0026-895X/87/04/0463-08$02.00/0

MOLECULAR PHARMACOLOGY, 32:463-470

Xenobiotic Metabolizing Enzymes Are Not Restricted to Parenchymal Cells in Rat Liver

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Received March 30, 1987; Accepted July 9, 1987

SUMMARY

To characterize the distribution and inducibility of drug metabolizing enzymes within different hepatic cell populations, the activities of aminopyrine *N*-demethylase, ethoxyresorufin *O*-deethylase, microsomal epoxide hydrolase and cytosolic glutathione transferase were measured in liver parenchymal, Kupffer, and endothelial cells isolated from untreated rats or rats pretreated with phenobarbital, 3-methylcholanthrene, or Aroclor 1254. Enzyme activities, measurable in all cases, were 2.3- to 5.7-fold higher in parenchymal cells than in Kupffer and endothelial cells. Phenobarbital increased aminopyrine *N*-demethylase, microsomal epoxide hydrolase, and cytosolic glutathione transferase activities, whereas 3-methylcholanthrene enhanced ethoxyresorufin *O*-deethylase, epoxide hydrolase, and glutathione transferase activities in the three cell populations. Aroclor 1254 consistently induced each of the enzyme activities in parenchymal, Kupffer, and endothelial cells. Western blot analyses revealed clear differences in the expression of proteins immunologically related to cytochrome P-450 PB-1, and glutathione transferases B and X in parenchymal cells compared with the corresponding Kupffer and endothelial cells. In contrast, only minor differences between the cell types were apparent in the expression of cytochromes P-450 PB-4, P-450 MC_{1a}, P-450 MC_{1b} and microsomal epoxide hydrolase. These studies establish that oxidative and postoxidative drug metabolizing enzymes are not restricted to parenchymal cells: similar but distinguishable complements of these enzymes are also found in Kupffer and endothelial cells.

The mammalian liver is composed of several different cell types. Parenchymal cells, which constitute about 90% of the total cell mass, only represent about 65% of the total cell number (1). The rest corresponds to nonparenchymal cells, mainly sinusoidal cells, but also hemopoietic, bile duct, and blood vessel wall cells. Four types of sinusoidal cells have been described: endothelial, Kupffer, fat-storing, and pit cells. They only account for about 6% of the total liver volume, but contribute considerably to the total number of liver cells (about 30%) (2, 3). Further, endothelial, Kupffer, and fat-storing cells contribute about 54, 29, and 17% of sinusoidal cells, respectively (4–6), whereas pit cells are very rare.

Parenchymal cells are involved in a) synthesis and secretion of several plasma proteins including albumin; b) carbohydrate, lipid, and amino acid metabolism; c) drug metabolism; d) bile secretion; e) elimination of nitrogen compounds after synthesis of urea. The main function of Kupffer cells is the removal of undesirable particles from the circulation (7, 8). Endothelial cells allow particles measuring up to 0.1 μm (e.g., chylomicrons and very low density lipoproteins) to enter the space of Disse through sieve-like fenestrae (9, 10). Both Kupffer and endothelial cells are important in the clearance of circulating lipoproteins, glycoproteins, lipopolysaccharides, and mucopolysaccharides and possess a well-developed lysosomal system for the degradation of these compounds (11, 12). Fat-storing cells are the main storage site of vitamin A in the liver (13) and may be involved in intralobular fibrogenesis (14, 15). Pit cells might have an endocrine function, as suggested by the morphology of their highly characteristic granules (16).

When a xenobiotic enters the hepatic circulation the first cells it encounters are the Kupffer and endothelial cells, which form the sinusoidal lining of the liver. Although the xenobiotic metabolizing capacity of parenchymal cells has been extensively investigated, there is not much information about the presence of drug metabolizing enzymes in nonparenchymal cells. Cantrell and Bresnick (17) showed that benzo(a)pyrene hydroxylase was present in rat liver nonparenchymal cells, although to a much lesser extent than in parenchymal cells; furthermore, this enzyme activity could be increased in both cell populations by previous administration of 3-methylcholanthrene or β-naphtoflavone. Govier (18) and Morland and

This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 302), the Alexander von Humboldt Foundation (P.S., W.M.L.), and the American Cancer Society (D.J.W.)

ABBREVIATIONS: GBSS, Gey's balanced salt solution; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Olsen (19) reported that nonparenchymal cells could acetylate several compounds (e.g., *p*-aminobenzoic acid and sulfonamides). Since the enzyme patterns in individual liver cell types is expected to represent an important factor in the control of cell-type specific toxicity, mutagenicity, and carcinogenicity, we investigated the distribution and inducibility of drug metabolizing enzymes within different cell populations of the rat liver, specifically the activities of aminopyrine *N*-demethylase, ethoxyresorufin *O*-deethylase, microsomal epoxide hydrolase, and cytosolic glutathione transferase in isolated liver parenchymal, Kupffer, and endothelial cells from untreated, phenobarbital-, 3-methylcholanthrene-, and Aroclor 1254-pretreated rats. Further, a qualitative analysis of proteins immunochemically[1] related to cytochromes P-450[2] PB-1, P-450 PB-4, P-450 MC_{1a}, and P-450 MC_{1b}, cytosolic glutathione transferases B and X, and microsomal epoxide hydrolase present in the three cell populations was performed by Western blotting.

Materials and Methods

Chemicals. Pronase E and phenobarbital were purchased from Merck (Darmstadt, FRG); collagenase from Boehringer (Mannheim, FRG); aminopyrine from Aldrich (Steinheim, FRG); ethoxyresorufin from Pierce (Rodgau, FRG); Nycodenz from Molter (Bammental, FRG); sodium dodecyl sulfate, diaminobenzidine, and bismethyleneacrylamide from Sigma (Taufkirchen, FRG); acrylamide from Serva (Heidelberg, FRG); nitrocellulose sheets from Schleicher and Schuell (Dassel, FRG); and horseradish peroxidase-conjugated antirabbit and antigoat immunoglobulins from Dakopats (Hamburg, FRG) and Paesel (Munich, FRG). Aroclor 1254 was a generous gift from Bayer AG (Leverkusen, FRG). [^{3}H]Benzo(a)pyrene4,5-oxide was synthesized as previously described (24) and had a specific activity of 1.2 mCi/mmol. All other chemicals employed were of the highest purity available.

Animals and pretreatments. Male Sprague-Dawley rats (200–240 g body wt) were purchased from Süddeutsche Versuchstierfarm (Tuttlingen, FRG), housed four per cage, and allowed free access to water and food until used. Phenobarbital in 0.85% NaCl was administered intraperitoneally for 3 consecutive days (100 mg/kg body wt/day). 3-Methylcholanthrene in corn oil was administered intraperitoneally for 3 consecutive days (25 mg/kg body wt/day). Aroclor 1254 in corn oil was administered as a single intraperitoneal dose (500 mg/kg body wt) 5 days before killing. Untreated rats received appropriate volumes of corn oil.

Isolation of rat liver parenchymal, Kupffer, and endothelial cells. Parenchymal and sinusoidal lining cells were isolated from different animals. The two sinusoidal lining cell types (Kupffer and endothelial cells) were isolated from the same livers.

Total liver cell suspensions were prepared using a collagenase perfusion method described by Glatt *et al.* (25), and parenchymal cells were isolated from the total liver cell suspension by differential centrifugation (26).

Nonparenchymal cell suspensions were prepared essentially as described by Knook and co-workers (27–29) by using pronase E, an enzyme that selectively destroys parenchymal cells (30). Briefly, the liver was first perfused *in situ* through the portal vein with GBSS at 37°C for 6 min at a constant flow rate of 10 ml/min; this was followed by a perfusion with 0.2% wt/vol pronase E dissolved in GBSS for 6 min. The liver was carefully excised, placed on a sieve, connected to a recirculating perfusion system, and perfused with 0.05% wt/vol collagenase and 0.05% wt/vol pronase E dissolved in GBSS for 30 min at 37°C. After perfusion, Glisson's capsule was removed and the partially digested contents were incubated for 30 min at 4°C (to inhibit the endocytosis of parenchymal cell debris by the nonparenchymal cells) in 100 ml GBSS containing 0.02% wt/vol pronase E and 0.05% collagenase. The pH of the mixture was monitored throughout the incubation and maintained at pH 7.4 with 5 N NaOH. The suspension was filtered through nylon gauze, and the filtrate was centrifuged at 300 *g* for 5 min. The nonparenchymal cells in the pellet were freed from erythrocytes and parenchymal cell debris by the following procedure. The cell pellet was resuspended in GBSS to a final volume of 11 ml and mixed with 14 ml GBSS without NaCl but containing 28.7% wt/vol Nycodenz to yield a final concentration of 17.2% wt/vol Nycodenz; this layer was covered with 2 ml GBSS. After centrifugation at 400 × *g* for 15 min at room temperature, the top of the 17.2% wt/vol Nycodenz layer was highly enriched with Kupffer and endothelial cells.

Kupffer and endothelial cells were further purified by centrifugal elutriation (28). A JE-6B elutriator rotor with a standard separation chamber (Beckman Instruments, Palo Alto, CA) was used in a J-6M/E Beckman centrifuge at a speed of 2550 rpm. The rotor, the elutriation medium (GBSS containing 0.5% wt/vol bovine serum albumin), and the samples collected during the separation were kept at 4°C to preserve the integrity of the cells. The nonparenchymal cells were loaded into the elutriation system with an initial flow rate of 10 ml/min. Lymphocytes were washed out of the separation chamber by increasing the flow rate to 13.5 ml/min. The endothelial and Kupffer cell fractions were obtained by using flow rates of 23 and 46 ml/min, respectively; for each fraction 150 ml eluate were collected. The fractions were centrifuged at 400 × *g* for 5 min, the supernatants discarded, the cell pellets frozen in liquid nitrogen and stored at −70°C until used.

Twelve isolations of Kupffer and endothelial cells from untreated, phenobarbital-, 3-methylcholanthrene-, and Aroclor 1254-pretreated rats were performed. For the enzyme assays ten samples of Kupffer and endothelial cells from each group were split into five pairs ($n = 5$). The remaining samples were used for the Western blotting. All determinations were performed in cell homogenates obtained by sonicating the cells for 30 sec at 60% duty cycle on a Branson cell disruptor (model B-15).

Viability, yield, purity, and characterization of the isolated rat liver cells. Cell viability was estimated by the capacity of the cells to exclude 0.25% trypan blue and at the cellular ultrastructure as observed by electron microscopy. Cell counts were performed with a hemocytometer. Kupffer cells, endothelial cells, and lymphocytes present in the different cell fractions were distinguished from each other by light microscopy after they had been stained for peroxidase and nonspecific esterase: in the rat, peroxidase is found exclusively in Kupffer cells (7) whereas, although esterase activity is present in all liver cells, lymphocytes do not exhibit this activity (31). Fat-storing cells were identified by fluorescence microscopy (4). The percentages of lymphocytes, Kupffer, endothelial, and fat-storing cells in the different cell fractions were afterwards confirmed by transmission electron microscopy as previously described (32).

To further verify that no contamination of the Kupffer and endothelial cell fractions were parenchymal cells or parenchymal cell debris occurred during the isolation procedure, parenchymal cells from 3-methylcholanthrene pretreated rats (thus possessing a high ethoxyresorufin *O*-deethylase activity) were added a) to the total liver cell suspensions from untreated rats during the 30-min *in vitro* incubation with pronase E and collagenase, b) to the nonparenchymal cell suspensions from untreated rats immediately before loading the elutriation chamber. In both cases, no increase of ethoxyresorufin *O*-deethylase activity in the elutriated Kupffer and endothelial cell fractions was observed (Table 1).

Enzyme assays. In all assays the amount of product was linear with both time and protein concentration. Aminopyrine *N*-demethylase

[1] The term "immunologically related protein" is used to designate those proteins in parenchymal, Kupffer, and endothelial cells that react on Western blots with antisera against proteins purified from the livers of male Sprague-Dawley rats. These studies do not, however, establish the precise degree of homology between immunoreactive proteins of the same apparent molecular weight thus detected in the different cell types.

[2] Cytochromes P-450 PB-4, P-450 MC_{1a}, and P-450 MC_{1b} identify major rat liver hemoproteins inducible by phenobarbital, isosafrole, and 3-methylcholanthrene, respectively (20–22). Cytochrome P-450 PB-1 is present in significant amounts in uninduced rat livers and is also inducible by phenobarbital (23).

TABLE 1

Quality control of sinusoidal lining cell isolation procedure

Ethoxyresorufin *O*-deethylase activity was measured in elutriated rat liver Kupffer and endothelial cells from untreated animals after addition of 20 × 10^6 liver parenchymal cells from 3-methylcholanthrene-pretreated rats a) to the total liver cell suspensions from untreated rats during the 30 min *in vitro* incubation with pronase E and collagenase and b) to the nonparenchymal cell suspensions from untreated rats immediately before loading the elutriation chamber. Values are expressed as means ± SD of four experiments per group.

Experimental condition	Cell type	Ethoxyresorufin *O*-deethylase activity
		nmol/min/mg protein
No addition	Kupffer	9 ± 3
	endothelial	7 ± 2
Addition during *in vitro* incubation	Kupffer	8 ± 3
	endothelial	9 ± 3
Addition before elutriation	Kupffer	9 ± 4
	endothelial	8 ± 2

activity was measured by determining formaldehyde production as described by Mazel (33). Ethoxyresorufin *O*-deethylase activity was measured fluorimetrically according to the method of Burke and Mayer (34). Epoxide hydrolase activity was determined radiometrically with [^{3}H]Benzo(a)pyrene4,5-oxide (35). Glutathione transferase activity was measured spectrophotometrically according to the method of Habig *et al.* (36) using 1-chloro-2,4-dinitrobenzene as substrate. Proteins were measured by the method of Lowry *et al.* (37) with bovine serum albumin as standard.

Gel electrophoresis. For SDS-PAGE the cells were thawed, resuspended in 10 mM potassium phosphate (pH 7.5) containing 5 mM EDTA, sonicated, and boiled in SDS-PAGE sample solubilization buffer (38). SDS-PAGE was performed according to Laemmli (38) with the following modifications: the stacking gel was 5% acrylamide and 0.1% bisacrylamide in 0.058 M Tris/phosphate (pH 6.7), 0.1% SDS, and the separating gel was 10% acrylamide and 0.2% bisacrylamide (15% acrylamide in the case of glutathione transferases B and X) in 0.38 M Tris/Cl (pH 8.9), 0.1% SDS. The transfer of proteins to nitrocellulose sheets and the immunological detection of proteins on nitrocellulose were performed as described previously (39, 40). Silver staining was done according to Oakley *et al.* (41). Western blot analyses were performed on parenchymal, Kupffer, and endothelial cells derived from two individual animals from each experimental group; no qualitative differences were found in the cells isolated from the two rats within each group.

Antibody preparations. Cytochrome P-450 isoeyzmes (PB-1, PB-4, MC_{1a}, and MC_{1b}), cytosolic glutathione transferases B and X, and microsomal epoxide hydrolase were purified from the livers of male Sprague-Dawley rats, and antibodies to these proteins were raised in rabbits using methods previously described (20–23). Cytochrome P-450 PB-1 is equivalent to cytochrome P-450 PB-C (42); cytochrome P-450 PB-4 corresponds to the major phenobarbital-inducible isoenzyme purified by other groups and termed cytochrome P-450 PB-B (43), P-450b (21), and P-450 fraction C (44). Cytochromes P-450 MC_{1a} and P-450 MC_{1b} correspond to the earlier described cytochromes P-450d (22) and P-450c (21), respectively, as judged by N-terminal sequence analyses and substrate specificities (C.R. Wolf, personal communication).

Statistics. Statistical analysis of the results was performed using Dunnett's test for multiple comparisons with a control (45).

Results

Characteristics of isolated cell populations. The yield, viability, purity, and protein content of parenchymal, Kupffer, and endothelial cells isolated from untreated rat livers are given in Table 2.

Based on previous reports in the literature (27, 28), parenchymal and nonparenchymal cells were isolated from different animals, to obtain higher yields of the latter. The total yield of isolated parenchymal cells after collagenase digestion of the liver was 264 ± 35 × 10^6 cells/rat liver (n = 4), which included 2 ± 1% endothelial cells, 3 ± 2% Kupffer cells, and 5 ± 4% fat-storing cells. Nonparenchymal liver cells were prepared from total liver cell suspension by incubation with pronase E, an enzyme that selectively destroys parenchymal cells (30). The nonparenchymal cell suspension used for the separation of Kupffer and endothelial cells by centrifugal elutriation contained 110 ± 24 × 10^6 cells/rat liver (n = 12; 59 ± 3% endothelial cells, 31 ± 3% Kupffer cells, and 8 ± 2% fat-storing cells). The recovery of Kupffer and endothelial cells after elutriation was 90 ± 4% and 87 ± 5%, respectively. The endothelial cell fractions were contaminated with 8 ± 3% lymphocytes and 4 ± 2% Kupffer cells. The Kupffer cell preparations were found to contain 10 ± 4% endothelial cells, 3 ± 2% fat-storing cells, and 0.2 ± 0.1% parenchymal cells.

Pretreatment of the animals with phenobarbital, 3-methylcholanthrene or Aroclor 1254 did not affect the yield, viability, and purity of the isolated parenchymal, endothelial, and Kupffer cell fractions. Aroclor 1254 significantly increased the protein concentrations of parenchymal, Kupffer, and endothelial cells by about 80, 30, and 25%, respectively; phenobarbital and 3-methylcholanthrene only increased protein concentration in the parenchymal cell population by about 25 and 15%, respectively.

Fig. 1 shows SDS-PAGE performed with rat liver parenchymal, Kupffer, and endothelial cell homogenates (2 μg protein/lane) from control, phenobarbital-, 3-methylcholanthrene-, and Aroclor 1254-pretreated rats; the protein bands were visualized by a silver staining technique (41). Parenchymal cells (Fig. 1, lanes *A–D*) possessed proteins that were barely or not at all detected in Kupffer and endothelial cells. However, both sinusoidal lining cell types (Fig. 1, lanes *F–M*) possessed several proteins in the low molecular weight region that were stained to a greater extent than in parenchymal cells. Cells from untreated and induced animals yielded similar protein banding patterns.

Aminopyrine N-demethylase activity and the presence of cytochromes P-450 PB-1 and P-450 PB-4 related proteins in isolated rat liver cells. Aminopyrine N-demethylase activity was three to five times higher in parenchymal cells than in Kupffer and endothelial cells derived from untreated animals (Table 3). After administration of phenobarbital or Aroclor 1254, a polychlorinated biphenyl mixture that exhibits both phenobarbital- and 3-methylcholanthrene-inducing properties (46), aminopyrine *N*-demethylase activity increased in parenchymal cells (about 5-fold) as well as in Kupffer (10- to 13-fold) and endothelial cells (5- to 9-fold).

Cytochrome P-450 PB-4 identifies a major rat liver hemoprotein inducible by phenobarbital (20). Antibodies against cytochrome P-450 PB-4 cross-react with cytochrome P-450 PB-5 due to the immunochemical relatedness of these two hemoproteins (20); cytochrome P-450 PB-5 is a minor form of cytochrome P-450 inducible by phenobarbital (20) and is equivalent to cytochrome P-450e (47). Rabbit antiserum against cytochrome P-450 PB-4 recognized two protein bands present at low levels in parenchymal but not in Kupffer or endothelial cells from untreated animals (Fig. 2A, lanes *a*, *e*, and *i*). After administration of phenobarbital or Aroclor 1254 two intensely

TABLE 2

Characteristics of the liver cell populations isolated from untreated rats

Cells were isolated as described in Materials and Methods. Values represent means ± SD of the number of rats given. Protein content is expressed as micrograms per 10^6 cells present in each cell fraction.

Cell type	No. of rats	Yield	Viability	Purity	Protein content
		$\times 10^6$ cells/rat liver	%		$\mu g/10^6$ cells
Parenchymal	4	264 ± 35	85 ± 7	90 ± 3	1478 ± 156
Endothelial	12	59 ± 10	95 ± 3	88 ± 4	69 ± 18
Kupffer	12	25 ± 6	93 ± 2	86 ± 4	109 ± 25

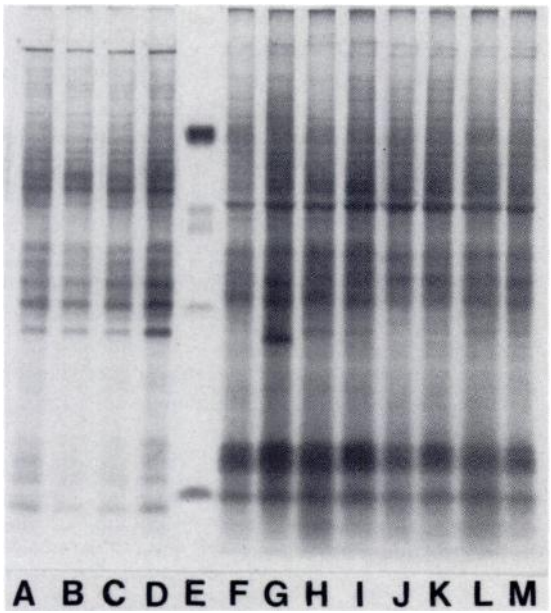

Fig. 1. SDS-PAGE profile of liver parenchymal, Kupffer, and endothelial cells isolated from untreated and induced rats. Each lane contains 1 μg total cell protein. *A–D*, parenchymal cells from untreated, phenobarbital-, Aroclor 1254-, and 3-methylcholanthrene-pretreated rats; *F–I*, Kupffer cells from untreated, phenobarbital-, Aroclor 1254, and 3-methylcholanthrene-pretreated rats; *J–M*, endothelial cells from untreated, phenobarbital-, Aroclor 1254-, and 3-methylcholanthrene-pretreated rats. A mixture of the following molecular weight marker proteins (1 μg each) was applied to the standard track E (from top to bottom): aldolase (M_r 15,8000), bovine serum albumin (M_r 68,000), chicken egg albumin (M_r 43,000), and chymotrypsinogen A (M_r 25,000). Gel electrophoresis and staining were performed as described in Materials and Methods.

stained bands were observed in all three cell types (Fig. 2A, lanes *b*, *c*, *f*, *g*, *j*, and *k*); the upper band corresponds to cytochrome P-450 PB-5, the lower band to cytochrome P-450 PB-4 (Fig. 3A). The induced protein bands with the lower molecular weight were electrophoretically distinct from the one expressed in parenchymal cells isolated from untreated and 3-methylcholanthrene-pretreated animals.

Cytochrome P-450 PB-1 is another isoenzyme inducible by phenobarbital (23). In parenchymal cells from untreated rats two protein bands immunochemically related to cytochrome P-450 PB-1 were observed (Fig. 2B, lane *a*); pretreatment of the animals with phenobarbital or Aroclor 1254 increased the staining intensity of the lower band, whereas 3-methylcholanthrene slightly increased that of the upper band (Fig. 2B, lanes *b–d*). However, in Kupffer and endothelial cells from control animals the band of higher molecular weight was either absent or present at much reduced levels (Fig. 2B, lanes *e* and *i*). The amount of the lower molecular weight protein in sinusoidal lining cells was very low when compared with parenchymal cells. After administration of phenobarbital or Aroclor 1254 the amount of the cytochrome P-450 PB-1-related protein in Kupffer and endothelial cells clearly increased, and another band with a much lower molecular weight was detected (Fig. 2B, lanes *f*, *g*, *j*, and *k*).

Ethoxyresorufin O-deethylase activity and the presence of cytochromes P-450 MC_{1a} and P-450 MC_{1b} related proteins in isolated rat liver cells. Microsomal *O*-deethylation of ethoxyresorufin is primarily catalyzed by those forms of cytochrome P-450 that are inducible by 3-methylcholanthrene (34). In untreated rats, ethoxyresorufin *O*-deethylase activity was five times higher in parenchymal cells than in Kupffer and endothelial cells (Table 3). Pretreatment of the animals with 3-methylcholanthrene or Aroclor 1254 induced ethoxyresorufin *O*-deethylase to a greater extent in parenchymal cells (24- to 27-fold) than in Kupffer (9-fold) or endothelial cells (7-fold).

Cytochrome P-450 MC_{1b} is a major rat liver hemoprotein inducible by 3-methylcholanthrene (21). The antibodies against cytochrome P-450 MC_{1b} used in this study show a very weak cross-reactivity with cytochrome P-450 MC_{1a} (Fig. 3C, lane *b*). No cytochrome P-450 MC_{1b}-related protein was observed in parenchymal, Kupffer, and endothelial cells from control or phenobarbital-pretreated rats (2C, lanes *a*, *b*, *e*, *f*, *i*, and *j*). After administration of 3-methylcholanthrene or Aroclor 1254 a major protein band was detected in all three cell types (Fig. 2C, lanes *c*, *d*, *g*, *h*, *k*, and *l*), and 3-methylcholanthrene seemed to induce this protein to a greater extent than did Aroclor 1254 in all three cell types. Two additional protein bands were observed in parenchymal cells from Aroclor 1254-pretreated rats (Fig. 2C, lane *d*); the protein with the lower molecular weight might be cytochrome P-450 MC_{1a} (Fig. 3C).

Cytochrome P-450 MC_{1a} and P-450 MC_{1b} share some but not all immunochemical determinants (48) and the rabbit antiserum against purified cytochrome P-450 MC_{1a} used in this study cross-reacts with cytochrome P-450 MC_{1b}. No cytochrome P-450 MC_{1a}-related protein was detected in the isolated liver cells of control or phenobarbital pretreated rats (Fig. 2D, lanes *a*, *b*, *e*, *f*, *i*, and *j*). After 3-methylcholanthrene or Aroclor 1254 treatment two bands were observed in all three cell types, but the intensity of the band corresponding to the higher molecular weight component in the sinusoidal lining cells was very low (Fig. 2D, lanes *c*, *d*, *g*, *h*, *k*, and *l*). The upper band recognized by the antibodies against cytochrome P-450 MC_{1a} in the parenchymal cells has been identified as cytochrome P-450 MC_{1b} (Fig. 3B).

Glutathione transferase activity and the presence of

TABLE 3

Drug metabolizing enzyme activities in parenchymal, Kupffer, and endothelial cells isolated from untreated rats or rats induced with phenobarbital-, 3-methylcholanthrene-, or Aroclor 1254

All enzyme assays were conducted in broken cell preparations. Values are expressed as means ± SD of four rats per treatment in the case of parenchymal cells, while ten samples of Kupffer and endothelial cells from each group were split into five pairs ($n = 5$).

Enzyme assay	Cell type	Untreated	Phenobarbital	3-Methylcholanthrene	Aroclor 1254
Aminopyrine N-demethylase[a]	parenchymal	15 ± 3	76 ± 9[c]	15 ± 4	67 ± 14[c]
	Kupffer	3 ± 2	39 ± 6[c]	2 ± 1	37 ± 6[c]
	endothelial	5 ± 2	44 ± 6[c]	8 ± 3	27 ± 8[c]
Ethoxyresorufin O-deethylase[b]	parenchymal	54 ± 12	67 ± 8	1317 ± 159[c]	1478 ± 287[c]
	Kupffer	10 ± 6	8 ± 3	89 ± 10[c]	95 ± 13[c]
	endothelial	10 ± 4	11 ± 5	67 ± 5[c]	72 ± 11[c]
Glutathione transferase[a]	parenchymal	1054 ± 138	1796 ± 278[c]	1520 ± 190[c]	1870 ± 359[c]
	Kupffer	301 ± 72	681 ± 155[c]	597 ± 123[c]	578 ± 101[c]
	endothelial	420 ± 119	789 ± 93[c]	692 ± 138[c]	774 ± 122[c]
Epoxide hydrolase[b]	parenchymal	3368 ± 479	5385 ± 614[c]	4479 ± 560[c]	5100 ± 716[c]
	Kupffer	592 ± 138	1562 ± 267[c]	944 ± 187[c]	1637 ± 203[c]
	endothelial	829 ± 145	1621 ± 194[c]	1107 ± 118[c]	1716 ± 220[c]

[a] Activity is expressed as nmol product formed/min/mg protein.
[b] Activity is expressed as pmol product formed/min/mg protein.
[c] Indicates significantly different from the corresponding control value ($p < 0.05$, Dunnett's test).

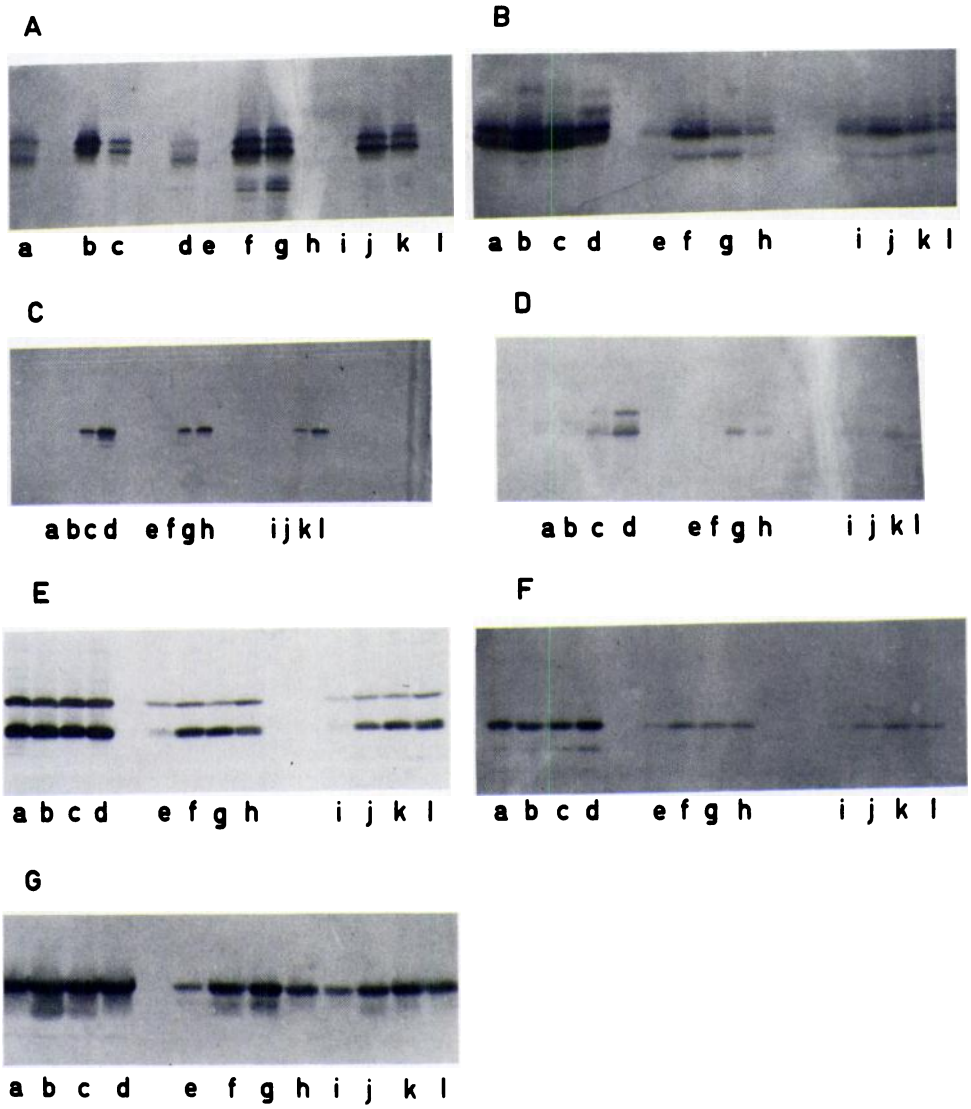

Fig. 2. SDS-PAGE profile of proteins immunologically related to cytochrome P-450 and cytosolic glutathione transferase isoenzymes and microsomal epoxide hydrolase in liver parenchymal, Kupffer, and endothelial cells from untreated and induced rats. Western blots of antibodies to cytochromes P-450 PB-4 (*panel A*), P-450 PB-1 (*panel B*), P-450 MC_{1b} (*panel C*), and P-450 MC_{1a} (*panel D*), glutathione transferase B(*panel E*), glutathione transferase X (*panel F*), and microsomal epoxide hydrolase (*panel G*), respectively, with parenchymal cells from untreated, phenobarbital-, Aroclor 1254- and 3-methylcholanthrene-pretreated rats (lanes *a–d*), Kupffer cells from untreated, phenobarbital-, Aroclor 1254, and 3-methylcholanthrene-pretreated rats (lanes *e–h*), and endothelial cells from untreated, phenobarbital-, Aroclor 1254-, and 3-methylcholanthrene-pretreated rats (lanes *i–l*); 6 μg of total parenchymal cell protein from Aroclor- and PB-induced rats were used in the Western blots exposed to antibodies to cytochrome P-450 PB-4 (*panel A, lanes a–d*), whereas all other lanes contain 60 μg total cell protein. Protein migration was from top to bottom. Gel electrophoresis and staining were performed as described in Materials and Methods.

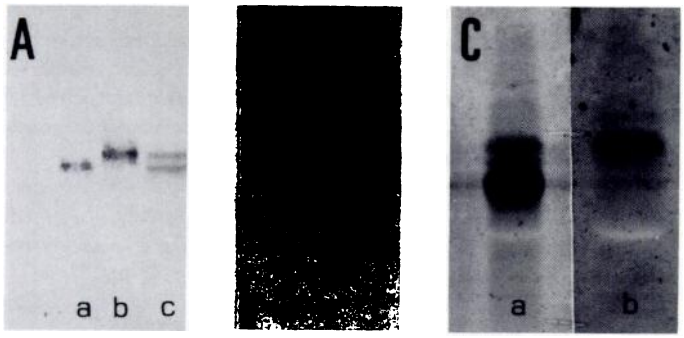

Fig. 3. Identification of several cytochrome P-450 isoenzymes present in liver parenchymal cells from Aroclor 1254-pretreated rats. Samples of SDS-solubilized total liver parenchymal cell protein (60 μg) from Aroclor 1254-pretreated rats and purified preparations of cytochrome P-450 forms (0.1 μg each) were analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. Protein migration was from top to bottom. *Panel A*, lanes *a*, *b*, and *c* contained cytochrome P-450 PB-4, cytochrome P-450 PB-5, and total parenchymal cell protein, respectively; the blot was incubated with antibodies to cytochrome P-450 PB-4. *Panel B*, lanes *a*, *b*, and *c* contained cytochrome P-450 MC_{1b}, cytochrome P-450 MC_{1a}, and total parenchymal cell protein, respectively; the blot was incubated with a mixture of antibodies to cytochromes P-450 MC_{1a} and P-450 MC_{1b}. *Panel C*, lanes *a* and *b* contained total parenchymal cell protein; lane *a* was incubated with antibodies to cytochrome P-450 MC_{1a} and lane *b* with antibodies to cytochrome P-450 MC_{1b}.

glutathione transferases B and X related proteins present in isolated rat liver cells. Glutathione transferase activity was measured with 1-chloro-2,4-dinitrobenzene as substrate. In untreated animals, glutathione transferase activity was 2.5 to 3.5 times higher in parenchymal cells than in sinusoidal lining cells (Table 3); endothelial cells possessed a higher glutathione transferase activity than Kupffer cells. Phenobarbital, 3-methylcholanthrene, and Aroclor 1254 enhanced glutathione transferase activity to a similar extent (1.5- to 2.3-fold) in all three cell types.

The "B-type" glutathione transferases are composed of the possible combinations of the subunits 1 (Ya; M_r 25,600) and 2 (Yc; M_r 28,000) (49, 50), corresponding to glutathione transferases 1-1, 1-2, and 2-2 in the nomenclature of Jakoby *et al.* (51). Antibodies against glutathione transferase B recognized two protein bands in all cell preparations (Fig. 2E), the upper band corresponding to the subunit Yc, the lower band to the subunit Ya. Lower amounts of these proteins were found in Kupffer and endothelial cells than in parenchymal cells from untreated animals (Fig. 2E, lanes *a*, *e*, and *i*). Phenobarbital, Aroclor 1254, or 3-methylcholanthrene did not significantly increase the staining intensity of either band in parenchymal cells (Fig. 2E, lanes *b–d*), whereas all three inducers increased the staining intensity of the lower molecular weight protein in Kupffer and endothelial cells (Fig. 2E, lanes *e–l*).

Glutathione transferase X is a homodimer composed of Yb subunits (52, 53). In all cases antibodies against glutathione transferase X yielded one band. In untreated animals, Kupffer and endothelial cells had lower levels of the glutathione transferase X-related protein than parenchymal cells (Fig. 2F, lanes *a*, *e*, and *i*). Phenobarbital, Aroclor 1254, and 3-methylcholanthrene increased the amount of this protein in the sinusoidal lining cells, whereas no significant induction was observed in parenchymal cells (Fig. 2F, lanes *b*, *c*, *d*, *f*, *g*, *h*, *j*, *k*, and *l*).

Epoxide hydrolase activity and the presence of microsomal epoxide hydrolase related protein in isolated rat liver cells. Epoxide hydrolase activity as determined by using benzo(a)-pyrene 4,5-oxide as substrate was higher in parenchymal cells than in Kupffer and endothelial cells (5.6- and 4.2-fold differences, respectively) (Table 3); although not statistically significant, endothelial cells possessed a higher epoxide hydrolase activity than Kupffer cells. Phenobarbital and Aroclor 1254 enhanced epoxide hydrolase activity to a greater extent than 3-methylcholanthrene in all three cells types. Furthermore, after administration of phenobarbital or Aroclor 1254, the increase of epoxide hydrolase activity was greater in the sinusoidal lining cells (2.0- to 2.8-fold) than in parenchymal cells (1.5-fold).

In the rat liver two forms of microsomal epoxide hydrolase have been identified (54, 55): microsomal epoxide hydrolase ch, which catalyzes the hydrolysis of cholesterol 5α,6α-oxide, and microsomal epoxide hydrolase b, with a high activity toward benzo(a)pyrene 4,5-oxide and styrene oxide. In addition, a cytosolic epoxide hydrolase with a high activity toward trans-stilbene oxide has been described (56). The antiserum used in this study reacts with microsomal epoxide hydrolase b but not with microsomal epoxide hydrolase ch or cytosolic epoxide hydrolase (55). Lower amounts of the microsomal epoxide hydrolase b-related protein were detected in Kupffer and endothelial cells than in parenchymal cells from control rats (Fig. 2G, lanes *a*, *e* and *i*). Phenobarbital, Aroclor 1254, and 3-methylcholanthrene induced this protein to a greater extent in Kupffer and endothelial cells than in parenchymal cells (Fig. 2G, lanes *b*, *c*, *d*, *f*, *g*, *h*, *j*, *k*, and *l*).

Discussion

The methods of cell isolation used in these experiments yielded Kupffer and endothelial cells populations that were essentially free from whole parenchymal cells and parenchymal cell debris. This was essential because of the generally high specific activity of parenchymal cell enzymes and the relatively low activities expected in the sinusoidal lining cells. The yield, purity, viability, and protein content of the sinusoidal lining cells isolated by pronase E treatment of the liver followed by centrifugal elutriation were similar to those obtained with other methods based on the use of pronase E and have been reported in the literature (13, 28, 29).

The cytochrome P-450 assays used in this study were selected according to their specificity for particular forms of the enzyme. Ethoxyresorufin *O*-deethylase activity is preferentially catalyzed by the forms of cytochrome P-450 that are inducible by 3-methylcholanthrene (34), whereas aminopyrine N-demethylation activity is more indicative of those forms of cytochrome P-450 that are inducible by phenobarbital (33). These two assays therefore provide a general indication of the corresponding cytochrome P-450 isozymes in parenchymal, Kupffer, and endothelial cells. In untreated animals, oxidative (aminopyrine N-demethylase and ethoxyresorufin O-deethylase) and postoxidative (epoxide hydrolase and glutathione transferase) enzyme activities were higher in parenchymal than in Kupffer or endothelial cells. In both sinusoidal lining cell types the oxidative enzyme activities were low, whereas the activities of the postoxidative enzymes were relatively high; furthermore, although no cytochrome P-450 PB-4, P-450 MC_{1a}, or P-450 MC_{1b} immunologically related proteins could be detected in Kupffer and endothelial cells isolated from untreated rats, Western blot analyses showed the presence of epoxide hydrolase and glutathione transferase-related proteins in both cell types. Hence,

Kupffer and endothelial cells may have a lower ability to oxidize xenobiotics to reactive electrophiles and a greater ability to conjugate or hydrolyze those products that might be formed.

The observations made in this study are consistent with previous immunohistochemical studies on the localization of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 within livers of untreated rats (57, 58): neither cytochrome P-450 PB-4 nor cytochrome P-450 MC_{1b} were detected in Kupffer and endothelial cells. However, although epoxide hydrolase and glutathione transferase activities have been measured and proteins immunochemically related to epoxide hydrolase and glutathione transferase have been recognized by Western blotting in Kupffer and endothelial cells, immunohistochemically it has not been possible to detect epoxide hydrolase (59) or glutathione transferase (60) within livers of untreated rats. This may be due to the fact that the concentrations of both enzymes per cell are below the detection limit of immunohistochemical techniques.

Phenobarbital induces *de novo* biosynthesis of several distinct isoenzymes of cytochrome P-450 in the mammalian liver, among them cytochrome P-450 PB-1 (23), P-450 PB-4 (20), and P-450 PB-5 (20). On administration of phenobarbital or Aroclor 1254 two cytochrome P-450 PB-4-related proteins were detected and aminopyrine N-demethylase activity increased in parenchymal, Kupffer, and endothelial cells; this increase is probably due to the induction of cytochrome P-450 PB-4 and not of P-450 PB-1, since purified cytochrome P-450 PB-4 catalyzes the N-demethylation of aminopyrine at about 10-fold the rate of cytochrome P-450 PB-1 (61).

Two cytochrome P-450 PB-1-related proteins were detected in parenchymal cells from untreated rats. Friedberg et al. (62) isolated two cDNA clones with an about 70% overall homology. The shorter cDNA hybrid-selected a mRNA that coded for a protein with a mobility on SDS gel equivalent to that of cytochrome P-450 PB-1, whereas the other cDNA selected a mRNA coding for a protein that was immunologically related to cytochrome P-450 PB-1 but had a higher molecular weight. This hemoprotein might be cytochrome P-450f (63): Gonzalez *et al.* (64) showed that cytochromes P-450 PB-1 (M_r = 55,400) and P-450f (M_r = 56,200) contain 490 amino acids and share 75% similarity with each other. Two cytochrome P-450 PB-1-related proteins were also observed in Kupffer and endothelial cells. One of these bands was of a distinctly lower apparent molecular weight; it cannot be eliminated that this protein is a degradation product. The other protein had a slightly different mobility in SDS-PAGE when compared with the two cytochrome P-450 PB-1-related proteins observed in parenchymal cells. It will be interesting to establish by S_1 nuclease experiments if there are differences in the mRNA populations coding for cytochrome P-450 PB-1-related proteins in parenchymal and sinusoidal lining cells.

Cytochrome P-450 MC_{1b}, the major rat liver 3-methylcholanthrene-inducible form of cytochrome P-450, catalyzes the *O*-deethylation of ethoxyresorufin (34). Although no band immunologically related to cytochrome P-450 MC_{1b} could be detected by SDS-PAGE in parenchymal, Kupffer, or endothelial cells from untreated rats, a distinct band was observed in parenchymal and sinusoidal lining cells after administration of 3-methylcholanthrene or Aroclor 1254. Although the staining intensity of this cytochrome P-450 MC_{1b} immunorelated protein was similar in all three cell types, after administration of 3-methylcholanthrene or Aroclor 1254, ethoxyresorufin *O*-deethylation was enhanced to a much greater extent in parenchymal cells (about 25-fold) than in Kupffer or endothelial cells (7- to 9-fold). One could argue that the amount of cytochrome P-450 MC_{1a}, which catalyzes the *O*-deethylation of ethoxyresorufin at about one-tenth the rate of cytochrome P-450 MC_{1b} (61), is extremely high in parenchymal cells when compared with Kupffer or endothelial cells; however, no such evidence was found in this study (Fig. 2D). Thus, the above-mentioned discrepancy remains presently unexplained.

In conclusion this study shows that in the rat liver not only parenchymal cells but also Kupffer and endothelial cells possess drug metabolizing enzymes and therefore may contribute to the hepatic disposition of xenobiotics. Western blot analyses revealed small but potentially significant differences in the expression of proteins immunologically related to cytochrome P-450 PB-1 and glutathione transferases B and X.

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