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Effects of Phenobarbital and 3-Methylcholanthrene Pretreatments on Monooxygenase Activities and Proportions of Isolated Rat Hepatocyte Subpopulations

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SUMMARY

Parenchymal cells, isolated from untreated (control) and phenobarbital- or 3-methylcholanthrene-treated rats, were separated into six subpopulations by Percoll discontinuous density gradient centrifugation. Short-term centrifugation in the medium provided well-preserved, viable cells in reasonably high yield, and the density distribution patterns of the cells were unimodal. The activity of 5′-nucleotidase (EC 3.1.3.5), a plasma membrane enzyme, was generally high in low-density hepatocytes, while glycogen contents were higher in high-density hepatocytes. Electron microscopy of the subpopulations revealed that low- and high-density cells had the characteristics of centrilobular and perilobular cells of the liver, respectively. With respect to the hepatocytes isolated from phenobarbital-treated rats, the cellular cytochrome P-450 content was high in the low-density fraction and low in the high-density fraction with a clear gradation according to density. 7-Ethoxycoumarin deethylase activity was higher and glutathione depletion by bromobenzene metabolism was more pronounced in low-density cells than in high-density cells. The contents of cytochrome P-450 (total) and the deethylase activities were a little higher in medium-density hepatocytes isolated from 3-methylcholanthrene-treated rats; therefore, the distribution patterns of the monooxygenase activity presented by the assay of subpopulations from phenobarbital- and 3-methylcholanthrene-treated rats were markedly different. The reduction of cellular glutathione by bromobenzene was less significant in hepatocytes from 3-methylcholanthrene-treated rats than that in hepatocytes from phenobarbital-treated rats. The proportion of the subpopulation was shifted to a lighter density range by treatment with phenobarbital and slightly by that with 3-methylcholanthrene.

INTRODUCTION

The biochemical and morphological heterogeneity of hepatic parenchymal cells within the lobules has been explored by many investigators. Novikoff (1) surveyed, by cytochemical staining, the quantitative differences in enzymes and cell components between pericentral and periportal regions. In recent years, much attention has been focused upon the localization of cytochrome P-450 in the liver lobule in relation to zonal necrosis due to toxic metabolites formed by monooxygenase-linked functions (2, 3). Centrilobular necrosis caused by bromobenzene, for example, was interpreted as being due to the active intermediate produced by cytochrome P-450, which is enriched in centrilobular cells. Therefore, cell damage is enhanced by simultaneous administration of PB[1] to rats (4). Microspectrophotometric search of a section of rat liver has indicated that the concentration of cytochrome P-450 is relatively higher in the pericentral region than in the periportal region, and that the concentration gradation across the lobule is markedly intensified by pretreatment of the animals with PB (5). Immunohistochemical studies proved that the concentration of cytochrome P-450 induced by PB is higher in the pericentral regions than in the periportal regions of the liver lobule, while that induced by 3-MC is more uniformly distributed (6).

Another approach to the study of zonal localization of enzymes in hepatocytes was introduced by Drochmans *et al.* (7), who separated rat liver cells into subpopulations according to density differences and proposed that low- and high-density hepatocytes could arise from the pericentral and periportal regions, respectively. Distinct proliferation of smooth-surfaced ER in low-density hepatocytes isolated from PB-treated rats has also been noted (8).

These lines of evidence seem to suggest that the heterogeneity and lobular distribution of hepatocytes can be correlated with the density of the cells. In this study, to

[1] The abbreviations used are: PB, phenobarbital; 3-MC, 3-methylcholanthrene; ER, endoplasmic reticulum; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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investigate further the significance of cellular heterogeneity on drug metabolism and drug interactions, isolated rat hepatocytes were separated into six subpopulations by a Percoll (9) discontinuous gradient and characterized in terms of cytochrome P-450 content, 7-ethoxycoumarin deethylase activity, and the extent of GSH depletion upon incubation with bromobenzene. Particular attention was paid to the effects of PB and 3-MC on the biochemical features of hepatocytes. Results from assays of two enzymes and electron micrographs of hepatocyte subpopulations are also presented.

EXPERIMENTAL PROCEDURES

Materials. BSA was purchased from Povit Productin N.V. (Amsterdam, Holland), collagenase from Boehringer GmbH (Mannheim, Federal Republic of Germany), 7-ethoxycoumarin from Sigma Chemical Company (St. Louis, Mo.), and GIBCO amino acid mixture (without L-glutamine) from Grand Island Biochemical Company (Grand Island, N. Y.). 7-Hydroxycoumarin and resorufin were obtained from Tokyo Kasei Company, Ltd. (Tokyo, Japan), and 3-MC and 7-ethoxyresorufin from Wako Pure Chemical Inc. (Tokyo). Sodium PB was obtained from Merck AG (Darmstadt, Federal Republic of Germany), and Percoll from Pharmacia Fine Chemicals Ab (Uppsala, Sweden). Resorufin was purified with a silica gel column using benzene/acetone (3:1) as the solvent. All other chemicals were reagent-grade.

Animals. Hepatocytes were obtained from 10- to 11-week-old male Wistar rats, weighing 300–330 g, which had been given laboratory chow and water ad libitum. One group (PB-treated) of rats was treated with sodium PB (80 mg/ml) in 0.9% NaCl solution injected i.p. for 3 days at a dosage of 80 mg/kg, and hepatocytes were isolated 24 hr after the last injection. Another group (3-MC-treated) was administered a suspension of 3-MC in sesame oil (40 mg/ml) i.p. for 3 days at a dosage of 40 mg/kg, and hepatocytes were obtained 48 hr after the last injection.

Isolation of hepatocytes. Isolated rat hepatocytes were prepared by a collagenase perfusion method as described previously (10), and the isolation was conducted daily at 10:30 a.m. to 11:00 a.m. The hepatocytes were washed by centrifugation at 300 rpm at 4° first with Buffer A containing 2% BSA and then with Buffer B containing 1% BSA. The viability of the hepatocytes was monitored by trypan blue exclusion and the lactic acid dehydrogenase latency test (11), which showed that the viability of the cells was 98–99% for the control and the PB-treated rats but 96–98% for the 3-MC-treated rats. The liver wet weight and yield of isolated hepatocytes are shown in Table 1. The liver weight was calculated by weighing the organs of rats of the same age and body weight, which were treated like those used for the isolation of hepatocytes. The freshly isolated cells obtained are referred to as "initial cells" in this text to distinguish them from the subpopulations separated by a Percoll density gradient, which is described below.

Buffers. Buffer A: Krebs-Henseleit buffer, containing 3.0 g of Hepes and benzylpenicillin (200 IU/ml) in 1000 ml (11). Buffer B: Krebs-Henseleit buffer containing 3.0 g of Hepes, 1.8 g of D-glucose, amino acid mixture (GIBCO), and benzylpenicillin (200 IU/ml) in 1000 ml. Buffer C: 8.0 g of NaCl, 0.4 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of Na_2HPO_4, 0.06 g of KH_2PO_4, 2.19 g of $NaHCO_3$, and 3.0 g of Hepes in 100 ml. After supplementation of the materials specified in the text, the pH of each buffer was adjusted to 7.4, except that of Buffer C, and O_2/CO_2 (95%:5%) gas was bubbled through the solution. The pH of Buffer C was adjusted to about 6.4 in order to have the pH of the buffered Percoll mixture (see below) equal 7.4.

TABLE 1

Yield of hepatocytes isolated from control, PB-treated, and 3-MC-treated rats

Values are means ± standard error of the mean.

	Control (n = 13)	PB-treated (n = 13)	3-MC-treated (n = 11)
Liver wet wt/body wt (%)	4.47 ± 0.09	5.22 ± 0.12	5.22 ± 0.14
No. of hepatocytes ($\times 10^7$)/g of liver	5.97 ± 0.23	3.89 ± 0.14	4.44 ± 0.12

Percoll density gradient. Nine volumes of Percoll and one volume of Buffer C were mixed (buffered Percoll). The solution was combined with Buffer B, containing 1% BSA in the various ratios shown in Table 2, and O_2/CO_2 (95%:5%) gas was bubbled through the mixture. A discontinuous gradient was prepared by successively layering 2-ml portions of the Percoll solutions of different densities given in Table 2 in a Beckman cellulose nitrate tube (16 × 102 mm). The densities of the series of the solution were determined at 4° with a digital precision density meter, DMA 02C (Anton Paar, Graz, Austria), using distilled water and ethylene glycol as the reference.

Fractionation of isolated hepatocytes. The initial cell suspension of 2.5 ml (10^7 cells/ml) was layered on top of the discontinuous Percoll gradient and centrifuged for 20 min at 2500 rpm. Six tubes were prepared for each run. Prior to fractionation, nonsedimenting cells, mainly damaged hepatocytes, in a layer on top of the gradient medium were discarded. The hepatocytes retained in each layer (F-1 to F-6) were transferred to 20-ml centrifuge tubes by aspiration; the six corresponding fractions were then combined. Percoll was removed by washing the cells twice with 6 ml of Buffer B containing 1% BSA by centrifugation (5 min at 800 rpm) and decantation. The pellet was suspended in 4 ml of the same buffer.

TABLE 2

Composition of Percoll discontinuous gradients for separation of hepatocyte subpopulations

	Buffered Percoll	1% BSA in Buffer D	Density[a]
	%	%	*g/ml*
F-1	50	50	1.070
F-2	65	35	1.087
F-3	70	30	1.093
F-4	75	25	1.099
F-5	80	20	1.105
F-6	85	15	1.110

[a] At 4°.

Centrifugation, fractionation, and washing were performed at 4°.

Cell size analysis. Isolated hepatocytes were suspended (5–10 × 10^5 cells/ml) in Buffer B, containing 1% BSA, and placed in a pair of Burker chambers. Eight photographs were taken of randomly selected sections of each chamber with ×20 magnification. The diameters of about 100 hepatocytes in the prints (final magnification, ×200) were measured with a micrometer with reference to a standard-scale (0.01-mm division) photograph, which had been taken simultaneously and processed in the same way.

Incubation of hepatocytes with bromobenzene. In a round-bottom flask, 15 μl of bromobenzene (18.9 mg/ml) in dimethyl sulfoxide (12) were mixed thoroughly with Buffer B, supplemented with 1% BSA, and portions of the cell suspension were added to bring the volume to 3.0 ml (1–5 × 10^6 cells/tube). For the control experiment, 15 μl of dimethyl sulfoxide were used instead of bromobenzene. The flask was incubated at 37° with shaking, and at time zero and 15 min, a 0.8-ml sample of the incubation mixture was transferred to the test tube with 0.7 ml of 13% trichloroacetic acid. After centrifugation for 20 min at 2500 rpm, a portion of the supernatant was used for the determination of GSH as described previously (13).

Assay of enzymes and cytochrome P-450. Isolated hepatocytes were disrupted by freezing and thawing the cell suspension three times, and portions were subjected to analyses of 5′-nucleotidase (EC 3.1.3.5), glutamate pyruvate transaminase (EC 2.6.1.2), and cytochrome P-450 (total). 5′-Nucleotidase was assayed as described by Gerlach and Hiby (14) using 0.1–0.2 ml of disrupted cells. The activity was expressed as the amount of inorganic phosphorus liberated from 5′-AMP per minute. Glutamate pyruvate transaminase activity was measured colorimetrically by the method of Bergmeyer and Bernt (15) with 50 μl of the disrupted cells (5 × 10^4 cells) per milliliter of reaction mixture. The glutamate pyruvate transaminase level was defined according to Karmen (16). The cytochrome P-450 (total) content was determined according to Omura and Sato (17) at 25° with a Shimadzu UV-300 spectrophotometer.

7-Ethoxycoumarin deethylase activity determination. Portions of disrupted cells (0.05–0.3 ml) were added to the incubation medium containing 100 μg of ethoxycoumarin and an NADPH-generating system in 50 mM Tris buffer (pH 7.4) with 150 mM KCl and 10 mM $MgCl_2$ (final volume 1.5 ml). After incubation for 4 min at 37° with shaking, the reaction was terminated by heating, and the 7-hydroxycoumarin formed was extracted with ethyl ether. The fluorescence of the extract at alkaline pH was determined at 455 nm with excitation of 370 nm (18). Blank values were obtained by the same procedure except that 7-ethoxycoumarin was omitted from the reaction mixture.

7-Ethoxyresorufin deethylase activity determination. The deethylase activity was assayed according to the method of Pohl and Fouts (19) with 150 μl of disrupted cells (1.5 × 10^5 cells) in 1.25 ml of incubation mixture. The reaction was initiated by adding NADPH and an NADPH-generating system 4 min after preincubation, and incubation was performed for 4 min at 37°.

Determination of glycogen. A 0.1-ml portion of the hepatocyte suspension was transferred to a test tube with 1.5 ml of ice-cold 0.4 N perchloric acid and centrifuged for 20 min at 2500 rpm. Glycogen was purified from the supernatant fraction and determined by the method of Handel (20), which is based on specific precipitation of a small amount of glycogen and colorimetric assay with anthrone. A calibration curve was constructed with 10–50 μg of glycogen per test tube.

Transmission electron microscopy. A suspension of isolated hepatocytes was fixed for 30 min at 4° with an equal volume of 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde and postfixed with 2% osmium tetroxide in 0.25 M sucrose-0.1 M phosphate buffer (pH 7.4) for 30 min. The fixed cells were dehydrated with graded ethanol, immersed in *n*-butyl glycidyl ether, embedded in epoxide resin (Epok 812), and cut with a Reichert ultramicrotome OmU4. The specimens were stained with lead citrate and analyzed by JEM-100 CX electron microscopy at 80 kV. Prior to the electron microscopy, subpopulations were surveyed by light microscopy (×400), which revealed that the major cells were round and uninuclear. The features of the cells appeared uniform except for those in Fraction 1 (F-1), which was contaminated by dead cells. Fatty droplets were frequently seen in the lower-density hepatocytes from PB- and 3-MC-treated rats. About 10 electron micrographs were randomly prepared from each section with low (×1600) and high (×8300) magnifications (original magnification).

RESULTS

Cell distribution. The distribution of hepatocyte subpopulations separated by discontinuous gradients obtained from control (n = 11), PB-treated (n = 11), and 3-MC-treated (n = 12) rats are shown in Fig. 1. (n represents the number of rats in each group; as seen in Fig. 1, the number of hepatocytes obtained in F-6, particularly for PB-treated rats, was often too low to be usable for the analyses in this study.) As shown in Fig. 1, the highest number of hepatocytes from control rats was in Fraction 4 (F-4) followed by those in F-5 and then F-3. On the other hand, the hepatocytes isolated from PB-treated rats were retained in a lower-density fraction, the number being highest in F-3 followed by that in F-4. The distribution pattern of the cells from 3-MC-treated rats was similar to that of the control. The pattern was not altered by further centrifugation for 40 min at 3000 rpm. The viability of the hepatocyte subpopulation was 98–99% according to the lactic acid dehydrogenase latency test (11), except for F-1, in which the viability for the control and the PB-treated groups averaged 97% but was 95–97% for the 3-MC-pretreated group. Recoveries of cells after fractionation and washing were 87 ± 4%, 93 ± 5%, and 79 ± 3% (mean ± standard error of the mean) for control, PB-treated, and 3-MC-treated rats, respectively. Certain cells could be lost during the separation procedures, and lower recovery of hepatocytes from 3-MC-treated rats may be due to removal of damaged cells remaining in the top layer of the gradient medium. As mentioned above, the viability of the initial cells from the 3-MC group was lower than those from other groups.

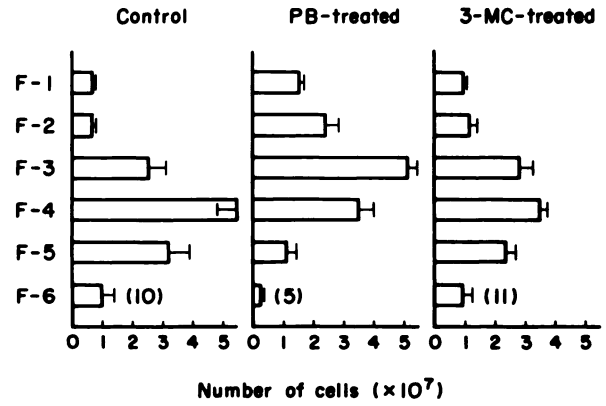

FIG. 1. *Numbers of hepatocytes in subpopulations*

Hepatocytes were isolated from control (n = 11), PB-treated (n = 11), and 3-MC-treated (n = 12) rats and fractionated into subpopulations (F-1 to F-6), as described in the text, using a Percoll density gradient. The numbers in parentheses for F-6 indicate the number of samples obtained in sufficient quantity for use in this determination. Values are mean ± standard error of the mean.

Cell size. The histogram of initial cells from control (n = 4), PB-treated (n = 4) and 3-MC-treated (n = 3) rats are shown in Fig. 2. The diameters of major cells in the control were close to 20 μm, while those of major cells from PB- and 3-MC-treated groups were close to 25 μm, although they were classified in the range of 20–25 μm. Generally, the mean diameter of low-density cells was larger than that of high-density cells; for example, in F-2 they were 21.5 ± 0.4, 26.4 ± 0.3, and 23.9 ± 0.5 μm, and in F-5, 20.8 ± 0.3, 24.1 ± 0.8, and 22.8 ± 0.1 μm for hepatocytes from control, PB-treated, and 3-MC-treated rats, respectively. (F-3 was found to be slightly larger than F-2 in the 3-MC experiment.) As given in Table 1, the yield of hepatocytes (cell number per gram of liver) from PB- and 3-MC-treated rats was 25–35% lower than that from the control. Note also that the mean diameters of the cells from the treated group were 15–20% larger than those from the untreated group (Fig. 2).

Cytochrome P-450 content of hepatocytes. Cytochrome P-450 (total) concentrations in freshly isolated hepatocytes (initial cells) were 0.24 ± 0.01, 0.94 ± 0.05, and 0.78 ± 0.03 nmole/10^6 cells for control (n = 11), PB-treated (n = 11), and 3-MC-treated (n = 12) rats, respectively. As shown in Fig. 3, Fractions 3 and 4 (F-3 and F-4) from the control rats retained a relatively high amount of cytochrome P-450. Although the contents were approximately 3-fold higher, a similar distribution pattern was obtained for the hepatocytes from 3-MC-treated rats. The pattern was markedly different when the subpopulations from PB-treated rats were compared: F-2 contained the highest amount of cytochrome P-450 and F-6 the lowest. No definite statement can be made concerning F-1, because of the presence of a small percentage of contamination by damaged cells in both control and treated groups. The number of hepatocytes in F-6, particularly those from PB-treated rats, was in some cases too low to allow their use in the analyses conducted in this study.

7-Ethoxycoumarin and 7-ethoxyresorufin deethylase activities. The metabolism of 7-ethoxycoumarin was markedly enhanced both by PB and 3-MC treatments (Fig. 4), averaging 8- and 12-fold, respectively. The deethylase activity and content of cytochrome P-450 (total) in subpopulations appear to be closely correlated. Similar patterns were obtained with the hepatocyte subpopulations from 3-MC-treated rats (n = 4) when deethylase activity was assayed using 7-ethoxyresorufin (21), a more specific substrate for cytochrome P-448 (Fig. 5). Interfractional variation of deethylase activities was approximately 2-fold in the subpopulations of 3-MC-treated rats, and this is comparable to the intercellular deethylase activities of ethoxyresorufin by individual cells isolated from 3-MC-treated rats (22).

Depletion of glutathione by bromobenzene. Bromobenzene is metabolized by the monooxygenase system to its active intermediate, which reacts with cellular GSH. Therefore, the drug-metabolizing activity of hepatocytes can also be estimated by measuring the GSH content of the cells. Table 3 shows the percentage of remaining GSH in hepatocyte subpopulations after 15 min of incubation in the presence of 0.6 mM bromobenzene. The

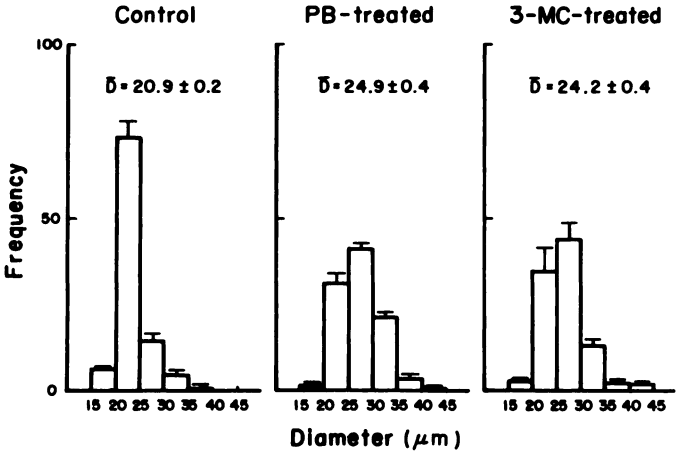

FIG. 2. *Size distribution histograms of initial cells isolated from control (n = 4), PB-treated (n = 4), and 3-MC-treated (n = 3) rats*

$\bar{D}$, mean diameter (micrometers) ± standard error of the mean.

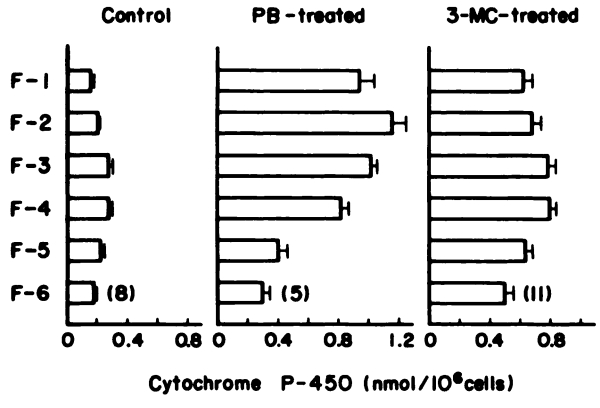

FIG. 3. *Cytochrome P-450 (total) content of hepatocyte subpopulations isolated from control (n = 11), PB-treated (n = 11), and 3-MC-treated (n = 12) rats*

Cytochrome P-450 concentrations were determined after disruption of the cells by freezing and thawing. See explanation in Fig. 1 on the number of F-6 samples.

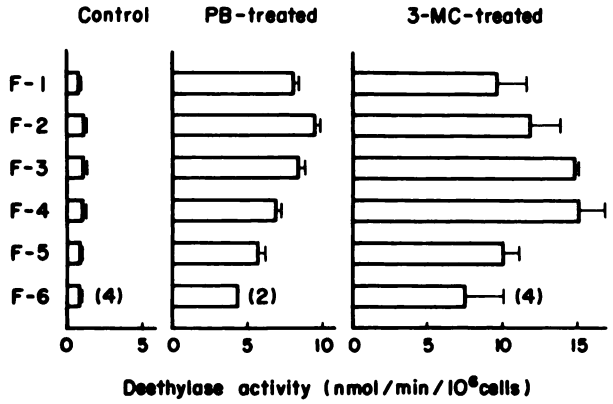

FIG. 4. *7-Ethoxycoumarin deethylase activities in hepatocyte subpopulations*

The hepatocytes were isolated from control (n = 5), PB-treated (n = 4), and 3-MC-treated (n = 5) rats. Values are means ± standard error of the mean. See explanation in Fig. 1 on the number of F-6 samples.

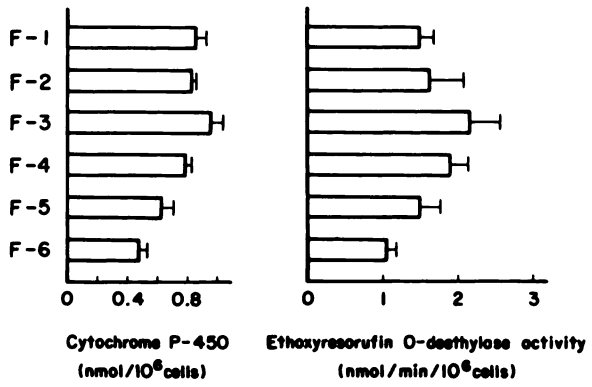

FIG. 5. *Cytochrome P-450 (total) content and 7-ethoxyresorufin deethylase activities in hepatocyte subpopulations isolated from 3-MC-treated rats (n = 4)*

Values are means ± standard error of the mean.

GSH levels of the initial cells were 45 ± 13 (n = 4), 60 ± 4 (n = 6), and 70 ± 4 (n = 6) nmoles/10^6 cells for the control, PB-treated, and 3-MC-treated rats, respectively. Not much change was found in the control experiment after incubation with bromobenzene. However, GSH levels in the hepatocytes prepared from PB- and 3-MC-treated rats were markedly influenced by incubation with bromobenzene. The level was reduced approximately 50% on the average in PB-treated rats, and the extent of reduction was correlated to the cytochrome P-450 content. In cells from 3-MC-treated rats, the GSH reduction was approximately 20%. After 15 min of incubation, in the presence or absence of bromobenzene, the viability of those from control and PB-treated rats remained at 97–99% and at 95–98% for 3-MC-treated rats. As stated above, the hepatocytes in F-1 showed somewhat lower values.

Glycogen content. The glycogen concentration within the liver lobule is reported to be generally higher in the pericentral region than in other regions (1, 23). With regard to the hepatocytes from control rats, F-5 and F-6 were apparently rich in glycogen, while F-1 to F-4 had similar lower glycogen concentrations (Fig. 6). Distinct gradation of the glycogen content from F-2 and F-6 was found with the hepatocytes isolated from PB- and 3-MC-treated rats. The glycogen levels of initial cells isolated from control, PB-treated, and 3-MC-treated rats were 97 ± 20 (n = 13), 120 ± 22 (n = 12), and 170 ± 29 (n = 9) μg/10^6 cells, respectively.

Enzyme activities. The activities of 5′-nucleotidase and glutamate pyruvate transaminase in hepatocyte subpopulations are shown in Fig. 7. Generally, 5′-nucleotidase was present in higher amounts in low- rather than high-density hepatocytes. In contrast, the activities of glutamate pyruvate transaminase were lower in the low-density cells than in high-density cells, although the fluctuations were appreciably large. 5′-Nucleotidase is defined as a marker enzyme of plasma membrane (24). Distinct gradation of glutamate pyruvate transaminase activity within the liver lobule was histochemically demonstrated by Shank *et al.* (25), although the feeding and ages of the rats were different from those in this study.

Electron microscopy. The F-1 and F-2 hepatocytes in the control experiment were clear and resembled each other; they were characterized by a relative abundance of smooth ER and few glycogen particles. Fatty droplets

TABLE 3

Cellular glutathione 15 min after incubation of hepatocytes in the presence of 0.6 mM bromobenzene

Values are means ± standard error of the mean. The GSH levels 15 min after incubation of hepatocytes in the absence of bromobenzene were essentially unchanged: the average remaining ratios were 98 ± 1%, 100 ± 1%, and 100 ± 1%, respectively, for control, PB, and 3-MC groups. The percentage of GSH remaining 15 min after incubation with bromobenzene is shown in parentheses (the 0-time value was taken as 100%).

Fraction	Control (n = 4)		PB-treated (n = 6)		3-MC-treated (n = 6)	
	0	15 min	0	15 min	0	15 min
	nmoles/10^6 cells		*nmoles/10^6 cells*		*nmoles/10^6 cells*	
F-1	41 ± 3	38 ± 4 (92)	54 ± 3	25 ± 2 (46)	62 ± 5	49 ± 4 (79)
F-2	62 ± 6	60 ± 6 (96)	71 ± 4	32 ± 2 (46)	80 ± 2	66 ± 2 (83)
F-3	53 ± 6	51 ± 6 (95)	62 ± 6	32 ± 2 (50)	78 ± 6	60 ± 5 (78)
F-4	47 ± 2	44 ± 2 (94)	66 ± 5	35 ± 3 (54)	84 ± 4	65 ± 3 (77)
F-5	52 ± 1	50 ± 2 (96)	68 ± 12	48 ± 10 (68)	85 ± 4	69 ± 3 (82)
F-6	61 ± 6[a]	58 ± 5[a] (96)	108[b]	91[b] (84)	104 ± 9	96 ± 11 (91)

[a] n = 3.
[b] n = 2.

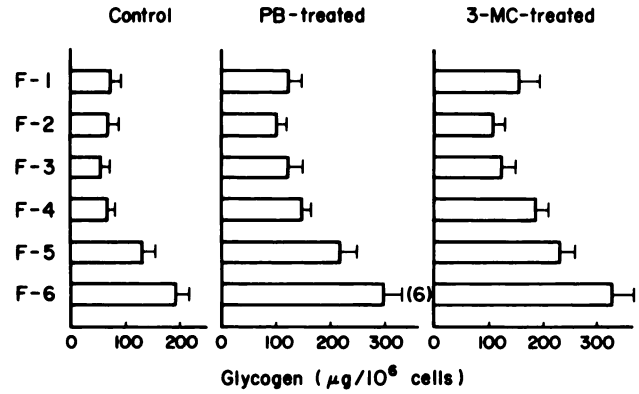

FIG. 6. *Content of glycogen in hepatocyte subpopulations*
The values for glycogen were obtained from control (n = 11), PB-treated (n = 11), and 3-MC-treated (n = 12) rat hepatocyte subpopulations. Values are means ± standard error of the mean. See explanation in Fig. 1 on the number of F-6 samples.

were frequently seen, but free ribosomes were few. Vast cytoplasmic areas and small or rodlike mitochondria were also noted. On the other hand, large, compact glycogen areas, prominent rough ER, and free ribosomes were found in higher-density hepatocytes (F-4 to F-6). Representative micrographs of hepatocytes retained in Fraction 2 (F-2) and Fraction 4 (F-4), from control rats, are shown in Fig. 8.

Survey of approximately 200 micrographs revealed that the effect of PB and 3-MC pretreatment of rats on the features of hepatocytes was notable, particularly in F-1, F-2, and F-3, although the gross features of the hepatocytes retained in the same density fraction resembled each other irrespective of whether they were from control or treated rats. With respect to the hepatocytes from PB-treated rats, proliferation of smooth ER in F-1 to F-3 and reduced rough ER were appreciable. The features of the hepatocytes from 3-MC-treated rats were

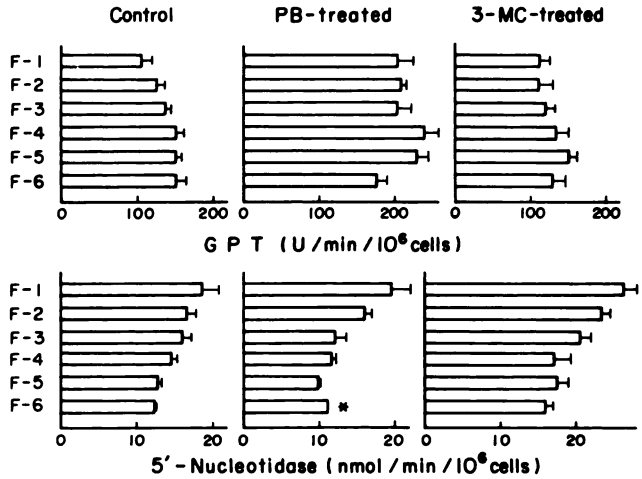

FIG. 7. *Activities of glutamate pyruvate transaminase (GPT) and 5′-nucleotidase in hepatocyte subpopulations isolated from control (n = 7), PB-treated (n = 4), and 3-MC-treated (n = 5) rats*
Values are means ± standard error of the mean. The *asterisk* indicates mean value, n = 2.

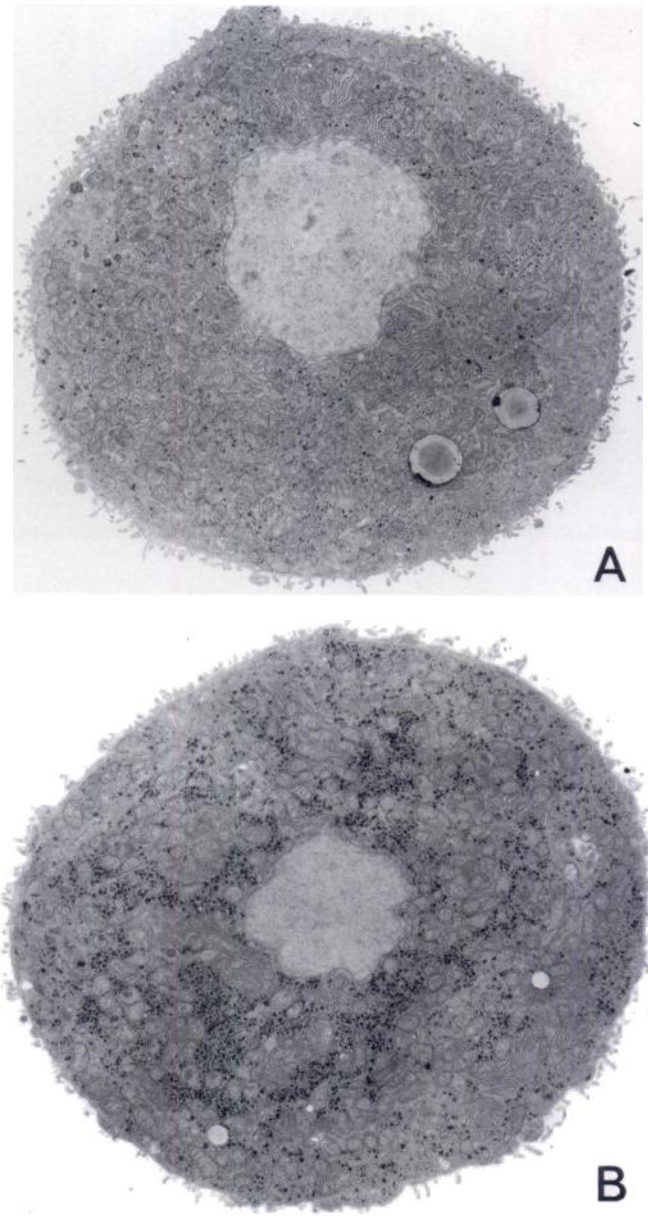

FIG. 8. *Transmission electron micrographs (low magnification) of isolated hepatocytes from control rats in F-2 and F-4*
A, Isolated hepatocytes in Fraction 2; B, isolated hepatocytes in Fraction 4 (×3,600).

similar to those of controls, except that free ribosomes and rough ER were notable in F-1 to F-3.

To display more clearly the details of cell structure, electron micrographs of hepatocytes obtained from control rats (F-2 to F-5) are presented at higher magnification (Fig. 9). Although the definition of the lobular zone is arbitrary, the profiles of lower- and higher-density cells presented similar characteristics of centrilobular and perilobular cells in the liver tissues, respectively.

DISCUSSION

The isolated hepatocyte system is an experimental model which retains *in vivo* biochemically and structurally organized functions and has been successfully utilized for the study of drug and general metabolism.

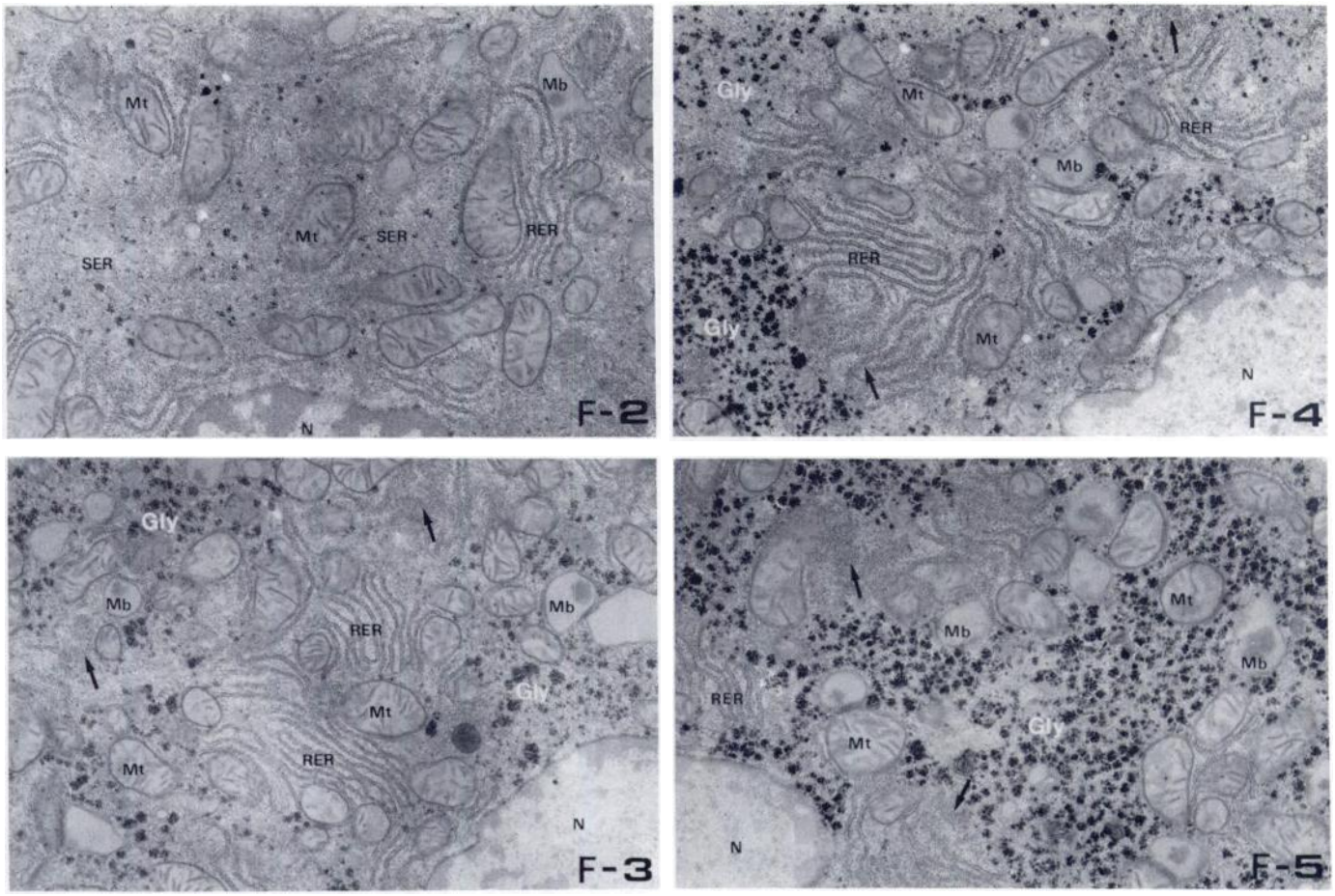

FIG. 9. *Transmission micrographs (high magnification) of isolated hepatocyte subpopulations in F-2 to F-5*

F-2, Isolated hepatocytes in Fraction 2; F-3, isolated hepatocytes in Fraction 3; F-4, isolated hepatocytes in Fraction 4; F-5, isolated hepatocytes in Fraction 5 (×22,000). *N*, nucleus; *SER*, smooth ER; *RER*, rough ER; *Gly*, glycogen; *Mt*, mitochondrion; *Mb*, microbody. Free ribosomes are indicated by an *arrow*.

Progress in this field is due to the recent exploration of isolation techniques to provide well-preserved viable cells in high yield (26, 27). However, as the available isolated hepatocytes are a mixture of heterogeneous parenchymal cells originating from different regions in the liver lobule, separation of specific regional cells should facilitate the study of hepatic metabolism. Attempts to accomplish this have been reported by several authors (7, 8). Treatment of animals with a drug such as PB, which enhances the heterogeneity of hepatocytes, appears to be useful for classifying the subpopulations by physical means such as density gradient centrifugation (8, 28).

In this study, we separated isolated rat hepatocytes into six subpopulations using Percoll discontinuous density gradient centrifugation and examined the biochemical functions by comparing the hepatocytes obtained from the control rats with those from PB-treated or 3-MC-treated rats. Percoll was chosen because it does not penetrate the cell membrane and can easily be removed with an ordinary washing procedure (9). Rapid isolation, inclusion of BSA in buffers supplemented with O_2/CO_2 (95%:5%) gas, and short-term centrifugation in Percoll density media provided viable cells in reasonably high yield. Since most of the damaged cells as well as nonparenchymal cells were retained in the upper layer of the Percoll density gradient, intact parenchymal cells were separated into subpopulations with unimodal fractionation patterns as reported by Seglen (29). The major cells were retained in the density media of around 1.09 to 1.10 g/ml; however, the cell density could be influenced by isolation techniques, density gradient media, animal age, medication to the animals, or fasting.

With respect to monooxygenase-linked functions, the profiles in the subpopulations obtained from PB- and 3-MC-treated rats differed markedly from those of control rats. After pretreatment of the rats with PB, the cellular cytochrome P-450 concentrations increased 4-fold on the average, and were reciprocally related to cell density. 7-Ethoxycoumarin deethylase activities and the extent of GSH depletion by bromobenzene metabolism were correlated with the cytochrome P-450 concentrations. The profiles of deethylase activities in the hepatocytes from 3-MC-treated rats differed from those of PB-treated rats, probably due to the different modes of induction of cytochrome P-448 by 3-MC (21, 30), which catalyzes deethylation more efficiently than does cytochrome P-450. The extent of GSH depletion by bromobenzene was less in the hepatocytes from 3-MC-treated rats than in those from PB-treated rats. This is consistent with the finding that pretreatment of rats with 3-MC results in

conversion of bromobenzene to its 2,3-epoxide, which is less toxic than the 3,4-epoxide predominantly formed in PB-treated rats (31, 32).

Histochemical and morphometric studies have proved that 5′-nucleotidase activity is higher in the centrilobular cells whereas glycogen is rich in perilobular cells (1, 23). Although the proportion of the subpopulations was influenced by the treatment of the rats, especially by PB, a gradation of 5′-nucleotidase and glycogen along with cell density was commonly detectable in control, PB-treated, and 3-MC-treated rats. At 10–11 a.m., when isolation of the hepatocytes was conducted, a considerable gradation of glycogen contents across the hepatic lobule from the periportal to the pericentral region was expected in the fed rats, since stored glycogen disappears first from the pericentral area during the daytime. Proliferation of lipid-rich smooth ER in the centrilobular cells (7, 8, 23) can also profoundly influence the density of hepatocytes. The mean diameter of the lower-density cells, particularly those from inducer-treated rats, was found to be larger than that of the higher-density cells. Since cell hypertrophy by PB administration is confined to the central region rich in smooth ER (33), the hepatocytes in this region are expected to be much lighter than those in other areas. Therefore, regional parenchymal cells are anticipated to be separable to a fair extent by density gradient centrifugation, but the separation profile of subpopulations should be different before and after induction. Such differences can be investigated directly by titrating the cells with antibodies to enzymes affected by the inducers. Electron micrographs of hepatocytes retained in the same density fraction generally resembled each other as to their gross features irrespective of whether they were from control or treated rats. F-2 was rich in smooth ER whereas F-5 was rich in glycogen granules with poorly developed smooth ER, although proliferation of smooth ER and an increased number of fat globules in low-density cells isolated from PB- and 3-MC-treated rats were noted. F-3 and F-4 showed patterns intermediary between those of F-2 and F-5. Thus, the morphological features of the subpopulations appear to conform to the hypothesis that low- and high-density cells correspond, with a high probability, to centrilobular and perilobular parenchymal cells, respectively (7, 8). One aspect of the present study is the finding that the yield of isolated hepatocytes appeared to be influenced by the treatment of rats with PB and 3-MC. A similar result was described by Sweeney *et al.* (34). Argyris (35) reported that after 3- or 4-day administration of PB to rats weighing 200–300 g, the number of hepatocytes decreased as measured by nuclear counts of the cells. Cellular enlargement accompanied by increasing ploidy of hepatocytes from PB-treated rats has been noted (36). These findings might indicate that mitotic arrest took place during the specific period of medication of adult rats. However, such a speculation must await closer investigation of the hepatocyte isolation procedures and morphometric analyses of the rat livers as specified above.

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