Biochemical Pharmacology, Vol. 42, No. 2, pp. 373–379, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

RAT ADULT HEPATOCYTES IN PRIMARY PURE AND MIXED MONOLAYER CULTURE

COMPARISON OF THE MAINTENANCE OF MIXED FUNCTION OXIDASE AND CONJUGATION PATHWAYS OF DRUG METABOLISM

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(*Received* 27 *August* 1990; *accepted* 9 *March* 1991)

Abstract—The stabilities of several drug oxidation and conjugation pathways in adult rat hepatocytes were investigated in two systems: a primary pure culture lasting 3 days and a primary mixed culture (hepatocytes co-cultured with epithelial cells) lasting 10 days. The cytochrome P450 content in hepatocytes drastically declined within 48 hr in both culture systems. Cytochrome P450-dependent mixed function oxidase was measured by the O-dealkylation of ethoxyresorufin (EROD) and of pentoxyresorufin (PROD). UPD-glucuronosyl transferase (UDP-GT) activity was measured using 1-naphthol and morphine as substrates. In both culture systems, the activities of enzymes belonging to the 3-methylcholanthrene-inducible family, namely EROD and 1-naphthol UDP-GT, were much better maintained than those of PROD and morphine UDP-GT, which belong to the phenobarbitone-inducible family: in pure cultures, EROD and 1-naphthol UDP-GT activities declined to 60% of initial values within 3 days; in mixed cultures, EROD activity was stable throughout the 10 day culture period, whereas that of 1-naphthol UDP-GT was stable until day 4 but had declined to 70% of the initial value by day 8. In contrast, PROD and morphine UDP-GT activities declined to approx. 30% of the initial values within 2 days in both culture systems, and had dropped to approx. 10% of the initial value within 8 days in mixed culture. Reduced glutathione (GSH) levels fluctuated, but remained high throughout culture. GSH conjugation declined to 40% of initial values within 3 days in pure culture, whereas it remained relatively constant in mixed culture. Comparison of these two culture systems therefore showed that although the inclusion of epithelial cells did prolong hepatocyte viability, there was a change in relative enzyme activities in both systems, suggesting a shift towards a more de-differentiated drug metabolism pattern.

Many carcinogens and cytotoxic xenobiotics are activated by cytochrome P450-dependent mixed function oxidation (MFO†) and detoxified by conjugation with glucuronic acid, sulfate or reduced glutathione (GSH). The extent of genotoxicity and cytotoxicity depends on the balance between these activation and detoxification processes. Primary cultures of hepatic parenchymal cells from experimental animals [1–3] and human subjects [4, 5] have become an established method for studying xenobiotic metabolism and mechanisms of cytotoxicity. However, a major problem inherent in primary cultured hepatocytes is the instability of a number of specific functions [6]. For example, MFO activity declines within the first 24–48 hr of culture [3, 7]. Furthermore, the oxidation and conjugation pathways are not tightly coupled [8] and, consequently, culture-dependent changes in either may substantially alter the disposal and consequent biological effect of a xenobiotic. There have been many attempts to maintain the levels of cytochrome P450 and its monooxygenase activities in culture by adding hormones, chemicals, ligands and/or inducers to the culture media [9–14]. Prolonged maintenance of active cytochrome P450 has also been reported in hepatocytes co-cultured with rat liver epithelial cells [15, 16], and in recent studies the maintenance of the enzymic functions dependent on cytochrome P450 [17] and that of the glutathione-*S*-transferase pathway [18, 19] during culture have been evaluated. In this article, the rates of two cytochrome P450-dependent reactions and three conjugation reactions were compared in a primary pure culture system of adult rat hepatocytes with those in a primary mixed culture system. MFO activity was measured using two homologous substrates, ethoxy and pentoxyresorufin, which can be used to identify the MFO activity of different isozymes of rat liver cytochrome P450 [20]. UDP-glucuronosyltransferase (UDP-GT) activity was measured using 1-naphthol and morphine, which are preferential substrates for the 3-methylcholanthrene-inducible and phenobarbital-inducible transferases respectively in rat [21]. Glutathione conjugation was investigated using 1-chloro-2,4-dinitrobenzene (CDNB), which is a

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† Abbreviations: MFO, mixed function oxidation; EROD, ethoxyresorufin O-deethylation; PROD, pentoxyresorufin O-depentylation; GSH, reduced glutathione; GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; UDP-GT, UDP-glucuronosyl transferase; MEM, minimum essential medium; ECOD, 7-ethoxycoumarin O-deethylation; AE, aldrin epoxidation.

substrate for at least four different isozymes of cytosolic glutathione-*S*-transferase (GST) in rat liver [22].

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM) and medium 199, fetal calf serum, glutamine, penicillin and streptomycin were obtained from Flow Laboratories, Bovine albumin, β-glucuronidase/aryl-sulfatase, *o*-phthalaldehyde, naphthol, glutathione-*S*-transferase and 1-chloro-2,4-dinitrobenzene were from Sigma, hydrocortisone hemisuccinate from Roussel-Uclaf, insulin from Novo and collagenase from Millipore Corp. Ethoxyresorufin and pentoxyresorufin were purchased from Molecular Probes, resorufin from Aldrich Chemie, UDP-glucuronic acid from Boehringer and morphine hydrochloride from Merck Chemical Division. [^{14}C]UDP-glucuronic acid and [^{14}C]napthol were obtained from Amersham.

METHODS

Preparation of hepatocytes and epithelial cells. Hepatocytes were isolated from male Sprague–Dawley rats by perfusion with collagenase as previously described [15]. They were then washed and resuspended in Earle's modified culture medium (a mixture of 75% MEM and 25% 199 medium) containing 10% fetal calf serum, 2 mM glutamine, 10 I.U./mL penicillin, 10 μg/mL streptomycin, 10^{-6} M hydrocortisone hemisuccinate and 0.1 I.U./mL insulin. Viability was assessed by Trypan Blue exclusion and the hepatocytes were diluted to give a final concentration of 750,000 viable cells/mL. Epithelial cells were prepared and cultured as described by Begue *et al.* [15]. Confluent, non-transformed monolayer cultures were used [15]. The cells were removed from the flasks with 0.25% trypsin, washed and resuspended in the culture medium described above, to give a final concentration of 10^6 cells/mL.

Culture of hepatocytes. Cells were seeded in 25 cm^2 Nunc plastic flasks with 3×10^6 viable cells per flask. In pure cultures, the medium was renewed 4–5 hr after seeding and then once daily. The mixed cultures were prepared as described by Begue *et al.* [15]. The medium was discarded 4–5 hr after seeding and 4×10^6 epithelial cells were added in an equal volume of fresh medium. The medium was then changed every 24 hr from day 1 to day 4 and day 7 to day 9.

Analytical methods. The measurement of cytochrome P450 was carried out on homogenates of freshly scraped cultured cells. Spectrophotometric analysis was performed according to Omura and Sato [23], by measuring the spectrum difference between carbon monoxide-treated, dithionite-reduced homogenate and dithionite-reduced homogenate, using a Kontron Uvikon spectrophotometer. MFO activities (EROD and PROD) were measured according to Burke *et al.* [20] and adapted to intact cells by the procedure of Edwards *et al.* [24] for ethoxycoumarin-*O*-deethylase (ECOD) activity determination. Briefly, the culture medium was discarded and 1 mL ethoxyresorufin or pentoxyresorufin (50 μM each in a 25 mM Hepes buffer pH 7.4 [24]) was added to the culture flasks. The reaction was stopped by adding 10 mL of liquid nitrogen, either immediately (blank flask) or after incubation for 30 min at 37° (assay flask). A standard flask received 1.27 μM resorufin. The cells were scraped off the flasks, sonicated and stored at −80° until analysis. To 500 μL of cell homogenate was added 700 μL of a deconjugation buffer (0.11 mM NaCl, 60 mM CH_3COONa) containing β-glucuronidase (420–560 units/mL) and aryl-sulphatase (21–56 units/mL). After 1 hr incubation at 37° in the dark, the samples were centrifuged and 1 mL of the supernatant was added to 1 mL of a 0.01 M NaCl solution. The fluorescence was read at an emission wavelength of 582 nm and an excitation wavelength of 535 nm, using a Perkin-Elmer spectrofluorimeter. MFO activities were expressed as pmol resorufin formed/min/flask.

UDP-GT and GST activities and GSH concentration were determined using scraped cell homogenates stored at −80° for up to 1 month until analysis. The glucuronoconjugation of morphine and 1-naphthol was measured using the radiometric methods described by Puig and Tephly [25] and Bock and White [26], which we adapted to native cell homogenates described below.

The incubation mixture used for the determination of 1-naphthol glucuronoconjugation contained 350 mM Tris–HCl, pH 7.4 (25 μL), 70 mM $MgCl_2$ (10 μL), cell homogenate (7.5×10^6 cells/mL, 50 μL) and 20 mM UDP-glucuronic acid (35 μL). The reaction was started by adding 25 mM [^{14}C]1-naphthol (1 mCi/mmol, 5 μL). The incubation was performed at 37° for 20 min and stopped by adding 2 mL chloroform. After adding 250 μL H_2O, the sample was centrifuged for 10 min at 3000 *g* to extract unreacted naphthol into the organic phase. The radioactivity of an aliquot (125 μL) of the aqueous phase containing the glucuronide was determined in Beckman Resolv scintillation fluid using an LKM β counter. The values for the non-enzymatic glucuronoconjugation blanks were substracted. The rate of naphthol glucuronoconjugation was calculated from the radioactivity of the original [^{14}C]1-naphthol solution. The incubation medium used for determination of morphine glucuronoconjugation contained 200 mM Tris–HCl, pH 8.4 (20 μL), 140 mM $MgCl_2$ (10 μL), cell homogenate (7.5×10^6 cells/mL, 50 μL) and 70 mM morphine (10 μL). The reaction was started by adding 20 mM [^{14}C]UDP-glucuronic acid (8 mCi/mmol, 35 μL). The incubation was performed at 37° for 10 min and stopped by adding 7 mL of ice-cold 1 M ammonium acetate, pH 9.2. The complete volume was then loaded onto a reverse phase C-18 minicolumn (Sep-Pak, Waters Associates) and the matrix was washed with 10 mL of 10 mM ammonium acetate, pH 9.2 to remove unreacted morphine. The morphine glucuronide was then eluted from the column with 5 mL of absolute ethanol and was measured as described above by scintillation counting techniques. UDP glucuronyltransferase activities were expressed as nmol naphthol glucuronide or nmol morphine glucuronide formed/min/flask.

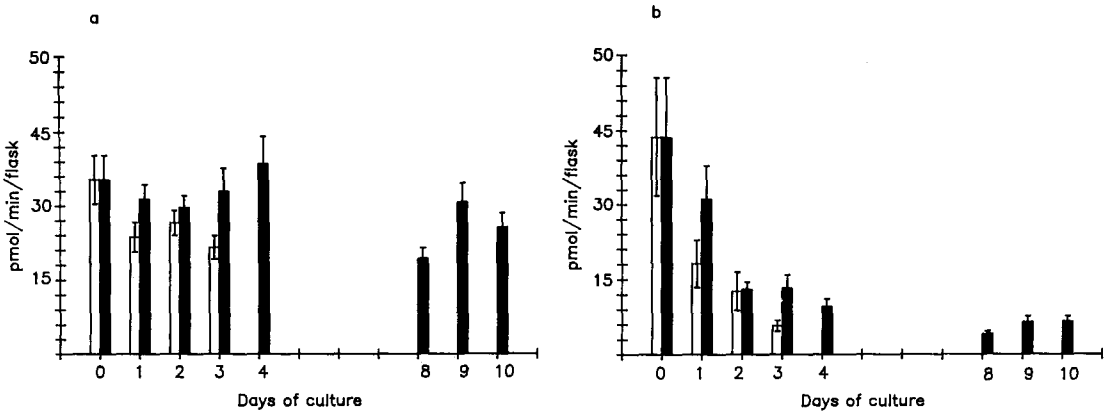

Fig. 1. Mixed function oxidase (MFO) activities of hepatocytes during culture. Ethoxyresorufin-O-deethylation (EROD) activity (a) and pentoxyresorufin-O-depentylation (PROD) activity (b) measured in intact hepatocytes, as described in Materials and Methods and expressed as pmol resorufin formed/min/flask. Enzyme activities in pure culture (□) and mixed cultures (■). On day 0, the activities were measured 4 hr after hepatocyte seeding. Values are expressed as the mean ± SEM of 8 or 9 independent experiments.

The glutathione conjugation of CDNB was measured by direct spectrophotometry as described by Habig and Jakoby [27]. The method was adapted to a Progress automate (Kone) and glutathione conjugation in the cell homogenate was measured from a calibration curve of purified GST and expressed as nmol CDNB conjugated/min/flask.

GSH concentrations were measured fluorimetrically by the method of Hissin and Hilf [28] and expressed as nmol/flask.

Preliminary experiments have shown that neither enzyme activities nor cytochrome P450 and GSH content could be detected in epithelial cell samples at the concentration used (4×10^6 cells per flask).

RESULTS

Cell survival

In our culture conditions, a pure population of male CD rat hepatocytes cultured on plastic could be maintained with maximal attachment for only 3 days. After this length of time, the cells started to detach from the dishes. In contrast, when co-cultured with rat liver epithelial cells, the hepatocytes remained attached to the plastic and in close contact with epithelial cells, for at least 10 days.

Cytochrome P450 content of hepatocytes during culture

The content of cytochrome P450 in hepatocytes 4 hr after sending was 0.130 ± 0.025 nmol/10^6 cells. After 24 hr, a 50–70% loss occurred in cytochrome P450 content in both pure (0.060 ± 0.010 nmol/10^6 cells) and mixed (0.045 ± 0.005 nmol/10^6 cells) hepatocyte cultures. Cytochrome P450 was no longer detectable from 48 hr of culture.

Mixed function oxidase activities of hepatocytes in pure culture and mixed culture

In pure cultures, the activity of EROD (Fig. 1a) declined to 60% of the initial activity (4 hr after seeding) within a day. In co-cultures, EROD activity was preserved throughout the 10 day culture period. The lower value observed on day 8 can be explained by the fact that the culture medium was not changed between day 4 and 7.

In contrast to EROD activity, hepatocyte PROD activity (Fig. 1b) fell irreversibly in both pure and co-cultures. The enzyme activity had fallen to approx. 30% of the initial value by 2 days of culture and to approx. 15% of the initial value within 3 days of pure culture or 8 days of co-cultures.

UDP-glucuronosyltransferase activities of hepatocytes during culture

The glucuronoconjugation of 1-naphthol (Fig. 2a) fell to approx. 60% of the initial value within the 3 days of pure culture. In co-cultures, the activity was stable for the first 4 days and was maintained at about 50% between day 8 and day 10 of co-culture. The glucuronoconjugation of morphine (Fig. 2b) sharply decreased to approx. 30% of the initial value by day 3 in both culture conditions and dropped to approx. 15% of the initial value within 8 days of co-culture.

Reduced glutathione content and glutathione-S-transferase activity of hepatocytes during culture

Intracellular GSH content (Fig. 3a) increased by 40% during the first day in pure culture, was equivalent to the initial value on day 2 and had decreased to 70% of the initial value on day 3 of culture. In co-cultures, the GSH content also increased by 15–35% between day 1 and day 3. Thereafter, GSH gradually decreased to approx. 50% of the initial value within 10 days.

GSH conjugation of CDNB (Fig. 3b) progressively decreased with time in pure culture. At day 3, the GST activity was 40% of the activity measured 4 hr after seeding. In contrast, GSH conjugation of

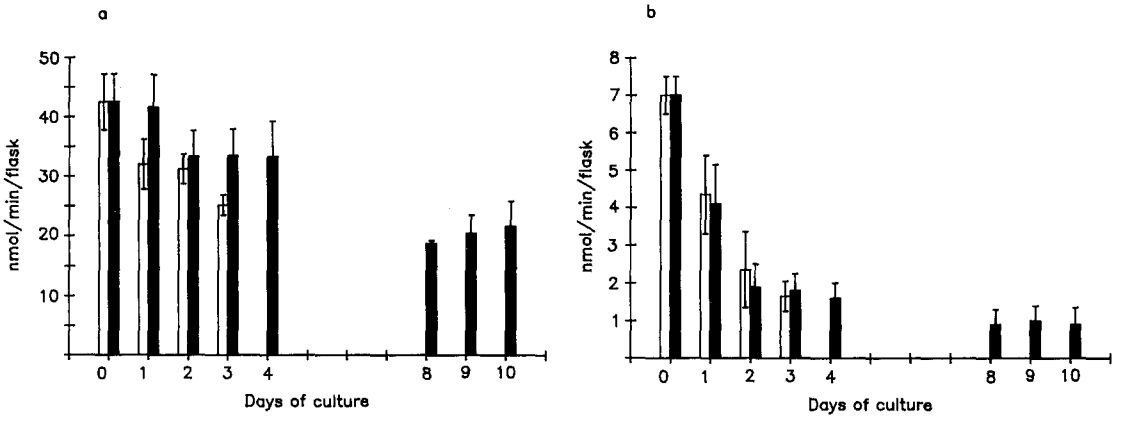

Fig. 2. UDP-glucuronosyltransferase (UDP-GT) activities of hepatocytes during culture. 1-Naphthol glucuronosyltransferase activity (a) and morphine glucuronosyltransferase activity (b) measured in hepatocyte homogenate as described in Materials and Methods and expressed as nmol naphthol glucuronide or morphine glucuronide formed/min/flask. Enzyme activities in pure cultures (□) and mixed cultures (■). On day 0, the activities were measured 4 hr after hepatocyte seeding. Values are expressed as the mean ± SEM of 3 to 5 independent experiments.

CDNB was stable during the first 8 days of co-culture, and 70% activity was maintained on day 9 and day 10.

DISCUSSION

The purpose of this study was to assess and compare the potential of rat hepatocyte primary pure and co-cultures as a system intended for the study of cytotoxicity, genotoxicity and the induction of drug metabolism by various chemicals. We therefore studied the P450-dependent mixed function oxidase (MFO) system, as well as conjugation reactions with glucuronic acid and glutathione, pathways known to be involved in activation and detoxification processes [5, 8, 28, 29]. The substrates chosen to investigate MFO reactions and UDP-GT activities were compounds with a hepatic microsomal metabolism which was known to respond *in vivo* in the rat to inducing agents such as phenobarbitone or 3-methylcholanthrene [20].

This study confirmed that the primary culture system for rat hepatocytes proposed by Guillouzo and co-workers [15, 30], namely their co-culture with another liver epithelial cell type, does indeed markedly prolong their viability compared to that of a pure hepatocyte population.

A complete loss of cytochrome P450 in both pure and mixed rat hepatocyte cultures within 48 hr was observed. Many investigators have described such a decline in primary pure cultured hepatocytes, although to a lower extent [9–12, 31, 32]. There are discrepancies between our results and those of Begue *et al.* [15] and Rogiers *et al.* [17] who reported significant maintenance of cytochrome P450 levels in mixed cultures. The differences can be partly explained by reactivity limits in this study due to the use of crude cell homogenate (and not the 10,000 *g* supernatant) for P450 determination.

Despite the rapid decline of the spectrophotometrically determined levels of the CO-binding holoprotein of P450, related MFO activities could be detected during the 3 days of pure culture and the 10 days of co-culture (see Fig. 1). Croci and Williams [29] reported similar results for other MFO activities in primary pure hepatocyte cultures. EROD activity decreased only slightly during pure culture and remained stable during co-culture. In contrast, in both culture systems, PROD activity strongly declined within 48 hr. It has already been reported that EROD activity is better maintained than PROD in primary pure hepatocyte cultures [3, 10]. The measurement of cytochrome P450 in pure cultured rat hepatocytes using specific antibodies has also revealed that the concentrations of different isozymes are maintained to differing extents in culture [33]. Furthermore, recently, Rogiers *et al.* [17] also reported a difference in the maintenance of two MFO activities, namely 7-ethoxycoumarin O-deethylation (ECOD) and aldrin epoxidation (AE) in both conventionally and co-cultured rat hepatocytes: while ECOD expression declined as a function of time in pure cultures and was well maintained in co-cultures, a dramatic loss of AE expression was noticed in both culture systems.

Differences between the maintenance of two different UDP-GTs were also observed: in both culture systems, morphine glucuronosyltransferase activity drastically decreased within 48 hr whereas glucuronoconjugation of 1-naphthol remained high (see Fig. 2).

The intracellular GSH content was maintained at high levels during both pure and co-culture (see Fig. 3). The GSH conjugation of CDNB gradually decreased during the 3 days of pure culture and remained high in co-culture. That GST activity towards CDNB remains well expressed during pure and mixed cultures has been reported earlier [17–19]. The work of Vandenberghe *et al.* [18] furthermore demonstrated that marked variations in the enzymic activity occurred in pure cultures

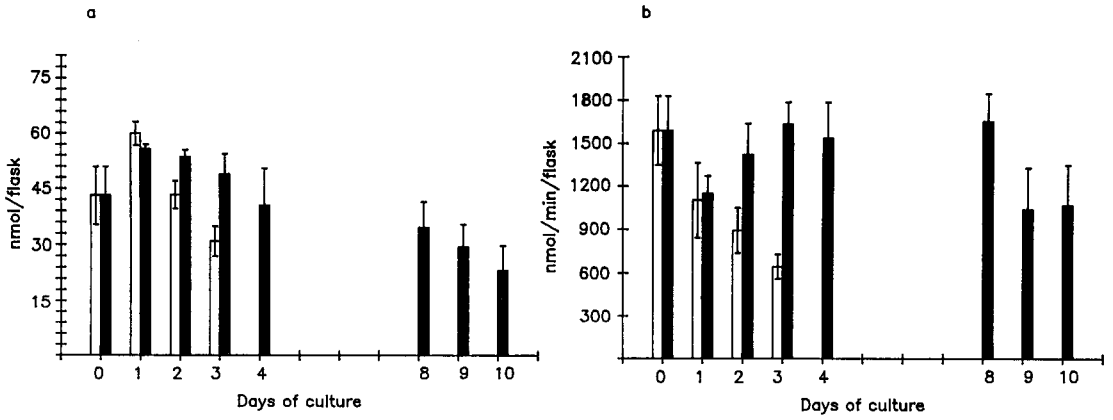

Fig. 3. Reduced glutathione (GSH) content and glutathione-*S*-transferase (GST) activity of hepatocytes during culture. Intracellular GSH content (a) and GST conjugation of CDNB (b) measured in hepatocyte homogenate as described in Materials and Methods and expressed respectively as nmol GSH/ flask and nmol CDNB conjugated/min/flask. Enzyme activity and GSH content in pure cultures (□) and mixed cultures (■). On day 0, the activities were measured 4 hr after hepatocyte seeding. Values are expressed as the mean ± SEM of 3 to 5 independent experiments.

depending on the medium composition, these variations being much less expressed in co-culture.

The two most interesting findings of this study were: (i) that the activities of enzymes belonging to the 3-MC-inducible family, namely EROD and 1-naphthol UDP-GT [20], were much better maintained in culture than PROD or morphine UDP-GT, which belong to the phenobarbitone-inducible family [20] and (ii) that the changes in the behaviour of these enzymes were similar in rat hepatocyte pure and co-cultures. It is thought that the shift towards cytochrome P448 (3-MC-inducible) predominance could reflect de-differentiation of adult hepatocytes [3]. Rogiers *et al.* [17] also suggested that de-differentiation occurred in pure and co-cultured adult rat hepatocytes, since AE activity, a sensitive parameter for de-differentiation [34] undergoes important decrease in function of culture time. Further studies are needed in order to find out which cytochrome P450 isoenzymes are involved in de-differentiation. However, it is noteworthy that EROD (this study) and ECOD [17], both depending on P450 IAI [17, 35] are well maintained while PROD (this study) and AE [17], depending on P450 IIB2 [17, 35] dramatically decrease in culture.

As 1-naphthol is considered to be more specific for the late fetal form and morphine for the neonatal form of UDP-GT [36, 37], the observed predominance of the former type of activity during culture is further evidence of possible de-differentiation of hepatocytes and development of a preneoplastic pattern of drug-metabolizing enzymes. However, Vandenberghe *et al.* [19] recently reported that in co-culture hepatocytes exhibit a more differentiated pattern of GST subunit gene expression than in pure culture. Furthermore, as shown in Table 1 co-cultured rat hepatocytes respond to phenobarbitone exposure by an apparently selective induction of cytochrome P450 MFO and UDP-GT activities, indicating that the characteristic response to phenobarbitone can still be elicited from hepatocytes in this culture system. AE has been reported not to respond to phenobarbitone in rat hepatocyte co-cultures [17] suggesting what very specific changes in the cytochrome P450 isoenzyme gene expression and regulation occur in co-culture.

Table 1. Enzyme activities in control and phenobarbitone-treated mixed hepatocyte cultures

	Control	PB
EROD	48 ± 6	120 ± 10
PROD	15 ± 2	49 ± 8
Naphthol UDP-GT	35 ± 5	34 ± 6
Morphine UDP-GT	2 ± 0.5	12 ± 2

Phenobarbitone (PB; 2 mM) was added 24 hr after initiating the culture and then daily. Enzyme activities were estimated after 4 days of culture. EROD and PROD were expressed as pmol resorufin formed/min/flask. UDP-GTs were expressed respectively as nmol naphthol glucuronide or nmol morphine glucuronide formed/min/flask. Values are expressed as the mean ± SEM of 4 to 5 independent experiments.

To summarize, adult rat hepatocytes co-cultured with rat epithelial cells maintain high levels of GSH content and GST, EROD and 1-naphthol UDP-GT activities for at least 10 days, whereas PROD and morphine UDP-GT activities rapidly decline. Although a pure population of rat hepatocytes retained total cell viability for only 3 days, a similar pattern of GSH content and enzyme activities could be observed. The overall impact of these differing behavior patterns on the disposal of chemicals cannot be forecast but, based on the fact that PROD and morphine UDP-GT activities both diminished whereas EROD and 1-naphthol UDP-GT remained stable, a shift to a more de-differentiated pattern of drug metabolism may well occur. Clearly, therefore, the changes reported here need to be taken into

consideration in studies of the metabolism of both endogenous and exogenous substrates by cultured hepatocytes.

Acknowledgement—The authors thank Mrs M. Ghosh for critical reading of this manuscript.

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