Biochemical Pharmacology, Vol. 40, No. 11, pp. 2525–2534, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

THE ISOENZYME PATTERN OF CYTOCHROME P450 IN RAT HEPATOCYTES IN PRIMARY CULTURE, COMPARING DIFFERENT ENZYME ACTIVITIES IN MICROSOMAL INCUBATIONS AND IN INTACT MONOLAYERS

H. M. WORTELBOER,* C. A. DE KRUIF, A. A. J. VAN IERSEL,† H. E. FALKE,‡ J. NOORDHOEK and B. J. BLAAUBOER

UTOX, c/o Research Institute of Toxicology (RITOX), University of Utrecht, P.O. Box 80.176, 3508 TD Utrecht; †National Institute of Public Health and Environmental Protection (RIVM), Laboratory of Toxicology, P.O. Box 1, 3720 BA Bilthoven; and ‡TNO-CIVO Toxicology and Nutrition Institute, Department of Biological Toxicology, P.O. Box 360, 3700 AJ Zeist, The Netherlands

(*Received* 10 *May* 1990; *accepted* 1 *August* 1990)

Abstract—Changes in the isoenzyme pattern of cytochrome P450 during culture were investigated in primary cultures of rat hepatocytes, measuring specific enzyme activities in microsomes prepared from cultured cells as well as in intact monolayers. Assays of 7-ethoxyresorufin O-deethylation (EROD), 7-pentoxyresorufin O-dependentylation (PROD), aniline 4-hydroxylation (AH) and the specific regioselective hydroxylation of testosterone were used as representatives of the activities of seven isoenzymes of cytochrome P450. The isoenzyme profile expressed as catalytic activities was qualitatively and quantitatively similar in microsomes obtained from freshly isolated hepatocytes in comparison with microsomes obtained from whole livers of untreated rats. There was a relatively high activity in EROD, AH and the oxidation of testosterone at the 7α, 2α, 6β, 16α and 17 sites (androstenedione). During culture, these microsomal enzyme activities declined at a similar rate to *ca.* 50% of the activities of microsomes prepared from freshly isolated hepatocytes after 24 hr and to 15% after 96 hr. The overall decline of cytochrome P450-dependent activities during culture was not accompanied with gross changes in catalytic profile. Determining the same drug-metabolizing activities directly in intact hepatocyte monolayers revealed a much higher metabolic rate for all measured P450-dependent activities. The profile of the catalytic activities was essentially the same as measured in microsomes prepared from cultured hepatocytes. The relatively low activity towards the 7α site of testosterone measured in intact hepatocytes, however, remained constant during culture. Determination of enzyme activities directly in intact hepatocytes is a convenient way of studying changes in monooxygenase activities of different P450 isoenzymes *in vitro*.

A wide variety of foreign compounds are metabolized by the hepatic biotransformation system. The activity of this system can be altered by many factors, including foreign chemicals. A pivotal role in biotransformation is played by cytochrome P450, a multigenic family of haemoproteins which determine the duration of action of many drugs and play a key role in chemical carcinogenesis and toxicity [1, 2]. The isoenzymes differ from each other in substrate specificity and catalytic activity [3]. Detailed description of the isoenzyme pattern of cytochrome P450 is desirable to provide a basis for studies on the monooxygenase-dependent metabolism and the subsequent effects of these biotransformation changes on biological effects of various xenobiotics.

Primary hepatocyte cultures represent a potentially useful model for studies on the effects of induction and different isoenzyme patterns of cytochrome P450 in correlation with toxicity of various compounds [4]. It is well known that the content and catalytic activities of cytochromes P450 decline during culture (for review see Ref. 5). Several attempts have been made to maintain either the content of cytochrome P450, the associated catalytic activities or both [5–10]. It is not clear whether the higher P450 levels and/or higher catalytic activities determined in these studies are the result of either stabilization or a more or less selective induction of one or more cytochrome P450 isoenzymes.

Monitoring specific biotransformation activities in addition to immunoblotting is a reliable way of describing changes in the population of cytochrome P450 isoenzymes [11, 12]. Burke *et al.* [13] investigated the selective dealkylation of phenoxazone alkyl derivatives, 7-ethoxyresorufin and 7-pentoxyresorufin, by two specific isoenzymes, namely P450IA1 (EROD§) and P450IIB1 (PROD). Aniline is 4-hydroxylated mainly by P450IIE1 and P450IA2 [14]. Furthermore, it has been shown that hydroxylation of testosterone is catalysed by different isoenzymes of cytochrome P450 with a high degree

* Correspondence to: H. M. Wortelboer, Research Institute of Toxicology (RITOX), P.O. Box 80.176, 3508 TD Utrecht, The Netherlands.

§ Abbreviations: AH, aniline 4-hydroxylation; DMSO, dimethylsulphoxide; EROD, 7-ethoxyresorufin O-deethylation; HBSS, Hanks' balanced salt solution; OHT, hydroxytestosterone; PBS, phosphate-buffered saline; PROD, 7-pentoxyresorufin O-dependentylation.

of regio- and stereoselectivity. Testosterone 7α-hydroxylation activity reflects the levels of P450IIA1 [15], 16β-hydroxylation activity the levels of P450IIB1, 2α-hydroxylation activity the levels of P450IIC11, and the formation of 6β-, 15β- and 18-hydroxytestosterone reflects the activity of P450IIIA [16–18].

In the present study we used different techniques to describe the changes in monooxygenase activity of P450 isoenzymes in primary rat hepatocytes during culture. Various specific activities were measured in microsomes prepared from primary cultured hepatocytes. Furthermore, to maintain the interrelationship between metabolic pathways in the intact cell [4, 19] and to avoid a possible selective destruction of isoenzymes during microsomal preparation from hepatocytes [20], we developed a more convenient way of determining catalytic activities directly in intact hepatocyte monolayers. The profiles of P450-dependent activities in intact cells as well as in microsomes from cultured cells are compared with the *in vivo* pattern as determined in microsomes prepared from whole liver.

MATERIALS AND METHODS

Materials

Newborn calf serum was purchased from Gibco Europe (Breda, The Netherlands). Testosterone, androstenedione, 11β- and 16α-hydroxytestosterone (11β- and 16α-OHT), Williams' medium E, insulin and hydrocortisone were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2α-OHT was a kind gift from Prof. D. N. Kirk (Queen Mary College, University of London), 15β- and 12β-OHT were gifts from G. D. Searle and Co. (Skokie, IL, U.S.A.). 6β-, 7α-, 16β- and 19-OHT were obtained from Steraloids (Wilton, NH, U.S.A.). β-Glucuronidase/arylsulphatase (EC 3.2.1.31/EC 3.1.6.1) was supplied by Boehringer (Mannheim, F.R.G.). All other chemicals were of analytical grade. The antibodies used in this study were all kind gifts. Monoclonal antibodies against P450IA1/2, P450IIB1/2, P450IIIA were from P. J. Kremers, Université de Liège, Belgium; a polyclonal antibody against P450IIE1 was from I. Johansson, Karolinska Institutet, Stockholm, Sweden; a polyclonal antibody against P450IVA1 was from G. G. Gibson, University of Surrey, Guildford, U.K.

Animals

Male Wistar rats (Riv.Tox(M)), RIVM, Bilthoven, The Netherlands), weighing 180–250 g, were fed *ad lib.* a TNO-CIVO Institute grain-based open-formula diet (TNO-CIVO, Zeist, The Netherlands) and had free access to drinking water.

Cell isolation and culture

Rat hepatocytes were isolated using the two-step collagenase perfusion technique described by Seglen [21] as modified by Paine *et al.* [22]. The cells were plated on 9-cm tissue culture dishes (Sterilin) at a density of 8×10^6 cells/dish in 10 mL Williams' medium E supplemented with 3% (v/v) newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/L gentamycin. Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°. After 4 hr in culture, media were replaced. Thereafter, media were refreshed every 24 hr.

Preparation of microsomes

Whole livers. Livers from control animals were perfused with ice-cold saline and homogenized in 0.15 M KCl containing 0.1 mM EDTA using a Potter-Elvehjem glass–Teflon homogenizer. Microsomes were prepared by centrifugation (2 × 20 min, 9000 *g*; supernatant 60 min, 105,000 *g*). The microsomal pellet was resuspended in sodium phosphate buffer (0.1 M, pH 7.8) containing 0.1 mM EDTA, frozen quickly in liquid N_2, and stored at −70°.

Cultured hepatocytes. Per treatment group seven culture dishes were washed in ice-cold PBS and scraped with a rubber policeman in 1 mL PBS/dish. Cells were centrifuged (3 min, 50 *g*); supernatant was replaced by 2.5 mL 0.15 M KCl containing 0.1 mM EDTA and cells were kept at −70° until preparation of microsomes. Cells were homogenized on ice by sonication using an MSE 100 W ultrasonic disintegrator. Sonication for 20 sec appeared to be optimal. After centrifugation for 2 × 20 min at 9000 *g* the supernatant was centrifuged for 60 min at 105,000 *g*. The microsomal pellet was resuspended by sonication (20 sec) in 700 μL 0.1 M sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA on ice, frozen quickly in liquid N_2 and stored at −70°. One milligram cellular protein yielded *ca.* 75 μg microsomal protein.

Enzyme assays

Cytochrome P450 *and protein.* Contents of protein and total cytochrome P450 were determined according to Rutten *et al.* [23] using a double-beam spectrophotometer (Pye Unicam 8800). Total amount of cytochrome P450 could not be determined in homogenized hepatocytes of 72 and 96 hr in culture because of the very low amount of cytochrome P450 in combination with the high levels of turbidity.

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD). Fluorimetric determinations of liver and hepatocyte microsomal 7-ethoxyresorufin O-deethylation (EROD) and 7-pentoxyresorufin O-depentylation (PROD) activities were performed according to the method of Burke *et al.* [24] using a Cobas-Bio centrifugal analyser, equipped with a spectrofluorimeter. Less than 200 μg microsomal protein was used in the incubation mixture of 0.1 M phosphate buffer (pH 7.4) with a final volume of 320 μL. Substrate was added in diluted DMSO (final DMSO concentration 0.1% v/v). Substrate concentrations of 5 μM were used.

Determinations directly in intact hepatocytes were as follows. Hepatocyte monolayers were washed twice with Hanks' balanced salt solution (HBSS) gassed with carbogen (95% O_2/5% CO_2, v/v). The incubation was initiated by adding 4 mL HBSS (37°) containing 5 μM substrate and 10 μM dicumarol. Dicumarol was added during incubation to prevent further metabolism of resorufin by the cytosolic enzyme DT-diaphorase [25]. To determine linearity of enzyme activity medium samples of 1 mL each were taken every 5 min during a period of 15 min (EROD) or 20 min (PROD). After dilution (1 : 1) of

the samples with incubation mixture fluorescence was determined using a Kontron SFM 25 fluorimeter. The fluorescence of resorufin appeared to be pH-dependent. A stable fluorescence was obtained after addition of 100 μL 0.1 M NaOH to the 2 mL diluted medium samples to bring the pH above 7.5.

Aniline 4-hydroxylation (AH). The AH activity of liver and hepatocyte microsomes was determined according to Chhabra *et al.* [26], using an aniline concentration of 15 mM. The reaction was stopped after 45 min at 37°, by adding 0.5 mL 20% TCA on ice. After centrifugation (5 min, 100 *g*), the formation of 4-aminophenol was determined spectrophotometrically at 630 nm, according to Imai *et al.* [27].

Determination of aniline 4-hydroxylation in intact hepatocytes using a short incubation time was not possible. The low activity of this reaction resulted in amounts of products below the limit of detection when incubation times comparable with the other measured reactions were used [28].

Testosterone hydroxylation. Determinations in microsomal preparations were carried out in 1 mL incubation mixture containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/mL), testosterone (250 μM) and 300–500 μg microsomal protein. Testosterone was added as methanolic solution (final methanol concentration 2% v/v). Incubation mixture and microsomes were mixed while standing on ice. Reactions were started by heating the mixture in a 37° water bath and stopped after 15 min by addition of 6 mL ice-cold dichloromethane.

For determinations in intact hepatocytes, monolayers were washed twice with HBSS gassed with carbogen. The reaction was started by adding HBSS (37°) containing 250 μM testosterone (added as methanolic solution, final concentration methanol 0.5% v/v) and stopped after 15 min by separating the medium and cooling it on ice. Cells were scraped in ice-cold PBS and both cells and medium were stored at −20° until extraction.

After extraction of the reaction products present in 1 mL medium or cell suspension with 6 mL dichloromethane, the aqueous phase containing the precipitated protein was removed and dichloromethane was evaporated under a stream of N_2 at room temperature. The residue was dissolved in 130 μL 50% (v/v) methanol/water.

HPLC analysis of testosterone metabolites. Testosterone metabolites were analysed according to Funae and Imaoka [29]. Analyses were performed on an HPLC system consisting of an LKB 2150 HPLC pump, a Kontron MSI 660 autosampler equipped with a 20 μL injection loop, an LKB 2151 variable-wavelength monitor and a Pye Unicam LC-XP gradient programmer. Testosterone oxidation products were separated using a Chromsep C18 (200 × 3 mm i.d., particle size 5 μm) reversed phase column preceded by a 10 mm C18 guard column (Chrompack, Middelburg, The Netherlands). The OHTs were eluted with aqueous solutions of 25% methanol from 0 to 10 min followed by a linear gradient to 40% methanol and 3.5% acetonitrile from 10 to 45 min, at a flow rate of 0.8 mL/min. Column temperature was kept at 50°. Column effluents were monitored at 254 nm. Metabolites were quantified by comparing their peak areas with those of authentic standards, using a Shimadzu C-R1A Chromatopac integrator. 11β-OHT was used as internal standard [30].

Western immunoblotting. Separation of microsomal proteins was carried out on a BioRad mini Protean II cell using the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) discontinuous system of Laemmli [31]. The resolved proteins were electrophoretically transferred to polyvinylidenedifluoride (PVDF) sheets (Millipore) according to the method of Towbin *et al.* [32] and immunochemically stained using antibodies to specific P450 forms.

RESULTS

Profile of cytochrome P450*-dependent activities in microsomes prepared from cultured hepatocytes*

To define optimal conditions for microsome preparation from hepatocytes different sonication times were used to homogenize the cells. The amounts of microsomal protein and cytochrome P450 recovered after 2–30 sec of sonication are shown in Table 1. No degradation of cytochrome P450 in the form of cytochrome P420 was detected in the spectral measurements. Longer sonication resulted in higher amounts of microsomal protein and cytochrome P450. In all further experiments a sonication time of 20 sec was used, because this time yielded the highest EROD and PROD activity per milligram of microsomal protein, although the PROD activity per nanomole P450 was somewhat less at that time. Recovery of cytochrome P450 in microsomes was *ca.* 30% as compared to the amount of cytochrome P450 in homogenized hepatocytes (*ca.* 14.3 and 48 pmol P450 per one million hepatocytes in microsomes and cell homogenates, respectively). Most of the cytochrome P450 got lost in the first 9000 *g* pellet, probably due to incomplete cell disruption.

The content of cytochrome P450 and the different catalytic activities in microsomes of freshly isolated hepatocytes were similar to those in microsomal preparations from whole livers of control animals (Fig. 1a and b). There was a relatively high activity towards aniline, a relatively low activity towards 7-ethoxyresorufin, and a negligible activity towards 7-pentoxyresorufin. The major oxidation products formed after testosterone exposure were 16α-OHT, androstenedione and 2α-OHT, followed by 6β-OHT and 7α-OHT. The formation of 15β-OHT, 16β-OHT and an unknown metabolite with a relative retention time of 0.83 (probably 18-OHT [16]) was negligible. In immunochemical studies no P450IA1, P450IIB1/2 and P450IVA1, and only minor amounts of P450IA2, P450IIC11, P450IIE1 and P450IIIA could be detected (results not shown). These results show that the procedures for preparation of microsomes from freshly isolated hepatocytes do not alter the P450 isoenzyme profile.

During culture AH activity and the formation of 2α-OHT, 7α-OHT and 16α-OHT declined similarly,

Table 1. The effect of sonication time during preparation of microsomes from freshly isolated hepatocytes on the recovery of microsomal protein, cytochrome P450 and two cytochrome P450-dependent catalytic activities

Sonication time (sec)	Protein (mg/mL)	P450 (pmol/mg)	EROD (pmol/min.mg protein)	EROD (pmol/min.nmol P450)	PROD (pmol/min.mg protein)	PROD (pmol/min.nmol P450)
2	3.70	186.39	37.44	200.86	5.40	28.97
5	4.19	216.06	55.56	257.15	6.56	30.36
10	4.44	235.16	51.54	219.17	6.40	27.22
20	5.67	275.72	62.40	226.32	6.62	24.01
30	6.62	294.52	60.96	206.98	5.96	20.24

Data are means of duplicate preparations.

showing an average activity of 55% of those measured in freshly isolated hepatocytes after 24 hr, and 18% after 96 hr in culture. A more rapid decline (to *ca.* 25% of freshly isolated cells) was measured for EROD activity and the formation of 6β-OHT during the first 24 hr in culture. The low levels of apoprotein of P450IA2 and P450IIIA in freshly isolated hepatocytes diminished during culture. After 24 hr in culture, the distribution of the measured catalytic activities did not change significantly (Table 2).

Optimal conditions for determination of cytochrome P450*-dependent activities in hepatocyte monolayers*

For determining the EROD and PROD activities in intact hepatocyte monolayers a substrate concentration of 5 μM appeared to be optimal (data not shown). EROD activity showed no lag time, while PROD activity revealed a lag time of 7 min. After that time the formation of resorufin was linear for at least 10 min (Fig. 2).

Formation of different hydroxy metabolites of testosterone in intact hepatocytes was linear over a 20 min incubation time (Fig. 3). No difference could be detected in the amount of testosterone metabolites in the media with or without deconjugation (data not shown). Testosterone itself was glucuronidated, but only to a small extent (less than 10%). Therefore, no deconjugation of the media samples had to be performed.

Extraction of reaction products of testosterone from both cells and media after a 15 min incubation period revealed that testosterone metabolites retained within the cells amounted to 9 to 18% of the content in the medium for all metabolites. Therefore, the metabolite pattern in the medium could be regarded as representative of the pattern in the complete incubation. Representative chromatograms of an extract of a monolayer incubation and of a microsomal incubation, both of 24-hr-old hepatocytes, are presented in Fig. 4a and b. A mixture of authentic standards is shown in Fig. 4c.

Profile of cytochrome P450*-dependent activities in hepatocyte monolayers*

Cytochrome P450-dependent activities determined in intact monolayers and expressed per milligram of cellular protein are shown in Fig. 5. If one compares these activities in microsomes (Fig. 1; activities expressed per mg microsomal protein) with the corresponding activities in monolayers (i.e. prepared from an equal amount of cells), the rate of product formation was 10 times higher in intact cells than in the microsomes, taking into account that in the preparation of microsomes there is a 30% loss of microsomal protein. The metabolic profile, however, appeared to be virtually the same (Fig. 5 vs Fig. 1c and d). Low activities were measured towards the substrates 7-ethoxyresorufin and 7-pentoxyresorufin. Moreover, formation of hydroxy metabolites of testosterone in intact hepatocytes showed the same pattern towards the specific metabolites 15β-OHT, 7α-OHT, 16β-OHT and 2α-OHT as determined in microsomes of cultured hepatocytes. Both the EROD and PROD activities measured in 4-hr-old primary cultures (4.3 ± 1.5 and 6.8 ± 1.9 pmol/min/

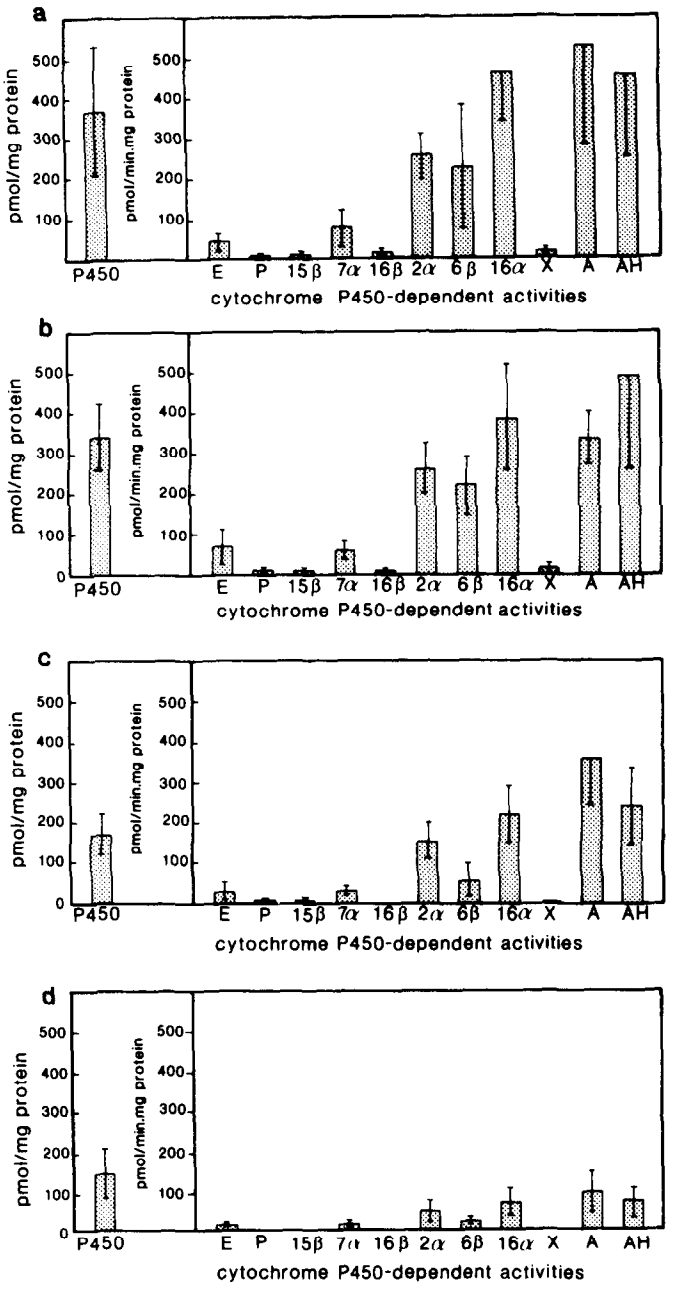

Fig. 1. Profiles of specific and less specific cytochrome P450-dependent activities in microsomes prepared (a) from whole livers of control animals, (b) from freshly isolated cells and from cultured cells after (c) 24 hr and (d) 96 hr in culture. Key: P450, total cytochrome P450 content; E, 7-ethoxyresorufin O-deethylation; P, 7-pentoxyresorufin O-depentylation; 15β, the formation of 15β-OHT; 7α, 7α-OHT; 16β, 16β-OHT; 2α, 2α-OHT; 6β, 6β-OHT; 16α, 16α-OHT; X, an unknown metabolite with $R_f = 0.83$; A, androstenedione; AH, aniline 4-hydroxylation. Values represent means ± SD, N = 5 rats *in vivo*, N = 10 rats *in vitro*.

mg cellular protein, respectively) were lower than in 24-hr-old cells (7.0 ± 1.9 and 18.4 ± 3.2 pmol/min/mg cellular protein, respectively). In contrast, a high degree of correlation between the decline in formation of testosterone metabolites measured in intact hepatocytes and in microsomes of cells during culture was seen for most of the metabolites of testosterone ($r = >0.938$ for all metabolites except 7α-OHT ($r = -0.055$) and androstenedione ($r =$ 0.788)). The rapid decline in hydroxylation at the 6β site of testosterone during the first 24 hr in culture was also seen in intact monolayers, as measured in microsomes from hepatocytes. The change in relative activity in the formation of 6β-OHT during culture is therefore not due to the preparation of microsomes. The formation of 7α-OHT and, to a lesser degree, androstenedione determined in intact hepatocytes remained at the same level during culture,

Table 2. Different cytochrome P450-dependent activities expressed as percentage of the summation of measured activity in microsomes prepared from primary hepatocytes during culture (data are means of N = 10)

Time in culture (hr)	Total P450 (pmol/mg protein)	Percentage of the sum of measured activity										Sum of measured activity (pmol/min.mg protein)
		EROD	PROD	15β	7α	16β	2α	6β	16α	X	A	AH	
0	343 ± 84	3.6	0.4	0.3	2.8	0.2	14.6	11.8	21.5	0.9	18.5	25.4	1842
24	170 ± 53	2.5	0.5	0.1	2.5	0.1	13.9	5.4	20.3	0.3	33.1	21.4	1091
48	176 ± 46	1.3	0.1	0.1	2.9	0.0	13.8	4.1	21.7	0.1	32.4	23.5	838
72	151 ± 46	1.8	0.1	0.2	3.3	0.0	14.5	4.7	22.5	0.0	30.8	22.0	520
96	136 ± 65	2.5	0.1	0.2	3.7	0.0	14.3	6.3	22.5	0.0	30.2	20.1	312

For abbreviations see legend to Fig. 1.

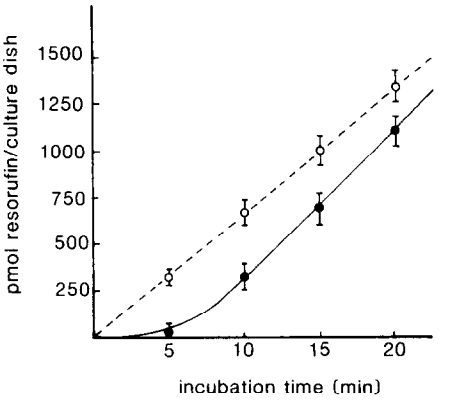

Fig. 2. Production of resorufin during EROD and PROD assay in monolayers of hepatocytes. (--○--) EROD; (—●—) PROD; values are means ± SD, each curve representing four different 24-hr-old hepatocyte monolayers.

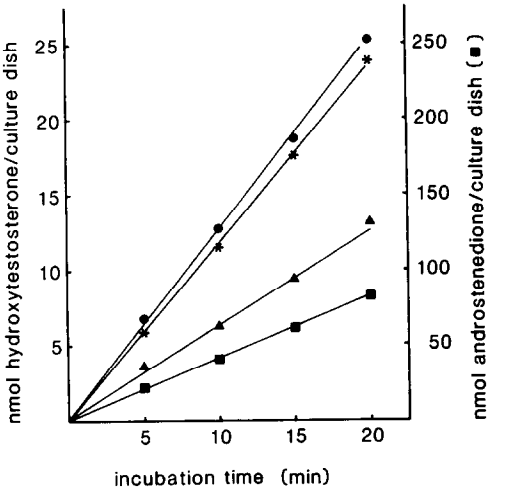

Fig. 3. Production of different metabolites of testosterone in monolayers of hepatocytes. (*) 6β-OHT; (●) 16α-OHT; (▲) 2α-OHT; (■) androstenedione; values are means of duplicate 24-hr-old hepatocyte monolayers.

which is in contrast to the decline measured in microsomes. As a result, small differences in the distribution of catalytic activities in intact hepatocytes occur during culture (Table 3).

DISCUSSION

In untreated rats isoenzymes of P450 are hard to determine immunochemically because their levels are usually below the detection limit. Although others [11, 12] could detect various isoenzymes (e.g. P450IA1/2, P450IIA1, P450IIB1/2, P450IIC6, P450IIC11, P450IIIA1) in untreated animals, we could detect only minor amounts of P450IA2, P450IIC11, P450IIE1 and P450IIIA in Riv.Tox(M)-Wistar rats. This is in full agreement with the low content of cytochrome P450 of 85 pmol/mg cellular protein in Riv.Tox(M) rats whereas these levels were

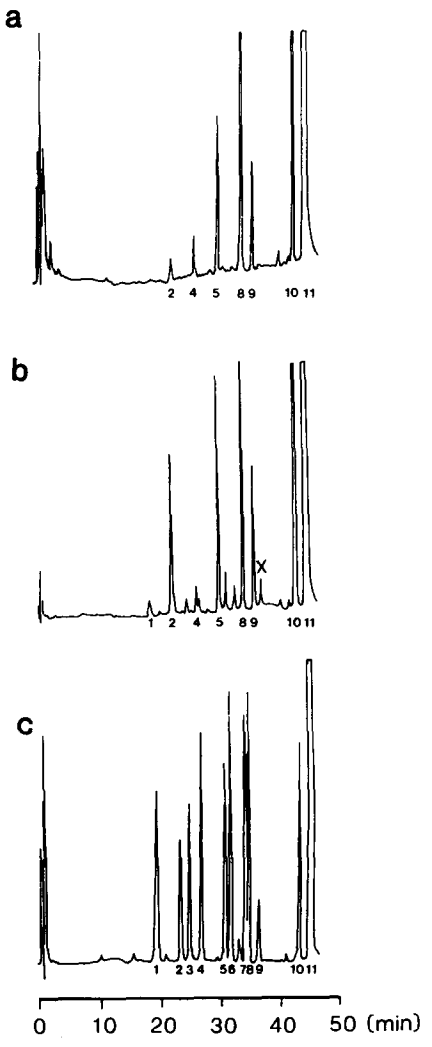

Fig. 4. Testosterone hydroxylation: representative chromatograms of (a) an extract of a microsomal incubation of 24-hr-old hepatocytes, (b) an extract of a cellular incubation of a 24-hr-old hepatocyte monolayer, and (c) a mixture of authentic standards (absorbance A_{254}). Key: 1, 15β-OHT; 2, 6β-OHT; 3, 19-OHT; 4, 7α-OHT; 5, 16α-OHT; 6, 16β-OHT; 7, 12β-OHT; 8, 11β-OHT (internal standard); 9, 2α-OHT; 10, androstenedione; 11, testosterone; X, unknown metabolite with $R_f = 0.83$. Peaks are appointed only when retention time correlated with a standard.

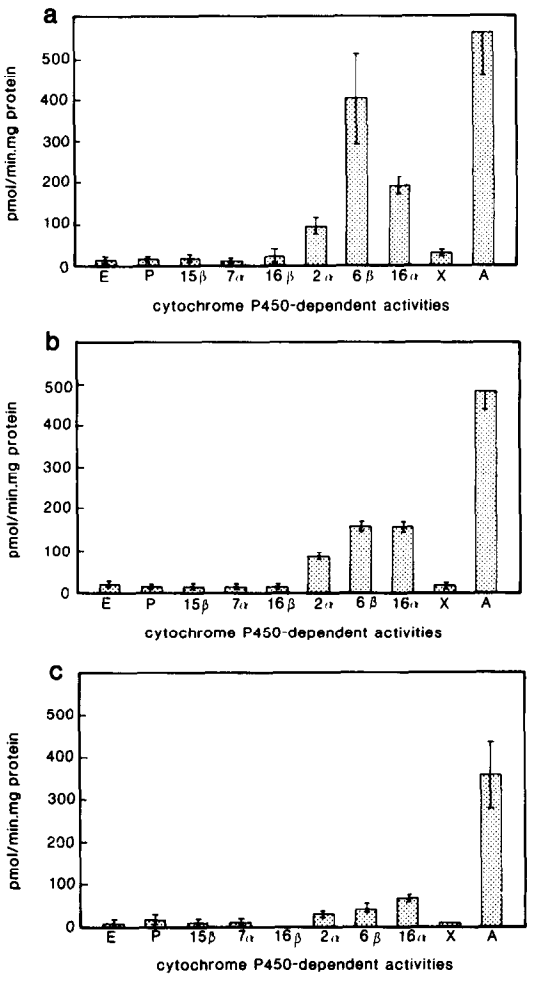

Fig. 5. Profiles of cytochrome P450-dependent activities measured directly in monolayers of cultured hepatocytes after (a) 4 hr, (b) 24 hr and (c) 96 hr culture. Values represent means ± SD, N = 3 rats. For abbreviations, see legend to Fig. 1.

225 and 500 pmol/mg cellular protein in other studies [11, 12]. Besides differences in strain, the lower content of cytochrome P450 is probably also due to differences in diet. In our study, we chose to feed the rats a semi-synthetic diet which most probably has a low induction potential. Because culture medium is supposed to be free of known inducers, we expect minimal changes in cytochrome P450 isoenzyme profile to occur in culture due to differences in environmental exposure of the cells *in vivo* and *in vitro*.

In cultured hepatocytes spectrally detectable cytochrome P450 is lost more rapidly than immunochemically detectable cytochrome P450 [11, 12]. Thus, levels of apoprotein may not directly reflect metabolic activity.

In this study we used a broad spectrum of specific catalytic activities of different isoenzymes of cytochrome P450 to describe the profile of isoenzymes in freshly isolated hepatocytes and in liver cells in culture for several days. In agreement with others [5, 33], no differences could be detected between cytochrome P450-catalysed activities measured in microsomes prepared from freshly isolated cells and whole livers. Furthermore, monitoring enzyme activities in microsomal preparations of cultured hepatocytes revealed the well-known rapid loss of cytochrome P450 (for review see Ref. 5), but also a decline of all cytochrome P450-dependent enzyme activities determined.

In contrast to previous suggestions that qualitative changes in the P450 isoenzyme profile with time in culture may occur favouring activity towards P450IA substrates [5, 33, 34], no such changes in the profile of cytochrome P450-dependent activities could be

Table 3. Different cytochrome P450-dependent activities as percentage of the summation of measured activity, expressed per milligram cellular protein in intact monolayers of hepatocytes during culture (data are means; N = 3)

Time in culture (hr)	Percentage of the sum of measured activity										Sum of measured activity (pmol/min.mg protein)
	EROD	PROD	15β	7α	16β	2α	6β	16α	X	A	
4	0.3	0.5	1.3	1.0	1.5	7.3	30.2	14.1	2.2	41.7	1349
24	0.7	1.9	1.2	1.3	1.2	8.3	16.5	16.1	1.5	51.2	963
96	0.7	1.0	1.4	1.8	0.4	5.6	7.7	12.3	1.0	68.0	529

For abbreviations see legend to Fig. 1.

detected in our study using e.g. 7-ethoxyresorufin as a substrate [13].

If we consider the battery of determinations as a good representation of total biotransformation activities, a comparison can be made of the distribution of different catalytic activities in the course of culture time (Table 2). During culture the distribution of most catalytic activities appeared to remain constant. However, the formation of 6β-OHT and androstenedione, expressed as a percentage of the summation of determined activities, changed during the first 24 hr in culture, but remained stable from 24 to 96 hr in culture.

It was of interest to determine whether the changes in catalytic activities as measured for the formation of 6β-OHT and androstenedione were due to selective destruction of isoenzymes during microsome preparations. Furthermore, because of the low recovery of cytochrome P450 during preparations of microsomes from hepatocytes (30%), more direct methods of determination of metabolic activities in intact hepatocytes would be desirable. Enzyme assays were developed for EROD, PROD and testosterone hydroxylation without disrupting intact monolayers. The advantages are obvious. Because no disruption of cells is needed, the endoplasmic reticulum remains intact and no possible enzyme damage due to homogenization occurs [35]. Smaller amounts of cells can be used for a single measurement because no cell material is lost during harvesting and microsome preparation. Our results show that the metabolic activities in intact cells were *ca.* three times higher than in microsomes. Furthermore, no extra cofactors are needed [19]. Using only a balanced salt solution and substrate, without extra cofactors, we consider such enzyme assays with intact hepatocytes more representative of the "*in vivo*" situation. During enzyme assays in intact cells, however, substrates and products should be able to pass the membrane and possible conjugation reactions have to be taken into consideration [4]. In our study, no conjugates of testosterone hydroxy metabolites could be detected in intact hepatocytes while testosterone itself was glucuronidated. Glucuronidation of testosterone metabolites in intact hepatocytes is apparently prevented by competition with the high amount of testosterone in the assay.

The high EROD, PROD and testosterone hydroxylation activities in primary cultured cells as compared with microsomal incubations prepared from cultured cells is remarkable. Whether this is due to enzyme damage and/or disturbance of physiological "routes" for substrates or product release, is not clear. In spite of this difference in metabolic activity, the same rapid decline of catalytic activities during culture was measured in microsomes from cultured hepatocytes as in intact cells. A few discrepancies were seen. After 24 hr in culture the EROD and PROD activities in intact cells were about twice as high as in 4-hr-old cultures. Such a difference between catalytic activity in 4- and 24-hr-old primary cultures was not seen in the hydroxylation of testosterone. In agreement with our study, Edwards *et al.* [36] reported the same low activity in ethoxycoumarin deethylation (ECOD) activity in 4-hr-old primary cultures as compared to 24-hr-old cultures. From 24 to 96 hr in culture, however, the EROD, PROD and ECOD [36] activities decreased at the same rate as all the other activities determined. The low activities measured after 4 hr in culture may be caused by less optimal cellular uptake of this type of substrates immediately after cell attachment because of other processes demanding energy.

The formation of 7α-OHT in intact cells remained at the same level during culture, indicating no decline of P450IIA1 [15] during culture. This is in remarkable contrast with microsomal incubations derived from cultured cells. Testosterone hydroxylation at the 6β site is catalysed by several cytochromes P450 when purified, namely P450IA1, P450IA2, P450IIC13 and P450IIIA [2, 18]. In microsomes, however, no significant contributions of P450IA and P450IIC13 to 6β-hydroxylation of testosterone could be detected [18]. Therefore, the decline of 6β-OHT during the first 24 hr in culture probably does reflect a decrease in P450IIIA. The latter is in agreement with a decline of P450IIIA apoprotein level during culture. A diminishing level of P450IA2 during culture was also reported by Guengerich [12].

In conclusion, metabolic activities measured in intact cultured hepatocytes are much higher than in microsomal incubations. This is only partly due to a low recovery of cytochrome P450. The unimpaired endoplasmic reticulum, the absence of possible enzyme damage due to homogenization and/or the possible more rapid release of products may contribute to this discrepancy. Thus, measurements of biotransformation activities in intact monolayers can be a convenient and reliable method to determine P450-dependent metabolic profiles.

REFERENCES

1. Nebert DW and Gonzalez FJ, P450 genes: structure,

evolution, and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
2. Conney AH, Induction of microsomal cytochrome P-450 enzymes. *Life Sci* **39**: 2493–2518, 1986.
3. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
4. Moldeus P, Comparison of model systems for metabolism. In: *Drug Metabolism: from Molecules to Man* (Eds. Benford DJ, Bridges JW and Gibson GG), pp. 309–316. Taylor and Francis, London, 1987.
5. Guillouzo A, Use of isolated and cultured hepatocytes for xenobiotic and cytotoxicity studies. In: *Isolated and Cultured Hepatocytes* (Eds. Guillouzo A and Guguen-Guillouzo C), Chap. 14, pp. 313–332. John Libbey Eurotext Ltd/INSERM, Paris, 1986.
6. Engelmann GL, Richardson AG and Fierer JA, Maintenance and induction of cytochrome P-450 in cultured rat hepatocytes. *Arch Biochem Biophys* **238**: 359–367, 1985.
7. Dich J, Vind C and Grunnet N, Long-term culture of hepatocytes: effect of hormones on enzyme activities and metabolic capacity. *Hepatology* **8**: 39–45, 1988.
8. Turner NA and Pitot HC, Dependence of the induction of cytochrome P-450 by phenobarbital in primary cultures of adult rat hepatocytes on the composition of the culture medium. *Biochem Pharmacol* **38**: 2247–2251, 1989.
9. Paine AJ, Williams LJ and Legg RF, Apparent maintenance of cytochrome P-450 by nicotinamide in primary cultures of rat hepatocytes. *Life Sci* **24**: 2185–2192, 1979.
10. Paine AJ and Hockin LJ, Nutrient imbalance causes the loss of cytochrome P-450 in liver cell culture: formulation of culture media which maintain cytochrome P-450 at *in vivo* concentrations. *Biochem Pharmacol* **29**: 3215–3218, 1980.
11. Steward AR, Dannan GA, Guzelian PS and Guengerich FP, Changes in the concentration of seven forms of cytochrome P-450 in primary cultures of adult rat hepatocytes. *Mol Pharmacol* **27**: 125–132, 1985.
12. Guengerich FP, Factors involved in the regulation of the levels and activities of rat liver cytochrome P-450. *Biochem Soc Trans* **12**: 68–70, 1984.
13. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
14. Ryan DE, Iida S, Wood AW, Thomas PE, Lieber CS and Levin W, Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J Biol Chem* **260**: 6385–6393, 1985.
15. Levin W, Thomas PE, Ryan DE and Wood AW, Isozyme specificity of testosterone 7α-hydroxylation in rat hepatic microsomes: is cytochrome P-450a the sole catalyst? *Arch Biochem Biophys* **258**: 630–635, 1987.
16. Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
17. Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK and Parkinson A, Regulation of testosterone hydroxylation by rat liver microsomal P-450. *Arch Biochem Biophys* **255**: 27–41, 1987.
18. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones: regioselectivity and stereoselectivity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
19. Thurman RG and Kauffman FC, Factors regulating drug metabolism in intact hepatocytes. *Pharmacol Rev* **31**: 229–251, 1980.
20. Wortelboer HM, de Kruif CA, de Boer WI, van Iersel AAJ, Falke HE and Blaauboer BJ, Induction and activity of several isoenzymes of cytochrome P450 in primary cultures of rat hepatocytes, in comparison with *in vivo* data. *Mol Toxicol* **1**: 373–381, 1989.
21. Seglen PO, Preparation of isolated rat liver cells. In: *Methods in Cell Biology* (Ed. Prescott DM), Vol. XIII, pp. 29–78. Academic Press, New York, 1976.
22. Paine AJ, Williams LJ and Legg RF, Determinants of cytochrome P-450 in liver cell cultures. In: *The Liver: Quantitative Aspects of Structure and Function* (Eds. Preisig R and Bircher J), pp. 99–109. Editio Cantor, Aulendorf, 1979.
23. Rutten AAJJL, Falke HE, Catsburg JF, Topp R, Blaauboer BJ, van Holsteijn I, Doorn L and van Leeuwen FXR, Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. *Arch Toxicol* **61**: 27–33, 1987.
24. Burke MD and Mayer RT, Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxazone and a homologous series of its *n*-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* **45**: 243–258, 1983.
25. Lubet RA, Nims RW, Mayer RT, Cameron JW and Schechtman LM, Measurements of cytochrome P-450 dependent dealkylation of alkoxyphenoxazones in hepatic S9s and hepatocyte homogenates: effect of dicumarol. *Mutat Res* **142**: 127–131, 1985.
26. Chhabra RS, Gram TE and Fouts JR, A comparative study of two procedures used in the determination of hepatic microsomal aniline hydroxylation. *Toxicol Appl Pharmacol* **22**: 50–58, 1972
27. Imai Y, Ito A and Sato R, Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J Biochem* **60**: 417–428, 1966.
28. Boot JH, van Holsteijn CWM, Seinen W and Blaauboer BJ, Effects of sulphydryl reagents on the formation of the aniline metabolite 4-aminophenol and its sulphate and glucuronide conjugates in primary cultures of rat hepatocytes. *Xenobiotica* **19**: 1267–1273, 1989.
29. Funae Y and Imaoka S, Purification and characterization of liver microsomal cytochrome P-450 from untreated male rats. *Biochim Biophys Acta* **926**: 349–358, 1987.
30. Hoeven van der Th, Testosterone oxidation by rat liver microsomes: effects of phenobarbital pretreatment and the detection of seven metabolites by HPLC. *Biochem Biophys Res Commun* **100**: 1285–1291, 1981.
31. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
32. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
33. Guzelian PS, Bissell DM and Meyer UA, Drug metabolism in adult rat hepatocytes in primary monolayer culture. *Gastroenterology* **72**: 1232–1239, 1977.
34. Lake BG and Paine AJ, The effect of hepatocyte culture conditions on cytochrome P-450 linked drug metabolizing enzymes. *Biochem Pharmacol* **31**: 2141–2144, 1982.

35. Howell KE, Devaney E and Gruenberg T, Subcellular fractionation of tissue culture cells. *Trends Biochem Sci* **14**: 44–47, 1989.
36. Edwards AM, Glistak ML, Lucas CM and Wilson PA, 7-Ethoxycoumarin deethylase activity as a convenient measure of liver drug metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochem Pharmacol* **33**: 1537–1546, 1984.