell growth (95,107). Loyer et al. (101) showed that in the presence of mitogens extracellular matrix substrates enter S-phase 48 hr after seeding: maximum DNA synthesis takes place at 84 hr and the maximum of mitotic figures is observed at approximately 96 hr. Human hepatocytes also exhibit a proliferative activity when stimulated with epidermal growth hormone, hepatocyte growth hormone, or human serum (108-110).

**EXTRACELULAR MATRIX COMPONENTS.**

The influence of extracellular matrix on the maintenance of liver-specific functions has also been widely analyzed (111-115) (Table 4). A variety of forms of extracellular matrices were tested. The efficiency of attachment of hepatocytes is increased when the cells are cultured in plastic dishes coated with matrix proteins. In most cases hepatocyte spreading is also enhanced and is associated with the loss of specific functions (112,116,117).

Maintenance of differentiated functions is prolonged when the cells are cultured on matrigel or in a sandwich, two conditions that favor the preservation of the globular cell shape. The most convincing improvement has been obtained using matrigel, a laminin-rich gel matrix isolated from a mouse sarcoma (54,113). Various functions, including induction of CYP2B1 and CYP2B2 by phenobarbital, are maintained for several days (54,55). However, the significance of matrigel effects is difficult to interpret because contamination with proteins, hormones, and/or growth factors has been reported (118,119). Among the various factors identified were transforming growth factor beta, epidermal growth factor, insulin-like growth factor-I, bovine fibroblast growth factor, and platelet-derived growth factor (118).

Rat hepatocytes also maintain a high albumin secretion rate for more than 40 days and a more cuboidal shape when cultured between two layers of type I collagen (120). Recently Lee et al. (114) reported that the continuous presence of l-proline in the medium of sandwiched rat hepatocytes was necessary and concluded that continued synthesis of endogenous collagen by hepatocytes was critical. However, further work is required to determine the precise role of l-proline and the mechanism of interaction between hepatocytes and neosynthesized collagen. Whether the cells must retain their cuboidal shape to express differentiated functions has been recently questioned. Caron (121) reported that rat hepatocytes spread on type I collagen and when exposed to matrigel diluted in the culture medium they showed increased transcription of the albumin gene without induction of a cuboidal cell shape.

Table 4. Matrix proteins affecting survival and liver-specific functions of hepatocytes in primary culture.

| Protein                              | Survival | Functions                                      | Reference                  |
|--------------------------------------|----------|------------------------------------------------|----------------------------|
| Collagens, fibronectin as substrates | +        | Early decrease or loss                         | Johansson and Hook (111), Michalopoulos and Piotot (112) |
| Matrigel                             | ++       | Many functions well-preserved for at least a few days | Bissell et al. (113)          |
| Collagen gel sandwich                | +        | Functions improved^b                          | Lee et al. (114), Dunn et al. (120) |
| Collagen for immobilization          | +        | Functions improved^b                          | Koebe et al. (115)            |

^Increased survival: +, a few days; ++ to +++, 1 week to several weeks. *In these two systems, hepatocytes still form a monolayer; results for a limited number of functions e.g., albumin production.
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Recently, improved maintenance and inducibility of CYP enzymes were obtained in rat hepatocytes cultured on crude liver membrane fractions (122). Saad et al. (123) concluded that these results could be explained by the presence of appreciable amounts of matrix components (e.g., laminin and type IV collagen) in the crude membrane preparations (123).

Cell–cell interactions. Another approach is to create cell–cell interactions in vitro. Indeed, the liver is an organ composed of many cell types, and both homotypic and heterotypic interactions may influence liver-specific functions (Table 5). When placed in pure culture hepatocytes reaggregate, but intercellular communication through gap junctions disappears within a few hours. Although various functions are dependent on cell density, whether at low or high density, hepatocytes show comparable survival times and exhibit phenotypic changes in conventional culture conditions, indicating that homotypic interactions are not critical in the maintenance of specific functions in vitro (83).

The first attempts to coculture hepatocytes with other cell types such as sinusoidal cells (124) and human fibroblasts (125) showed only limited improvement of hepatocyte survival and function. A considerable step forward in long-term maintenance of differentiated hepatocytes was made by adding another liver epithelial cell type (126). When cocultured with rat liver epithelial cells probably derived from primitive biliary cells, hepatocytes from various species including humans survive for several weeks and retain various liver-specific functions; these include production of plasma proteins, expression of both phase I and phase II drug metabolizing enzymes, and taurocholate uptake (52,71,127–135). Cocultured hepatocytes remain able to transcribe specific genes at higher values (still representing 20 to 40% of initial values) than those measured in cells cultured in the presence of 2% dimethyl sulfoxide or on matrix proteins (69). The maintenance of differentiated functions is associated with early deposition of matrix proteins primarily between the two cell types (71,126,128) and with preservation of communication through gap junctions (136). These two features could be related because in pure hepatocyte cultures the addition of proteoglycans improves gap junction activity (137). The enhanced survival and function of hepatocytes cocultured with rat liver epithelial cells have been confirmed by many investigators (134,138–143); the induction of CYP2B1 and CYP2B2 by phenobarbital has also been demonstrated (137,144). Moreover, other cells have been effective, including both liver endothelial cells and nonhepatic cells (145–147). Studies from our laboratory (148,149) have shown that the presence of an integral plasma membrane glycoprotein is a prerequisite to obtain long-term survival of differentiated hepatocytes in coculture. This glycoprotein is expressed by both hepatocytes and rat liver epithelial cells and is also found in some other tissues (148,149). One could postulate that nonhepatic cells that express this glycoprotein are potentially capable of interacting with hepatocytes. However, it is important that these cells cease to divide when they form confluent cell monolayers with hepatocytes and that, like rat liver epithelial cells (69), they can be separated from parenchymal cells. As in conventional culture, the levels of functions in coculture are dependent on the composition of the nutrient medium (150). As a rule some qualitative and quantitative changes occur, and the reappearance of fetal-like functions is not totally prevented in coculture (65). Moreover, it is important to point out that our coculture system is corticosteroid dependent (151) and that not all liver epithelial cell lines are similarly efficient to stimulate expression of liver-specific functions in parenchymal cells. Therefore, it is critical to evaluate the influence of each liver epithelial cell line on hepatocyte function, e.g., by measuring albumin production in the cocultures over a 2-week period.

Specific functions are also better preserved in hepatocytes cultured in aggregates. These structures are obtained by seeding the cells into a dish so they cannot adhere to the surface but instead regaggregate with each other to form multilayer spheroids. Such three-dimensional cultures have been prepared from fetal (152), neonatal (153,154), and adult (155) hepatocytes. Dishes coated with poly(2-hydroxyethyl methacrylate) and positively charged primaria dishes are used for spheroid formation. Long-term secretion of albumin and transferrin (155) and deposition of matrix proteins (153) have been demonstrated. Spheroids frequently contain nonparenchymal cells, and it is not clear whether these cells are essential for long-term survival of hepatocytes. Compared to monolayer hepatocyte cocultures, spheroids suffer from some major limitations: Small aggregates tend to fuse to form larger aggregates, cells in the center of aggregates do not survive, the number of nonparenchymal cells and hepatocytes per aggregate cannot be precisely estimated, and the survival of hepatocytes is usually more limited (152–155).

In summary, although adult hepatocytes can express various liver-specific functions for several weeks when placed in sophisticated culture conditions, they do not fully mimic the in vivo situation. Some phenotypic changes occur early during the isolation process and after seeding, probably as a consequence of an adaptation of parenchymal cells to a new environment. However, even if they are lost rapidly after cell seeding, some functions remain inducible, as shown for sulfotransferase activities (67). Moreover, there is currently no experimental condition that allows normal hepatocytes to actively proliferate and therefore be subcultivated.

Table 5. Influence of cell–cell interactions on survival and liver-specific functions of hepatocytes in primary culture.

| Factors                        | Survivala | Functions                                                                 | Reference                                      |
|--------------------------------|-----------|---------------------------------------------------------------------------|------------------------------------------------|
| Homotypic factors (hepatocytes + hepatocytes) | −         | Various functions are related either positively or negatively to cell density | Guguen-Guilhouzo (83)                          |
| Heterotypic factors            |           |                                                                           |                                                |
| Hepatocytes + Kupffer cells    | −         | No improvement                                                           | Wanson et al. (124)                            |
| Hepatocytes + primitive biliary cells | ++       | A number of functions are well maintained at high levels                | Guguen-Guilhouzo et al. (126,128), Begue et al. (127) |
| Hepatocytes + liver endothelial cells | ++ or − | Limited improvement                                                       | Morin et al. (138)                            |
| Hepatocytes + 3T3 cells         | ++        | Various functions better preserved                                        | Mendoza-Figueroa et al. (145)                  |

aIncreased survival: −, no; +, a few days; ++ to ++++, 1 week to several weeks. b All the cells interacting with hepatocytes express a liver-specific regulating protein on their plasma membrane (148).
IN VITRO HEPATOTOXICITY

Isolation and Culture of Hepatic Nonparenchymal Cells. Kupffer and endothelial cells can be obtained by the collagenase–protease method and further separated by elutriation (156). Maintenance and propagation of liver endothelial cells in culture have been difficult, though to some extent successful (157).

Stellate cells are purified from nonparenchymal cells first isolated by the pronase–collagenase method, using a single-step density gradient centrifugation with Nicodenz (Nyegaard and Co., Oslo, Norway) at a final concentration of 11.4% (158). Freshly isolated stellate cells display a morphologic appearance similar to that observed in vivo. During the first 3 days after seeding, stellate cells appear homogenous and show characteristic morphology with lipid droplets surrounding the nucleus. During the following days they divide and transform into myofibroblastlike cells expressing desmin and iso-α-actin and producing increased amounts of collagen.

Both intrahepatic biliary and gallbladder epithelial cells can be isolated and cultured. Intrahepatic biliary cells have been obtained by immunoisolation with an antibody that recognizes a cell-surface glycoprotein localized only on these cells in the liver; they can be maintained for a short period using standard culture conditions (159). Gallbladder epithelial cells are obtained from gallbladder mucosa by collagenase treatment (160,161); they attach to various substrates and rapidly proliferate. Gallbladder biliary cells express specific markers such as γ-glutamyltranspeptidase and cytokeratins and maintain mucus secretion for at least 1 week in culture (162).

Liver Cell Lines. Characteristics of liver cell lines include unlimited subcultivation and cell availability in large numbers. Hepatocyte cell lines can be obtained from hepatomas or after transfection of normal hepatocytes with viral or cellular DNA. Various hepatoma cell lines have been established from rodent or human hepatomas; however, none of them express the full spectrum of drug metabolizing enzymes. Moreover, marked deviation of the phenotype and genotype usually occurs with time in culture. Similarly, none of the immortalized rodent and human hepatic cell lines established to the present have retained all tissue-specific functions and can be considered to reflect the in vivo situation.

Nonparenchymal cell lines have also been obtained. Thus, rat liver epithelial cells, probably derived from primitive biliary cells and isolated by trypsinisation of the liver of young rats, transform in vitro and can be cultured indefinitely. They express only a few hepatic functions; e.g., CYP2E1 and epoxide hydrolase activity (163).

Subcellular Fractions. Subcellular fractions, i.e., tissue homogenates, microsomes, and purified organelles such as mitochondria and nuclei, are also useful in studying mechanisms of drug- and chemical-induced hepatotoxicity. The liver 9000 x g supernatant fraction and microsomes fortified with cofactors have long been recognized as a good metabolizing system for activation of xenobiotics in in vitro mutagenicity testing procedures (164). Microsomes are also used to detect lipid peroxidation induced by hepatotoxins. Isolated mitochondria are used to study the effects of chemicals on oxidative phosphorylation, ATP synthesis, and β-oxidation of fatty acids. Isolated nuclei are used in run-on experiments for gene transcription analyses. Like perfused liver and isolated hepatocytes, subcellular fractions are functional only for a short period.

Genetically Engineered Cells. Yeasts, bacteria, insect cells, and nonhepatic mammalian cells have been transfected with cDNA-encoding human CYPs and express transiently or stably one or several human CYPs. They are useful to identify CYPs involved in the metabolism of chemicals, covalent binding, and drug inhibition studies (165). More recently, cells transfected with the cDNA for various phase II enzymes have also been obtained (166,167). An unlimited number of transfected cells can be prepared in an inexpensive way.

In Vitro Liver Models in Toxicity Testing

The evaluation of the safety of a chemical before human exposure represents a critical step. Chronic toxicity studies during the development of a new drug are time consuming and costly. They are usually performed on two animal species: a rodent and a nonrodent such as dog or monkey. Because marked interspecies differences frequently exist in both the rates and the routes of drug metabolism, particularly between humans and animals, toxicologic data obtained in two animal species cannot be extrapolated to the human situation without risk.

For these reasons as well as for ethical considerations, there is considerable interest in reducing animal usage and toxicity testing costs. Moreover, it is desirable to clearly establish human risk before human exposure to chemicals. This explains why in vitro approaches have received increased attention in recent years. Because the liver is the primary target organ, in vitro liver systems, especially the isolated hepatocyte model, have been widely used for toxicity testing. They have the potential to provide information about how chemicals are metabolized and how the liver can be adversely affected by a toxic agent. This implies that in vitro liver preparations are metabolically competent and produce the same metabolites as in vivo. Isolated hepatocytes, either in suspension or in primary culture, are used for screening evaluation of different classes of chemicals; cytotoxicity can be assessed by nonspecific and/or liver-specific end points. However, the most frequent use of these models is for mechanistic studies of the action of hepatotoxins.

Cytotoxicity Measures

A wide array of morphologic and biochemical markers are available for obtaining information at the cellular and molecular levels to detect and measure chemical-induced perturbations (168). Several classifications can be proposed: e.g., parameters that estimate plasma membrane integrity and parameters that estimate cellular effects; parameters that assess irreversible cytotoxicity (cell death) and reflect reversible changes; or nonspecific and liver-specific end points.

Nonspecific End Points. Nonspecific end points are usually used to estimate irreversible cellular damage. The choice of cytotoxicity indicator depends on a number of factors that include correspondence with the lesion being induced in vivo, reliability, reproducibility and sensitivity, convenience, cost, and species specificity. Nonspecific criteria may monitor morphologic changes, membrane integrity, subcellular perturbations, and metabolic activities. Light microscopic examination is useful to support biochemical end points; cell rounding, cell detachment, blebbing indicative of cell membrane and cytoskeleton disturbance, vacuolization, and accumulation of lipid droplets can be visualized, whereas transmission electron microscopy is required to detect subtle alterations of organelles.

End points for in vitro cytotoxicity evaluation include the following: Morphologic parameters

• Light microscopy: cell shape, nuclear and cytoplasmic alterations; accumulation of vacuoles; lipid droplets, formation of blebs; cell attachment and detachment (cell count)
• Electron microscopy: alterations of organelles
Biochemical parameters
- Plasma membrane integrity: protein or DNA content; trypan blue exclusion test; enzyme leakage (lactate dehydrogenase [LDH], transaminases); release of $^{51}$Cr neutral red uptake
- Alterations of cell permeability: leakage of ions and small molecules ($K^+$ concentration; $Ca^{2+}$ influx); accumulation of exogenous molecules (sucinate, etc.)
- Metabolic parameters: 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) test (reduction of a formazan salt); ATP content; protein neosynthesis; lactate/pyruvate ratio; glutathione (GSH) content; lipid peroxidation; covalent binding

**PLASMA MEMBRANE DISTURBANCES. Loss of plasma membrane integrity.** Assays for loss of membrane integrity are indicators of irreversible cell injury; they include cell count (percentage of attached cells), trypan blue exclusion, and cytosolic enzyme leakage.

Cell death (necrosis) is the consequence of loss of plasma membrane integrity assessed by trypan blue uptake and leakage of cytosolic enzymes. The trypan blue exclusion test is widely used to estimate the percentage of viable cells in isolated hepatocyte suspensions. Several enzymes are used for monitoring cell viability. The extracellular LDH to total LDH ratio correlates better than that of other enzymes with morphologic changes observed by phase-contrast microscopy and cell viability assessed by trypan blue exclusion (169,170). This greater sensitivity of LDH can be explained by the intracellular compartmentalization of transaminases; thus 80 to 85% of aspartate aminotransferase is found in mitochondria. However, LDH is not a constant reliable indicator of loss of plasma membrane integrity and cell death; it is a less reliable and less sensitive parameter for human hepatocytes than for their rodent counterparts (171,172). Reliable values are obtained by the determination of intracellular LDH (172), an index also used to count viable hepatocytes (171,173). However, in some cases, it could be advisable to also determine if viable cells have detached.

The release of $^{51}$Cr is another end point of cell death; it measures membrane integrity. This index has some limitations because the cells must be preloaded with the isotope and $^{51}$Cr is not entirely released by lysed cells.

Most of the tests used to estimate irreversible cellular damage can be conducted in 96-well plates with 30,000 cells/well. A number of tests used to evaluate cell density in the cultures are now miniaturized and automated (174). Dose–response curves are calculated from the results obtained, then the results are summarized as median inhibition concentration (IC$_{50}$) values; i.e., the concentrations of test chemicals that modify the response of test wells by 50% (175). IC$_{50}$ values are sometimes used, as they are considered the lowest concentrations causing statistically significant cytotoxic effects (176). However, the IC$_{50}$ value is usually considered the best indicator of in vitro cytotoxicity because it is taken from the middle of the dose–response curve (176).

Another index, i.e., the maximum non-toxic concentration over a given incubation period, can also be calculated and is useful for designing long-term toxicity studies with repeated additions of the chemical (177). The most widely used tests quantitate some cellular components such as DNA and proteins or estimate the number of viable cells (e.g., by the reduction of MTT by mitochondrial succinate dehydrogenases or the uptake of neutral red in lysosomes, the latter being frequently more sensitive).

**Reversible plasma membrane alterations.** Other indices of toxicity may reflect reversible injury. Thus increased permeability of plasma membranes to ions and small molecules precedes total loss of cell viability. A loss of intracellular $K^+$ and alteration of $Ca^{2+}$ ion flux are usually observed. Changes in O$_2$ consumption estimated after addition of exogenous succinate also precede irreversible injury.

**SUBCELLULAR CHANGES.** With the exception of direct action of a toxic compound on the cellular membrane, subcellular changes also occur prior to loss of plasma membrane integrity. Subcellular changes can be visualized by functional changes and electron microscopic examination. The functional indicators are based on evaluation of energy conservation, e.g., ATP content and metabolic competence, e.g., lactate/pyruvate ratio, and protein synthesis. Both secreted and intracellular neosynthesized proteins can be estimated. Metabolic disturbances must be significant and reproducible to be taken into consideration. They often far precede irreversible cellular injury. Whether the effects are reversible is also an important question that is rarely answered (178).

**OTHER INDICATORS OF REVERSIBLE CELULAR DISTURBANCES.** Other indicators of reversible cellular disturbances exist that indicate a potential for injury without insight into particular site(s). The most frequently used indicators that fit into this category are GSH content, lipid peroxidation, and covalent binding of reactive compounds to cellular macromolecules.

**Glutathione levels.** The tripeptide GSH is the most efficient protection of the cells against reactive metabolites and free radicals. GSH is an essential cofactor for GSH peroxidase and removes hydroperoxides; it is converted to its oxidized form (GSGG). GSH is also involved in the conjugation and inactivation by GST, of reactive metabolites formed by oxidation reactions. Intracellular GSH levels are usually measured by a fluorimetric method. GSGS is toxic to the intracellular medium. It is converted back to GSH by the GSH reductase pathway. The most common pathway of GSH depletion in drug toxicity is excessive consumption of GST without recovery. One of the consequences of GSH depletion can be the modification of the sulphhydril groups to arylating and oxidizing species (179). Several xenobiotics induce an oxidative stress that results in $S$-thiolation of proteins, presumably by thiol–disulphate exchange (180). Two mechanisms of detoxification of proteins have been identified, both ultimately requiring GSH and enzymatic reduction by reduced nicotinamide adenine dinucleotide phosphate: one involves GSH and GSH reductase and the other involves thioredoxin and thioredoxin reductase, a thiol reductant showing a broad specificity for reduction of disulfides (180).

GSH is also present in mitochondria; it prevents the effects of oxidants generated during oxidative phosphorylation on sensitive thiol groups (181). Mitochondria rely on GSH peroxidase, and hence on GSH, to detoxify hydroperoxides.

Both GSH and GSGS can be measured enzymatically or detected by high-performance liquid chromatography, but both methods lack sensitivity. Consequently, intracellular GSH levels are usually measured by a fluorimetric method (182).

**Lipid peroxidation.** Lipid peroxidation results from interaction of unsaturated lipid components with oxygen free radicals or excess H$_2$O$_2$ generated by toxic compounds during metabolism. Various analytical methods are used to measure lipid peroxidation products in vitro; they are based on the detection of intermediates, reactants, and end products of lipid peroxidation. The methods include measurement of malondialdehyde formation using the thiobarbituric acid reaction, spectrophotometric detection of lipid-conjugated...
dienes, measurement of evolved gaseous hydrocarbons (ethane and pentane), quantification of fluorescent pigments, and chemiluminescence (183). Two widely used markers of lipid peroxidation are extracellular free malondialdehyde formation (although it appears to be related to only 10% of the total lipids oxidized estimated on the ultrafiltrate of culture medium by size-exclusion chromatography) and conjugated dienes evaluated by the second-derivative ultraviolet spectroscopy of the cell lipid extract (184). A promising method is the detection of free radicals by spin trapping in whole cells by electron paramagnetic resonance (185).

Covalent binding. Covalent binding of reactive compounds to cellular macromolecules has been considered to be an important mechanism of hepatotoxicity by xenobiotics (186). However, cellular targets (mainly proteins) and their intracellular location remain obscure and the significance of covalent binding of reactive chemicals to proteins in the appearance of cell death is not obvious. Moreover, it has been reported that hepatocytes treated with acetaminophen, covalent binding ascribed to denatured cell material is about twice the level of binding measured in viable cells (187), making the use of appropriate controls essential and making overestimation of covalent binding likely when a fraction of treated cells is irreversibly damaged.

Liver-Specific Criteria. A number of markers representative of the various liver-specific functions can also serve as parameters of functional disturbances in isolated hepatocytes exposed to xenobiotics. The most widely tested markers include synthesis of liver-specific plasma proteins such as albumin and transferrin and acute-phase proteins, glyconeogenesis, and glycogen synthesis (188); urea synthesis (189); lipoprotein synthesis; induction or inhibition of specific CYPs (190); and bile acid secretion.

Various specific alterations of organelles can also be observed in liver parenchymal cells by electron microscopic examination, e.g., proliferation of smooth endoplasmic reticulum, increased number of peroxisomes associated with induction of the peroxisomal β-oxidation pathway (191), and characteristic accumulation of concentric membranes in lysosomes.

These criteria are usually much more sensitive than end points of the plasma membrane integrity; they compare to criteria for metabolic competence. However, with a few compounds major differences can be observed. Thus, cycloheximide inhibits protein synthesis but not urea synthesis, whereas norvaline does the opposite (192).

Choice of Experimental Conditions

Experimental conditions must be carefully defined; they can greatly affect the results. Among the major parameters to be considered are the functional capacity of the cells, composition of the medium, the physicochemical properties of the test compound, duration of treatment, and choice of end points.

Retention of Xenobiotic Metabolic Capacity by Isolated Hepatocytes. A number of compounds are hepatotoxic only after biotransformation. The production of reactive metabolites is dependent on a balance of activation and inactivation pathways; therefore, it is critical to demonstrate that isolated hepatocytes are capable of producing the same metabolites as those formed in vivo. The parent xenobiotic and metabolites can be measured in both media and cell extracts and their intracellular covalently bound percentage can be estimated.

A number of studies have shown that hepatocytes retain their ability to generate metabolites identical to those found in vivo. Consequently, this model is suitable for demonstrating interspecies variability and potential drug interactions (127,193–202). The results with human cells were either close to or quite different from those obtained with hepatocytes from the most frequently used animal species.

A good in vivo/in vitro qualitative correlation for metabolites formed by both phases I and phase II pathways is usually found. However, various parameters such as chemical concentration and the duration of the incubation with the chemical may greatly affect the metabolic profile. Some drugs are rapidly and actively metabolized whereas others are transformed much more slowly. Conjugated metabolites can be detected only after 6 to 8 hr of incubation or more (127). In a recent study we found that minor metabolites were detected only after two or three daily exposures to the compounds (203). The choice of drug concentrations to be tested is also important. Some metabolites can be missed if the concentration is too low; if the concentration is too high cellular toxicity can result. It is appropriate to test at least two concentrations, with the highest close to the maximum nontoxic concentration. However, for some drugs, in vitro biotransformation is low and does not reflect the in vivo situation whatever culture conditions are used (204). Such striking quantitative differences are sometimes minimized when metabolites are expressed per cell, as shown by Berthou et al. (204) for caffeine. In addition, the metabolic profile obtained with hepatocytes can be quite different from a metabolic profile obtained from a given compartment in vivo (e.g., urine or plasma) and it misses metabolism in nonhepatic tissues in vivo. In vivo/in vitro quantitative differences can be much more pronounced in one species compared to another. The new drug S-3341 was poorly metabolized both in humans and in primary human hepatocyte cultures (4 and 20%, respectively), but was much more biotransformed in vitro than in vivo in the rat (63 vs 20%) (205).

There is evidence for stereoselective and competitive metabolism of drugs, and the implications on the induction of liver cell damage should be considered. Le Corre et al. (206) have analyzed stereoselective metabolism of disopyramide in human hepatocyte cultures. The metabolic rate of S(+)/R(-) disopyramide was 2.5-fold higher than that of R(-) disopyramide. The stereoselective index S(+)/R(-) was similar to that found in vivo (1.51 ± 0.11 vs 1.55 ± 0.10). Hepatocyte cultures also represent a powerful system to demonstrate or predict drug interactions (200,207). Exposure of human hepatocytes to various concentrations of erythromycin estolate results in a dose-dependent inhibition of cyclosporine metabolism; both compounds are transformed by CYP3A4. Erythromycin estolate that is hepatotoxic in vivo forms stable complexes with CYP3A both in vivo and in vitro (208,209).

The capacity of hepatocytes to form metabolites decreases with time in culture; as expected from measurement of drug metabolizing enzymes, it drops much earlier in rodent hepatocytes than in their human counterparts (198). For example, no change in the metabolic rate and percentage of the two major metabolites formed from ketotifen was observed in human hepatocytes during the first 4 days of culture; the metabolic rate markedly decreased in rat hepatocyte cultures between day 2 and day 4.

The use of more sophisticated culture conditions is required for prolonged maintenance of drug metabolism activity in cultured hepatocytes; this is of particular importance when the cells must be induced in vitro before addition of the test drug. By using the coculture system, i.e., hepatocytes mixed with rat liver epithelial cells, Villa et al. (210) demonstrated that erythromycin estolate induced CYP and formed inactive complex with the iron of
the induced CYP in rat hepatocytes. In cocultured human hepatocytes, caffeine and theophylline metabolism were increased 5.8 ± 2.3- and 3.3 ± 1.1-fold, respectively, after a 3-day treatment with 3'-methylcholanthrene (211). Glucuronidation of ketotifen was still demonstrated after 3 weeks of coculture, whereas no conjugate was detected in 6 days pure culture (127). Huge interindividual variations are frequently observed in drug metabolic profiles in human hepatocyte cultures, making it critical to test at least two different cell populations and evaluate functional capacity of each cell population by measuring various phase I and phase II enzyme activities. Such a metabolic analysis may determine whether the cells that are being used in vitro are from an individual with particular metabolic characteristics related to factors such as genetic polymorphism, for instance, or to environmental factors (e.g., heavy smokers, drug abusers, etc.).

Composition of the Medium. The composition of the culture medium may greatly affect the response of isolated hepatocytes to cytotoxic agents. Cytotoxicity of sodium salicylate to cultured rat hepatocytes is dependent on the concentration of bovine serum albumin in the medium (212). Various compounds bind proteins. Villa et al. (190) found that the toxicity of erythromycin estolate (known to bind proteins) in rat hepatocytes increased in a medium deprived of proteins. The addition of calcium to the medium has been a matter of debate. Indeed, extracellular Ca2+ has paradoxical beneficial and detrimental effects to isolated hepatocytes exposed to toxic compounds. Calcium chloride in the incubation medium (3.6 mM) enhanced carbon tetrachloride (CCl4) toxicity in rat hepatocytes (213-216) and it was hypothesized that calcium entry into the cell may be involved in liver cell damage. In contrast, Smith et al. (216) report that several cytotoxic agents caused less injury to cultured hepatocytes in the presence of 2.6 mM calcium than under calcium-free conditions. Edmonson and Bang (217) demonstrated that in the absence of extracellular calcium, isolated hepatocytes rapidly release LDH and fail to accumulate γ-aminobutyric acid. Stacey and Klaassen (218) reported that isolated hepatocytes exposed to cadmium and copper amphoterins show no change in viability with the addition of 1 mM calcium chloride; however, omission of Ca2+ reversed cytotoxicity of the calcium ionophore A23187. Recent evidence shows that in many chemical toxicities an increase in intracellular free calcium can be dissociated from the toxicity generated by that agent (219,220), supporting the idea that the rise in intracellular calcium is a consequence of cell membrane injury. By using imaging fluorescence microscopy, the temporal relationships between changes in Ca2+ and cell death were evaluated in individual cells. Sakaida et al. (219) showed that oxidative stress produced by tert-butyl hydroperoxide induced three distinct phases of increased cytosolic-free calcium concentration that occurred somewhat asynchronously in primary rat hepatocyte cultures. Harman et al. (220) reported that in acetaminophen-treated mouse hepatocytes, intracellular calcium concentration increased well after the appearance of cell surface blebs and shortly before cell death.

Drug Incubation Conditions. When evaluating the toxicity of a test chemical to isolated hepatocytes, the investigator must be aware that a number of problems regarding chemical incubation conditions affect the quality and accuracy of the results. Before starting the experiment on hepatocytes a number of questions must be addressed: What solvent can be used to dissolve the compound? Is the compound volatile? Are reactions occurring between the compound and the medium that will affect pH, osmosality, or turbidity? Will reactions result in the formation of precipitates?

Duration of Treatment. The duration of incubation with chemicals can be a critical parameter because of the lack of extrahepatic elimination of toxic compounds and the accumulation of compounds in the culture medium. Paine and Hockin (221) studied the effects of three groups of toxins on cultured rat hepatocytes maintained in conditions that resulted in either a low or a high concentration of CYP. As predicted, toxins that are not metabolized by CYP, i.e., ethionine and galactosamine, were equally toxic regardless of the CYP concentration. In contrast, the two other groups of toxins had nonpredictable behavior. Indeed, compounds that are activated by CYP (CCl4, bromobenzene, cyclophosphamide, paracetamol) and those that are detoxified by CYP (pentobarbital, strychnine) were also equally toxic regardless of the CYP content of the cells after a 24-hr incubation. Only when the exposure was limited to 1 to 4 hr and followed by a 20-hr incubation in a toxin-free medium was CCl4 more toxic to cultured hepatocytes containing high levels of CYP.

Short-term assays, however, do not reflect the common in vivo situation in which drugs often produce liver damage after repeated administration for weeks or months. For long-term toxicity, in vitro models in which hepatocytes retain their metabolic activities are required. Cocultures of hepatocytes with rat liver epithelial cells offer an attractive approach for estimating chronic toxicity of chemicals (177,205). Various drug concentrations can be tested beginning when cell confluency is reached and with daily renewal. The highest concentration used is the maximum nontoxic concentration, defined after a 24-hr incubation of the compound with pure hepatocyte cultures. Depending on the chemical tested, four situations can be observed: no cytotoxicity to both cell types is found; only hepatocytes are damaged; only rat liver epithelial cells are damaged; and both cell types are sensitive to the chemical. As in acute toxicity testing, major differences in the sensitivity of end points can be observed (177). Because rat liver epithelial cells used in coculture do not metabolize drugs, comparisons of the effects of similar drug concentrations on pure cultures of these cells make it possible to determine whether a drug requires biotransformation to be cytotoxic. The coculture model thus has a double interest: it allows the detection of a cumulative chemical-induced toxic effect as well as the ability to distinguish between compounds that require biotransformation to induce cellular injury. Thus amitryptiline, but not clonidine, exerted a cumulative toxic effect in rat hepatocyte cocultures (177).

Choice of End Points. A single end point is usually sufficient to estimate irreversible injury induced by toxic compounds. For example, comparable results were obtained with primary rat hepatocyte cultures exposed to various compounds by measuring extracellular and total LDH ratio, total cellular protein content, MTT assay, and neutral red uptake (174). When metabolic competence is evaluated, IC50 values can be much lower than those calculated from parameters of plasma membrane integrity and false negative compounds have been reported. The advantage of criteria reflecting both irreversible cellular damage and altered metabolic competence is that they allow determination of metabolic disturbances with concentrations of the toxic agent much lower than those required for cell death.

Detection of Cytotoxic and Genotoxic Compounds

Comparative Screening of Cytotoxic Compounds. Isolated hepatocytes, either in suspension or in culture, represent a
unique experimental approach to screening various compounds at several concentrations using different criteria on the same cell suspension. However, they are not suitable for screening idiosyncratic toxins having a hypersensitivity origin because these involve an immune reaction.

Only short-term studies not exceeding 2 to 4 hr can be performed with suspensions of freshly isolated parenchymal cells. Moreover, it must be considered that the cells are altered, primarily because of the use of collagenase and mechanical agitation. Among the altered functions are lower 5'-nucleotidase and alkaline phosphatase activities and response to hormones. Consequently, it is probably more effective to allow hepatocytes to recover from the trauma of isolation and regain metabolic properties. However, one has to face major changes affecting various phase I and phase II enzymes after a few hours of culture. This explains why most studies on attached parenchymal cells have been conducted over a short period of time not exceeding 24 hr and with the addition of a single dose of the test chemical. Comparison of the data obtained with nonhepatic cells such as HeLa cells indicates that hepatocytes are usually more sensitive to compounds that require metabolic activation (222). Normal hepatocytes also are more sensitive than hepatoma cells, which exhibit lower drug metabolizing enzyme activities (174). Species differences in the sensitivity of the cells are also frequently observed.

The suitability of isolated hepatocytes as a screening procedure early in drug development is more convincing when structurally related compounds are tested, as we have shown for neuroleptic agents (170) and macrolide antibiotics (190). Chemicals associated with hepatotoxicity in humans are usually more cytotoxic to rat or human hepatocytes in vitro than nonhepatocytotoxic compounds, particularly those that require hepatic biotransformation to exert cellular damage.

It is obvious that the utility of in vitro systems depends on confidence in extrapolating data to the in vivo situation. For such comparisons the most useful parameters are those that assess end point toxicity for which in vivo data (histology, elevated serum enzyme levels, median lethal dose [LD50]) are most readily available.

Several in vitro toxicity studies on cultured hepatocytes have been designed to establish comparisons with in vivo hepatotoxic responses. In general, the in vitro/in vivo correspondence in discriminating between hepatotoxicants and nonhepatotoxicants is good. Story et al. (169) tested 34 chemicals for cytotoxicity in cultured rat hepatocytes by measuring transaminase release after 2 and 5 hr of exposure. A good correlation between in vitro cytotoxicity and in vivo hepatotoxic response was found. Tyson et al. (223) investigated 23 chemicals and found good correspondence for all but two (thioacetamide and allyl alcohol) on the basis of enzyme release. Inconsistencies can be related to the nature of the chemical tested, the incubation condition, and the end point tested. Indeed, a simple protocol for in vitro cytotoxicity testing cannot be used for all kinds of compounds. Fautrel et al. (174) tested 30 coded liquid or coded solid compounds on primary rat hepatocyte cultures using 96-well plates and did not find a good in vitro/in vivo correlation for all compounds tested. Obviously in agreement with other works, their study indicates that volatile and liposoluble compounds require appropriate culture conditions. Tyson et al. (224) found that relative hepatotoxic potentials for five haloalkanes measured in hepatocyte suspensions and in vitro were ranked correctly if partition coefficients were factored into the data to reconcile the differences between solvent volatility and retention in the two systems. Obviously more information is needed on compounds for which no correlation is found between in vivo and in vitro data. This will be of great help in designing more suitable in vitro tests and for our knowledge about their limitations.

IC50 values have been compared with in vivo lethal doses. In a multicenter study we compared neutral red uptake IC50 values obtained with primary rat hepatocyte cultures and oral, ip, or iv LD50 values. A significant correlation was found only between IC50 and iv LD50 values (225). Similarly, Jover et al. (176) found a good correlation between MIT IC50 on cultured human hepatocytes and human acute lethal blood concentration for 10 chemicals.

Another important question is the correlation between in vitro toxic concentrations and the in vivo toxic doses. Such a correlation must take into account the pharmacokinetics of the drug; it remains an open question that requires further investigation.

Screening of Genotoxic Compounds. In vitro liver models have long been used to assess the genotoxic potential of chemical and physical agents on the basis of their ability to covalently bind to and damage DNA, induce DNA repair responses, mutate specific genetic loci, induce phenotypically altered cells, or induce chromosome or chromatid-level aberrations (226–228). Metabolism of chemicals is frequently a necessary prerequisite.

Because they retain the critical activation/inactivation balance, isolated hepatocytes are well suited for quantitation of DNA repair elicited by exposure to genotoxic agents of a wide variety of structural classes. Only a few chemicals, including cimetidine (229), p-naphthylamine, 2,6-diaminotoluene, and 5-methylchrysene (230), give a qualitatively different response between the human and rat hepatocytes. In the last study, 25 compounds were tested; compounds found to be genotoxic in vitro were negative in vitro in only one case out of eight. Extensive validation has been performed with rat hepatocytes (231) and the hepatocyte primary culture/DNA repair test is now widely accepted by testing and regulatory agencies. Because the unscheduled DNA synthesis (UDS) assay requires small numbers of cells, it represents the suitable method to test genotoxic compounds on human hepatocytes (230). Various chemicals induce UDS in human hepatocytes; they include nitrosamines, 2-acetylaminofluorene, benzo[a]pyrene, aflatoxins, and dinitropyrenes. Aflatoxin B1, a suspected human hepatocarcinogen, induces a UDS response at or below 1 μM. Surprisingly, although the UDS response is usually similar in human and rat hepatocytes (232), marked differences are found when human hepatocytes are compared to hepatocytes of other animal species (233).

Because clonal growth of hepatocytes remains unsuccessful, direct mutagenesis assays with hepatocytes are not possible. To obviate this difficulty, hepatocytes can be cocultured with actively proliferating cells such as human fibroblasts (234) or V-79 Chinese hamster fibroblasts (235). Moreover, the low growth capacity of primary hepatocytes makes their wide use for assays such as the micronucleus and sister chromatid exchange questionable.

The UDS test with isolated hepatocytes is becoming more significant because it is frequently a component of test batteries. Intralaboratory and interlaboratory reproducibility is good; however, this test detects DNA alterations, not mutations.

Evaluation of Hepatoprotective Agents

Various antioxidants and scavengers of free radicals have been tested on in vitro liver preparations and act as in vivo. During the last few years, new naturally occurring
substances of plant origin and from synthetic compounds have also been evaluated. These studies have led to the selection of the most active molecules and have shown that the beneficial effects could be dependent on the toxin. Moreover, structure–activity correlations have been sometimes established and a few molecules reportedly have their own toxicity (236,237).

Their effects may depend on the species studied and the duration of treatment. (+)-Cyanidanol-3 was effective on rat hepatocytes intoxicated by aflatoxin B1 when added at least 5 min before the mycotoxin, but was without effect on human hepatocytes (236). The significance of in vivo and in vitro experimental data to the human situation may be questioned. Potential antidotes must be added before or just after toxin administration to be effective. Such conditions do not usually mimic the human situation. Repeated administration of the hepatoprotective agent is a more reasonable approach to obtain improvements in patients suffering from chronic liver diseases, as expected with malatolite. This synthetic compound, which reportedly protected rats intoxicated with CCl4, was without effect on both rat and human hepatocytes even after repeated treatment in vitro (238) and on patients with primary biliary cirrhosis as shown by a multicenter study (239).

Primary hepatocytes of human and rodent origin have also been used recently to analyze the mechanism of action of important classes of chemoprotective agents that modulate metabolic processing of carcinogens, such as diethiolethiones, isothiocyanates, and flavones. By using primary human hepatocytes, we have shown that oltipraz, a synthetic derivative of 1,2-dithione-3-thione, and sulforaphane, an isothiocyanate found in broccoli, are both transient inhibitors of CYPs and inducers of GSTs (240,241). However, not all GST forms are equally induced. Thus oltipraz mainly induces GST alpha forms that are not involved in conjugation of GSH to the carcinogenic exo-8,9-epoxide metabolite of aflatoxin B1 (240).

**In Vitro Studies on Characteristic Liver Lesions and Associated Biochemical Mechanisms Induced by Toxic Compounds**

Most in vitro investigations on toxic compounds have been directed to the reproducibility of liver-specific lesions and biochemical changes associated with the lesions.

**Characteristic Liver Lesions.** A number of investigations have tested in vitro liver cell models for their ability to reproduce steatosis and cholestasis and induce specific organelle injury.

Isolated hepatocytes reproduce in vivo steatosis. Impairment of fatty acid metabolism and protein synthesis has been reported in rat hepatocyte suspensions exposed to drugs known to produce steatosis in vivo, e.g., tetracycline (242) and methotrexate (243).

Isolated hepatocytes in suspension, hepatocyte couplets, and primary hepatocyte cultures have been used to demonstrate the cholestatic potential of various compounds that induce cholestasis in vivo (244). Both morphologic and functional approaches have been used. The use of fluorescent-labeled substrates and imaging techniques allows visualization of changes in bile canalicus structures (244,245). Hepatocyte couplets possess a bile canalicus that is functional for a few hours. Phallolidin, bile acids such as taurolochololate and chenodeoxycholate, ursodeoxycholate, and taurocholate inhibited canalicular secretion, whereas noncholestatic compounds were ineffective (246).

Compounds that induce cholestasis by acting on other liver cell types, e.g., paracetamol, galactosamine, and erythromycin, as well as chlorpromazine, were also ineffective (246). The absence of effect of chlorpromazine could be explained by an incubation period that was too short (2 hr), as this drug is reportedly cholestatic only after biotransformation (247). By contrast, its cholestatic action has been shown in primary rat hepatocyte cultures after a longer exposure period (248). Alteration of actin filaments by phallolidin treatment has been observed in both models (244,246).

Tauroursodeoxycholate, which prevents liver lesions in experimental cholestasis (249), partly prevented changes in pericanalicular actin microfilament distribution by taurolocholate or erythromycin estolate in isolated rat hepatocyte couplets when added at equimolar concentrations with either compound (250).

Specific alterations of organelles have been noted with various compounds. Rat or human hepatocytes exposed to amiodarone show the formation of multilamellar inclusion bodies in the absence of other structural changes (251,252). The major metabolite desethylamiodarone also induces the formation of such inclusions (252). Perhexiline maleate and antibiotics such as erythromycin and trospectin sulfate (253) are other compounds that induce accumulation of concentric membranes in lysosomes in cultured hepatocytes. A linear correlation was obtained between amiodarone intracellular concentration and the percent volume density of inclusion bodies (252). Myelinoid inclusion bodies were observed with concentrations only two to five times as high as the usual drug levels in amiodarone-treated patients. Peroxisome proliferation after treatment of rats with several hypolipidemic agents, including clofibrate, nafenopin, and beclofibrate, has been reproduced in vitro (254–256). Specific mitochondrial alterations have been observed in rat hepatocytes both in vivo and in vitro after treatment with ditercalinium (257).

**Biochemical Mechanisms.** There are many examples illustrating the use of isolated hepatocytes to determine the mechanisms involved in xenobiotic-mediated hepatotoxicity. Similar chemical- or drug-induced biochemical changes in vivo and in vitro have been demonstrated for various drugs, particularly the classical hepatotoxins such as paracetamol, CCl4, and halothane, as well as for metals.

Hepatotoxicity of paracetamol is induced by the formation of N-acetyl-p-benzoquinoneimine (258). When this reactive metabolite, formed by a CYP-mediated pathway, is not completely detoxified by conjugation with glutathione, it binds to cellular proteins and induces liver injury. Species differences in sensitivity to paracetamol toxic effects have been reproduced in isolated hepatocytes. In addition, by using isolated hamster hepatocytes Boobs et al. (259) have shown that binding of the reactive metabolite to cellular proteins and loss of cell viability could be dissociated. After a 90-min incubation with paracetamol, hamster hepatocytes were washed to remove all unbound drug and refreshed with paracetamol-free medium. A rapid loss of cell viability was observed and was prevented by the addition of N-acetylcysteine. This suggested that the reducing agent was able to repair oxidative damage induced by N-acetyl-p-benzoquinoneimine. The target proteins that interact with paracetamol-reactive metabolites have been analyzed by Bartolome et al. (260). At least two proteins of 44 and 58 kDa, respectively, were covalently associated with paracetamol by using antibodies directed against paracetamol-bound antigens.

CCl4 is another hepatotoxin that has been studied extensively. A comparative study in vivo and in primary rat hepatocyte
cultures led to the conclusion that the biochemical changes induced by this toxic compound were generally similar in both models but more rapid in vitro (261). Decreased protein and lipoprotein synthesis (262), increased lipid peroxidation (263), and potentiation of CCl4-induced liver injury by pretreatment of the animals with 2,5-hexanenedione, isopropanol, and chlordecone (264,265) have been reproduced in isolated rat hepatocytes. As in vivo, the CYP inhibitor SKF-525 inhibited toxicity induced by CCl4 (266). In some studies in vivo observations were not reproduced in in vitro models, probably because of inappropriate experimental conditions (263). Evidence shows that CCl4 used at relatively high doses induces membrane injury through a direct solvent action (267).

The anesthetic halothane causes two types of liver damage: transient liver dysfunction in about 20% of anesthetized patients or, in rare cases, a severe hepatitis resulting from a cell-mediated immune response. Both forms are thought to be related to metabolism of the anesthetic that occurs through an oxidative and a reductive pathway.

Several studies have established a correlation between hepatic centrilobular injury and low oxygen tension; greater liver damage has been associated with the formation of unstable reactive metabolites through the reductive pathway. Cytotoxicity in isolated hepatocytes from halothane has been demonstrated (172,268,269). Rat cells are more sensitive than their human counterparts (172). In a recent study using enriched perportal and centrilobular hepatocyte subpopulations in primary culture, we found that the latter were the most sensitive to the anesthetic and that increased reductive metabolism of halothane induced by decreasing oxygen concentration is not a critical parameter for the occurrence of liver damage in these cells (270). In immune-mediated halothane hepatitis, neoantigens are generated under oxidative conditions and involve the formation of highly reactive intermediate trifluoroacetyl groups capable of covalently binding to liver proteins (271). Isolated hepatocytes have also been used to identify immunogenic moieties that are generated after exposure to halothane and are responsible for this immune response. An interesting approach has been proposed by some groups; it involves using hepatocytes from pretreated animals and analyzing cytotoxic effects of sera from patients with halothane-induced hepatitis. Strong evidence was provided that in vivo administration of halothane could generate neoantigenic determinants on hepatocyte plasma membranes that are very similar, if not identical, to those generated in patients with halothane hepatitis (272). However, the use of animal hepatocytes could have some limitations. In a recent study (273), we prepared a hapten-specific antifluoroacetyl antibody that reacted with novel antigens in primary human hepatocyte cultures exposed to halothane. These antigens, located in the endoplasmic reticulum and on the plasma membrane, had molecular masses similar to those of trifluoroacetyl protein antigens expressed in vivo (273). The cellular distribution of these antigens was compatible with their generation by CYPs and their accessibility to immune effectors in patients. In a recent study CYP2E1 was found to be a cell surface autoantigen in halothane hepatitis (274). As already noted with clometacin, another drug known to induce immunological hepatitis in humans (275), primary human hepatocytes could represent a valuable model for detecting circulating antibodies in the sera of patients with halothane hepatitis and for characterizing the antigens recognized by these antibodies.

Isolated hepatocytes are also widely used to study metal-induced toxicity [reviewed by Klaassen and Stacey (276)]. Some metals such as cadmium and mercury are not essential for life and are considered only from a toxicologic point of view, whereas other metals such as iron and copper are essential and only their excess is responsible for tissue damage.

Several studies have shown that cadmium is cytotoxic to isolated hepatocytes (276,277) and an approximate comparison of metal doses in vivo and in vitro indicates that cell death occurs at similar levels. Recent studies have shown that cadmium pretreatment exerts a protective effect against the genotoxicity of 2-acetylaminofluorene in cultured rat hepatocytes (278). This effect could be related to an increase of metallothionein (thought to be involved in protection mechanisms) after cadmium treatment. A protection against cadmium toxicity in vitro has been reported with zinc, lead, and iron (279).

Investigations of iron toxicity in isolated hepatocytes after a short period of exposure did not demonstrate that this metal was cytotoxic (280). However, Morel et al. (281) demonstrated that ferric iron in its complex form with nitrilotriacetic acid induced a dose- and time-dependent toxicity to primary rat hepatocyte cultures. The cells were exposed to 5, 20, or 100 mM ferric iron up to 48 hr; cytotoxicity was demonstrated by production of free radicals and LDH release.

The use of chelators to remove metals is an important application of in vitro hepatocyte systems in the assessment of metal cytotoxicity (282,283). As an example, pyoverdin Pr, an iron chelator isolated from pseudomonas fluorescens, was recently found to prevent cytotoxicity induced by ferric nitrolactate in rat hepatocyte cultures. It appears to be as effective as the well-known chelator desferrioxamine B (284). Metals induce toxicity by a variety of mechanisms. In addition to peroxidation, which is frequently elicited, inhibition of mitochondrial function or adenosine phosphatases and interaction with sulphydryl groups have been reported (277).

Future Strategies

Liver Cell Models

The advantages and limitations of different liver preparations are well documented; the potential use for pharmacotoxicologic research is summarized in Table 6. Because the isolated perfused liver model can be used only for a few hours, two other intact

| Table 6. Applications of human in vitro liver preparations in pharmacotoxicologic studies. |
|---------------------------------------------|----------------|----------------|----------------|----------------|
| Assay                                      | Slices         | Suspended hepatocytes | Cultured hepatocytes | Microsomes     |
| Metabolic profile                          | +             | ±               | +              | ±             |
| Comparative interspecies metabolism        | ±             | ±               | ±              | ±             |
| Kinetic studies                            | ±             | ±               | ±              | ±             |
| Drug-drug interactions                     | ±             | ±               | ±              | ±             |
| Induction studies                          | ±             | ±               | ±              | ±             |
| Toxicity screening                         | ±             | ±               | ±              | ±             |
| Mechanistic studies                        | ±             | ±               | ±              | ±             |
| Unscheduled DNA synthesis test             | ±             | ±               | ±              | ±             |

Abbreviations: ±, possible to some extent; +, currently used; −, not suitable. *Isolated organs not included because of lack of availability. #Short-term study.
systems, i.e., slices (285) and isolated hepatocytes (286,287), appear to be more powerful models to mimic the in vivo situation. Isolated hepatocytes either in suspension or in culture are used more frequently. However, one must face the problems of availability and instability in primary culture of normal hepatocytes and the inability of permanent hepatocyte cell lines to express all of the functions exhibited by their normal counterparts in vivo.

Normal Hepatocytes. The major limitation in the use of normal hepatocytes is the availability of human samples and, to a lesser degree, of large mammal livers. Technical, safety, and ethical problems are associated with the use of human liver tissue. The opportunity to obtain hepatocytes from some animal species such as monkey or dog is also limited, primarily for ethical and economical reasons. Because of the progress in liver transplantation in humans, nonpathologic whole organs available for cell isolation are now extremely scarce. Pathologic and injured livers can be used; however, cell yields depend on the extent of the lesions, e.g., steatosis. Cirrhotic livers are poorly dissociated by collagenase treatment. Consequently, human hepatocytes are now more frequently prepared from surgical tissue pieces; this requires careful evaluation of their functional activities before any data can be correctly interpreted.

Human liver samples must be treated as biohazardous. Ideally, possible infection of the donor by hepatitis and/or human immunodeficiency viruses should be determined before handling and transport of liver tissue.

The scarcity and unpredictability of human liver tissue lead to the question of whether liver tissue and liver cell banks should be established and made available to those willing to conduct investigations with human liver. Although cell recovery is still limited, there is now evidence that both human and animal liver parenchymal cells can be cryopreserved for months or even years. However, it is not conceivable that supplies of human liver tissue will dramatically increase in the future if one abides by ethical and legal aspects, and consequently no large tissue or cell bank that will allow distribution of samples through a large area could be established. One way to accommodate both ethical aspects and the need for functionally characterized samples might be to set up a database and a tissue bank under the control of an institutional organization at the national level, with cells or tissues distributed as reference samples.

Another critical problem is the functional stability of normal adult hepatocytes in primary culture. Recent investigations have clearly established that three groups of factors, i.e., soluble factors, extracellular matrix components, and cell–cell interactions, improve in vitro maintenance of liver-specific functions. However, whatever the culture conditions used, hepatocytes exhibit phenotypic changes within a few days in vitro. The coculture model that associates hepatocytes with rat liver epithelial cells remains the most powerful in vitro system (288). Knowledge of factors required for hepatocyte replication in vitro is another important challenge for the future. Therefore, significant progress must be made to improve experimental conditions—progress that will lead to prolonged maintenance of liver-specific functions and/or to more than a few cycles of replication in vitro. Such improvements would be particularly advantageous for long-term hepatic toxicology and carcinogenesis studies.

Hepatocyte Cell Lines. The development of immortalized hepatocyte cell lines represents a promising alternative to nonproliferating or poorly replicating normal hepatocytes. Development of immortalized animal hepatocyte lines that retain various specific liver properties is proving difficult. Immortalized human hepatocytes could be the optimal choice, but they appear to be still more difficult to obtain and maintain in culture with some liver-specific functions (289). This means that optimal conditions for immortalizing and culturing adult hepatocytes must be further defined.

An interesting new approach is to establish hepatocyte lines from transgenic mice. Immortalized nontransformed fetal hepatocyte lines obtained by dissociation of the liver before visible pathologic changes reportedly retain highly differentiated functions for many months when cultured in a sophisticated medium (290).

Presently, attempts are being made to transfect human hepatocyte cell lines with cDNA encoding CYPs in which expression is missing and to obtain transgenic mice expressing human drug-metabolizing enzymes.

In Vitro Toxicity Tests

Considerable information has been published regarding the use of the isolated hepatocyte, either in suspension or in primary culture, for in vitro toxicity testing (286,287). Although carried out with different methodologies, these studies give us a good idea about advantages and limitations of the model for screening and mechanistic analysis of potentially toxic compounds.

The interlaboratory validation schemes previously conducted using classical experimental conditions and various in vitro end points indicate that no single protocol (i.e., one culture condition) can be used for testing every compound (174) and that problems of interpretation can be greatly magnified when tests involving different mechanisms of toxicity and end points are compared (291).

Early phenotypic changes occurring in hepatocytes after seeding are a major limitation for long-term toxicity screening. However, the use of more sophisticated culture conditions (e.g., cocultures) could overcome this drawback.

Consequently, the following strategy is proposed for short-term and long-term toxicity studies. Compounds could first be tested on nonhepatic cells (e.g., fibroblasts, cell lines) using nonspecific end points over a short period not exceeding 20 to 24 hr, and IC50 values would be calculated. This study would elicit information on compound properties such as solubility and intrinsic toxicity. These data would be useful for testing the compound on primary rat hepatocytes in a second step. When liver-specific chemical-induced cellular lesions are suspected, appropriate liver-specific indicators would be used. Long-term cytotoxicity would be estimated using sophisticated culture systems (e.g., cocultures). The cultures would be exposed daily for either a few days or 1 to 2 weeks. If liver-specific toxicity is demonstrated with animal hepatocytes, and particularly if species differences exist in metabolic pathways of the test compound, the use of human hepatocytes would be desirable. Another step could be carried out in vitro using mixed cultures of hepatocytes and nonhepatic target cells (e.g., kidney cells). This last model system would determine whether metabolites formed by liver cells would be toxic for other cells. These cultures could be performed in static conditions (or preferably in perfusion conditions), thereby better mimicking in vivo xenobiotic clearance (292).

In addition to intact hepatocyte models, liver S9, microsomes, and genetically engineered cells are also of interest, particularly for the study of mutagens. One may wonder whether the rat liver S9 could be replaced by microsomes from genetically engineered cells expressing human CYPs in appropriate concentrations in the
Ames test. It could also be possible to mimic the human situation, including the genetic defects in some CYPs.

Conclusions

Over the last few decades, major progress has been made in the development of reproducible methods for the preparation and culture of differentiated hepatocytes and liver slices of both animal and human origin. The usefulness of isolated hepatocytes for drug metabolism and toxicity studies has been demonstrated. It is time to standardize methodologies that could be validated and accepted by the scientific community and by regulatory agencies. However, it should be considered that if a great deal of information can be obtained from in vitro models, the appropriate strategy in the course of drug development is their use in alternance with in vivo studies. Moreover, some limitations exist related to the occurrence of early phenotypic changes in cultured hepatocytes, their low proliferating capacity, and the scarce availability of human hepatocytes. The major challenges of the coming years will be to further improve culture conditions for primary adult hepatocytes and to obtain immortalized, untransformed hepatocytes retaining liver-specific functions. The use of new technologies (e.g., scanning laser cytometer, confocal microscopy) should also allow the development of new, reliable, and sensitive toxicity tests.

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