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HETEROGENEOUS DISTRIBUTION OF DRUG METABOLISM IN ELUTRIATED RAT HEPATOCYTES*†

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Abstract—Centrifugal elutriation was used to separate isolated rat hepatocytes into five fractions containing cells of different sizes. These fractions were compared with regard to cell size, morphology and function. Analyzed by flow cytometry, the small cells were found to be enriched in fraction 1 and the large cells in fraction 5. Evaluation by light and electron microscopy indicated that the fractions contained single hepatocytes of normal structure. The cytochrome P-450 content and the 7-ethoxycoumarin *O*-deethylase activity were assessed in hepatocytes from untreated animals, those treated with phenobarbital, and those treated with phenobarbital plus allylisopropylacetamide. In both untreated and phenobarbital-treated animals, cytochrome P-450 content and 7-ethoxycoumarin *O*-deethylase activity rose significantly from fraction 1 to fraction 5. The P-450 content gradually rose up to 2-fold. The enzyme activity rose 5-fold, and it increased steeply between fractions 2 and 3. The cytochrome P-450 content in phenobarbital-plus-allylisopropylacetamide-treated animals was decreased in all fractions but more extensively in fraction 5 than in fraction 1.

The transport functions and the metabolic activities of hepatocytes vary depending upon their location within the hepatic lobule [1]. The centrolobular region shows by a variety of techniques, including immunofluorescence [2], an enrichment over the periportal region in smooth endoplasmic reticulum [3], cytochrome P-450 content [4, 5], and cytochrome P-450-dependent enzyme activities [6, 7]. Centrifugal elutriation, a technique which separates cells largely according to size, produces cells with a better cell viability than cells separated by other means [8–10]. This technique allows a structure–function comparison between hepatocytes of varying cell sizes. We isolated rat hepatocytes and separated them into five fractions by centrifugal elutriation in order to compare these subpopulations of cells by morphological and functional criteria. Our findings indicate that both total cytochrome P-450 content and 7-ethoxycoumarin *O*-deethylase activity were enriched in large hepatocytes as compared with small hepatocytes.

MATERIALS AND METHODS

Animal preparation and operation procedures. Nonfasting male Sprague–Dawley rats weighing 300–400 g and fed standard laboratory chow were used in all experiments. One group of animals received no treatment. A second group of animals received sodium phenobarbital daily (Eli Lilly Co., Indianapolis, IN, 80 mg/kg/day in 0.9% saline) by i.p. injection for 4 days. Their hepatocytes were isolated on the morning of day 5. A third group received phenobarbital for 4 days and on the morning of day 5 a single subcutaneous injection of allylisopropylacetamide (AIA) (Hoffmann-La Roche, Nutley, NJ 400 mg/kg, as a 20 mg/ml solution in normal saline). Their hepatocytes were isolated 2 hr later. The hepatocytes were isolated according to the two-step *in situ* perfusion procedure of Seglen [11]. Following nembutal (45 mg/kg, i.p.) anesthesia, the abdomen was opened through a midline incision and the portal anatomy was exposed. The portal vein was cannulated with a 14 gauge polypropylene catheter, immediately flushed with 1 ml of heparinized Krebs–Henseleit buffer, and the catheter was secured in place. The anterior thoracic rib cage was excised, a PE 100 polyethylene tubing (Clay Adams Division, Becton Dickinson & Co., Parsippany, NJ) was inserted into the intrathoracic portion of the inferior

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vena cava above the diaphram, and the right renal vein was ligated. The time interval between the interruption of the portal circulation and the onset of artificial perfusion was less than 1 min.

Perfusion apparatus, medium and experimental conditions. The operative procedure and the artificial perfusion, which took approximately 25 min, were performed in a constant temperature (37°) room. The medium was recirculated through the liver by a Harvard peristatic pump (model 1203, Harvard Apparatus, Millis, MA) to and from a receptacle that was suffused with 5% CO_2 and 95% O_2 flowing at 3 l/min. The perfusate flow rate was maintained at 25 ml/min.

The perfusion medium consisted of two Krebs–Henseleit solutions. The perfusion was begun *in situ* with 250 ml of calcium-free Krebs–Henseleit buffer (pH 7.4, 37°, 295 mOsmoles) with 5 mM glucose. Then the perfusate was replaced by magnesium-free Krebs–Henseleit buffer containing 2.5 mM calcium, 5 mM glucose, and 0.05% collagenase (Sigma Chemical Co., St. Louis, MO). After perfusion with 400 ml of this medium, the digested liver tissue was removed, weighed, and gently teased apart with a Teflon probe into cold (4°) carbogenated Krebs–Henseleit buffer. The tissue suspension was collected in 50 ml polyethylene conical tubes (Corning Laboratory Supplies, Corning, NY) and placed on ice.

Preparation of isolated hepatocytes. All glassware was siliconized. The tissue suspension was initially filtered through a coarse nylon mesh. The resulting suspension was then centrifuged (International Clinical Centrifuge model CL, International Equipment Co., Needham, MA) at 70 *g* for 1 min, the supernatant fraction was removed by aspiration, and the cells were resuspended in cold carbogenated buffer. This procedure was repeated three times and then the cells were allowed to sediment at 1 *g* for 15 min. Following sedimentation, the cell suspension was filtered through a 100 micron mesh and then a 62 micron nylon mesh (Tetko Inc. Monterey Park, CA). Following filtration, the cell suspension was assessed under an inverted microscope (Zeiss JCM 405) for the presence of nonparenchymal cells and the number of single hepatocytes. Viability was assessed by trypan blue exclusion in a hemocytometer and by the release of lactate dehydrogenase (LDH activity media/LDH activity sonicated cells) [12, 13].

Preparation of hepatocytes for centrifugal elutriation. Prior to elutriation, the cells were suspended in Krebs–Henseleit buffer with 5 mM glucose that had been equilibrated with carbogen. Cell number was estimated by a Coulter Particle Size Distribution Plotter, model B (Coulter Electronics, Inc. Haileah, FL). Elutriation was performed according to Wanson and coworkers [8–10]. The J-21-B centrifuge and the JE-6 elutriator rotor were from Beckman Instruments, Inc. (Palo Alto, CA). The elutriation process was carried out at 4°. The cell suspension, containing 5–10 × 10^7 cells in 15 ml, was slowly injected into the mixing chamber. The cells were introduced into the separation chamber at a flow rate of 15 ml/min, while the rotor was spinning at 840 rpm (100 *g*). The elutriated fraction harvested at this loading flow rate was analyzed separately as it contained largely cellular debris and nonviable hepatocytes. The cells concentrated in the rotor chamber were then submitted to a counterflow of increasing flow rates from 20, 25, 30, 35 and 45 ml/min corresponding to the elutriated fractions 1 to 5. The hepatocytes in the five elutriated fractions were concentrated by centrifugation at 1000 rpm for 3 min. The supernatant fraction was aspirated, and the cells were resuspended in cold carbogenated Krebs–Henseleit buffer. Viability was again assessed by trypan blue exclusion and by the release of lactate dehydrogenase. The fractions were briefly assessed by inverted microscopy for cell size distribution and quantitated by a Coulter counter. Resuspended fractions, as well as nonfractionated cells, were prepared for light and electron microscopy, flow cytometry, and intracellular enzyme assay.

Preparation for light and electron microscopy. Centrifuged pellets of isolated hepatocytes were fixed in a mixture of 1.5% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hr, rinsed in 0.15 M cacodylate buffer, postfixed in 2% osmium tetroxide in 0.15 M cacodylate buffer (pH 7.4) for 30 min, dehydrated in ethanol, and embedded in Epon. One micron thick sections stained with toluidine blue were examined by light microscopy. Thin sections were cut on a Reichart ultracut ultramicrotome, stained double with uranyl acetate and lead citrate, and examined in a Zeiss EM-10 transmission electron microscope. In untreated and phenobarbital-treated animals, ultrastructural morphometry was carried out to determine mitochondrial volume. Fifteen micrographs of mononuclear hepatocytes were taken randomly at a magnification of 4000 from blocks containing cells from fraction 1 and fraction 5. The photomicrographs were then enlarged to a magnification of 12,000. Mitochondrial volume (fractional volume of hepatocyte cytoplasm occupied by mitochondria) was estimated by point counting using a double period square lattice test screen and a mathematical formula as described by Weibel [14].

Flow cytometric analysis. Flow cytometric analysis using narrow forward angle light scatter as an indication of relative cell size [15] was performed both on freshly isolated rat hepatocytes and on subpopulations of hepatocytes prepared by centrifugal elutriation. All samples were suspended in carbogenated Krebs–Henseleit buffer at a concentration of 1–3 × 10^6 cell/ml. Immediately prior to analysis, samples were filtered through a 100 micron nylon mesh to remove aggregates and debris. Flow cytometry was carried out using an Ortho Cytofluorograf 50H (Ortho Diagnostic Systems, Westwood, MA), equipped with a model 2150 data processing system. A detailed description of the cytofluorografic technique has been published [16]. In brief, a 5W argon laser (488 nm) was used for the generation of light scatter signals. Scattered light was collected over a narrow angle forward of the beam. Histograms of cell number versus forward light scatter were developed over an intensity range of 0–1000 channels for a computer gated population that further excluded hepatocyte doublets, red blood cells, and small debris. The light scatter histogram produced by untreated freshly isolated hepatocytes was arbitrarily divided into four regions as follows:

Region	*Channel No.*	*Relative cell size*
1	1–86	small
2	87–179	↓
3	180–316	↓
4	317–1000	large

The channel selections producing these regions were preset for comparative analysis of all subsequent experiments. The mean intensity light scatter produced by cells falling within each region was calculated and expressed as mean channel number. Because the cytofluorograf utilized was not equipped with a volume sensing device, the mean channel number of forward light scatter for each region was used as an indicator of relative cell size. Five to ten thousand cells from each sample were analyzed, and the percentage of cells falling in each of the preset regions was recorded on the light scatter histogram. Fixed calf thymocyte nuclei were used for instrument standardization. Prior to each analysis, polystyrene beads (5, 10 and 20 micron size) were measured, and their mean channel number was determined. The mean channel number plotted against bead size in microns was linear over the size range analyzed.

Fluorescein diacetate (FDA) staining. In vitro, hepatocytes of untreated animals were incubated in carbogenated Krebs–Henseleit buffer containing 1×10^{-5} M FDA for 5 min at 37°. These cells were then washed with fresh buffer and viewed with a fluorescence microscope (Leitz Ortholux model, West Germany) equipped with an HB-200 mercury arc illumination, a 490 nm interference type excitation filter, a numerical aperture 1.2–1.4 dark field condenser, and a 530 nm barrier filter.

In vivo, livers of untreated rats were perfused with a carbogenated Krebs–Henseleit buffer containing 1×10^{-5} M FDA. Immediately following the perfusion, the liver was excised, and random pieces of tissue from four lobes were frozen in liquid nitrogen. Cryostat sections were prepared, sealed with a cover slip, and refrigerated prior to viewing by fluorescence microscopy. In additional experiments *in vivo*, some rat livers were initially perfused *in situ* with the carbogenated FDA buffer, following which the hepatocytes were immediately isolated, elutriated, and prepared for fluorescence measurement by flow cytometry. In control experiments, the autofluorescence of hepatocytes or liver sections, respectively, was examined.

Assays. Cellular protein was determined by a modification [17] of the method of Lowry *et al.* [18], with bovine serum albumin as a standard. Cytochrome P-450 was determined on the 10,000 *g* supernatant fraction of the sonicated cell suspension, and the cytochrome P-450 content was quantitated by the method of Omura and Sato [19]. 7-Ethoxycoumarin *O*-deethylase activity was determined on the 10,000 *g* supernatant fraction of the sonicated cell suspensions, and quantitated by the method of Ullrich and Weber [20] using the extraction procedure for the product 7-hydroxycoumarin (umbelliferone) described by Creaven *et al.* [21].

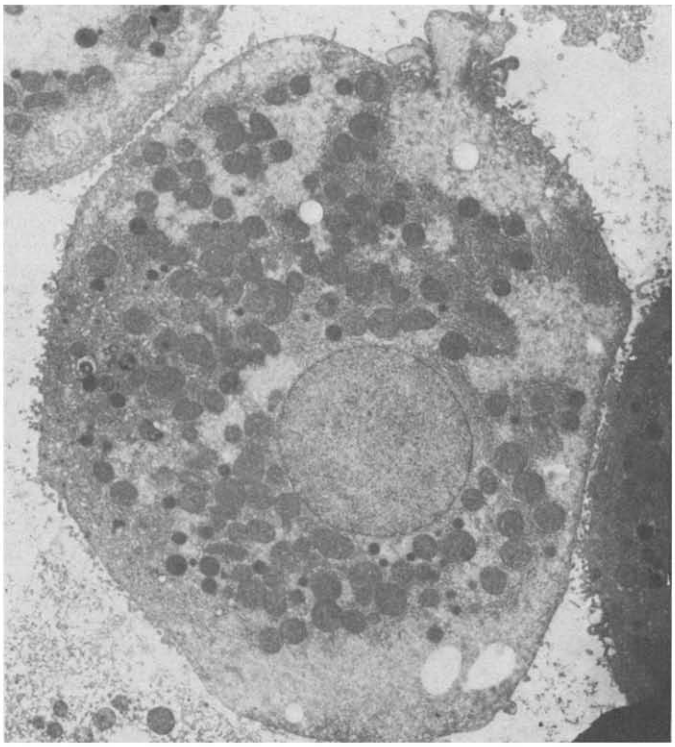
Analysis of data. Student's *t*-test of differences between two sample means was used to assess the significance between parameters [22], and statistical indices were expressed as mean ± S.E.M.

RESULTS

Yield, viability and recovery. The total yield of isolated hepatocytes following collagenase digestion was $3.1 \pm 0.3 \times 10^8$ cells (N = 39) and $1.8 \pm 0.2\ 10^7$ cells/g of liver. The percentage of trypan blue exclusion by the cells was tested before and after elutriation; 90.4 ± 0.7% (N = 38) of the cells excluded the dye prior to elutriation. Non-viable cells were removed by the elutriation process during chamber loading. The percentage of cells excluding the dye increased from fraction 1 (93%) to fraction 5 (98%). The percentage of lactate dehydrogenase released was 17.0 ± 2.8%, 11.4 ± 2.2% and 4.3 ± 2.1% in freshly prepared cell suspensions and elutriated fractions 1 and 5 respectively. Thus, the percent release of lactate dehydrogenase directly correlated with the percent trypan blue exclusion, a finding observed by others, inasmuch as 93% trypan blue exclusion approximated 15% LDH release [23]. The recovery of hepatocytes following elutriation was calculated for each experiment and was 89.5 ± 3.3% (N = 21).

Light and electron microscopy. The untreated hepatocytes were round in shape and studded on their surface with short microvilli. Some cells were relatively electron dense (dark cells) and containing well-preserved organelles distributed throughout the cytoplasm in the usual manner. These dark cells were more numerous in fractions 1 and 2. Other cells were of low electron density (light cells) and contained mitochondria of a high density that were enriched in the perinuclear region. The smooth endoplasmic reticulum was moderate in amount and was more conspicious in the cellular periphery of these light cells. In both light and dark cells, the rough endoplasmic reticulum was present in moderate amounts. The Golgi complexes, lysosomes and perioxisomes appeared morphologically intact. Glycogen particles were deposited in small amounts in the dark hepatocytes, but were virtually absent in light hepatocytes. A comparison of the number of double nucleated hepatocytes in the elutriated fractions showed a significant increase in double nuclei in fraction 5 (7.7 ± 1.4%) relative to fraction 1 (4.3 ± 1.4%). Fractions 1 through 5 showed pure hepatocytes, and there were only occasional double hepatocytes (Fig. 1, A and B). The morphology of the hepatocytes from the phenobarbital-treated animals was similar to that of hepatocytes from untreated animals except that the smooth endoplasmic reticulum was noticeably increased in amount and occasionally formed aggregates which displaced other organelles. The morphology of the hepatocytes from phenobarbital-plus-AIA-treated animals did not differ from the hepatocytes from phenobarbital-treated animals. In untreated and phenobarbital-treated animals, the morphometric measurement of mitochondrial volume indicated that the small cells (fraction 1) had significantly ($P < 0.001$) larger mitochondrial volume than the large cells (fraction 5). In untreated animals, the percentage volume of cytoplasm occupied by mitochondria was 25.9 ± 0.6%, N = 15, in fraction 1, and 23.0 ± 0.4% in fraction 5. In phenobarbital-treated animals, the percentage volumes were 24.4 ± 0.6% and 19.0 ± 0.4% in fractions 1 and 5 respectively.

(A)



(B)

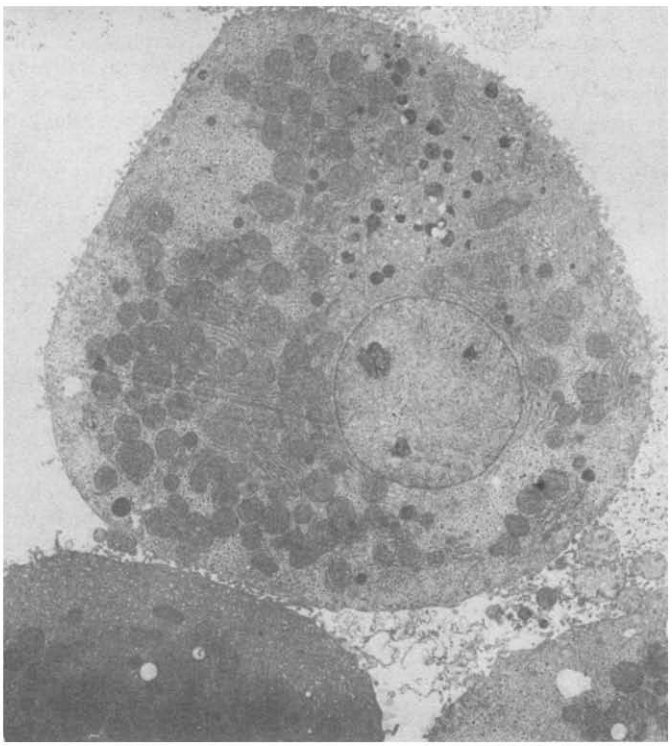


Fig. 1. Transmission electron micrographs (low magnification) of isolated hepatocytes in fraction 1 (A) and fraction 5 (B) from control rats. Magnification: ×3100 respectively.

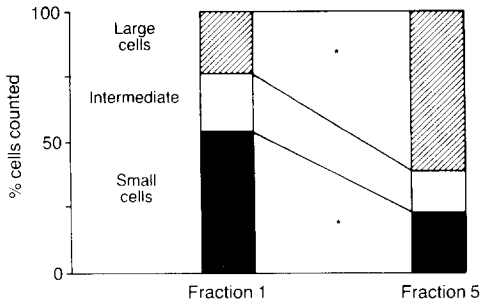


Fig. 2. Cell number distribution in elutriated hepatocyte fractions 1 (enriched in small cells) and 5 (enriched in large cells) from phenobarbital-treated rats as analyzed by flow cytometry. The bars [diagonal, large cells (Region 4); open, intermediate cells (Regions 2 + 3); and closed, small cells (Region 1)], represent the mean percentage of the total cells counted (N = 5000–10,000) in each fraction for five experiments. An asterisk (*) indicates $P < 0.01$, comparing the difference between the percentage of large cells in fractions 1 and 5 and the percentage of small cells in fractions 1 and 5 respectively.

Elutriation and flow cytometry. Isolated hepatocytes from untreated, phenobarbital-treated, and phenobarbital-plus-AIA-treated animals were separated into five fractions by centrifugal elutriation. To assess whether phenobarbital or phenobarbital-plus-AIA treatment was associated with a shift of cells into different elutriated fractions, we compared the number of cells in each elutriated fraction from these two experimental groups with the number of cells in elutriated fractions from untreated animals. The number of cells in each elutriated fraction was similar for the three groups. However, following phenobarbital treatment, the size of cells, not the number, increased significantly ($P < 0.01$) for both small and large cells. The hepatocyte size (mean channel number) was 48.2 ± 1.5 (N = 5) for the small cells (fraction 1) in untreated animals and 58.2 ± 2.1 for the small cells in phenobarbital-treated animals. For the large cells (fraction 5), the hepatocyte size was 724.6 ± 40.0 in the untreated animals and 847 ± 42.2 in phenobarbital-treated animals. This increase in cell size was equivalent in all subpopulations of elutriated hepatocytes, and it was similar in degree to that observed in freshly prepared hepatocytes of phenobarbital-treated animals (not shown). The distribution in the number of elutriated hepatocytes from phenobarbital-treated animals for fractions 1 and 5 is depicted in Fig. 2. The small cells were significantly ($P < 0.01$) enriched in fraction 1, and the large cells were enriched in fraction 5. Similar findings were observed for elutriated hepatocytes from untreated animals.

Functional characteristics of elutriated hepatocytes from untreated and phenobarbital-treated rats. In studies with untreated animals, the cytochrome P-450 content was found to have increased from fraction 1 to fraction 5 by a factor of two [98.7 ± 7.6 μmoles/mg protein (fraction 1) and 180.6 ± 72.3 (fraction 5), N = 5]. For phenobarbital-treated animals, the cytochrome P-450 content in elutriated hepatocytes is shown in Fig. 3. For these animals, the total cytochrome P-450 content also increased from fraction 1 to fraction 5 by a factor of two [109.7 ± 14.9] (fraction 1) and 248.7 ± 67.8 (fraction 5), N = 4]. 7-Ethoxycoumarin *O*-deethylase activity (Fig. 1B) increased from fraction 1 to fraction 5 by a factor of five [0.086 ± 0.023 (fraction 1) and 0.415 ± 0.063 (fraction 5), N = 3]. In contrast to the gradual increase in the cytochrome P-450 content, the activity of 7-ethoxycoumarin *O*-deethylase increased abruptly from fraction 2 to fraction 3.

Effect of AIA treatment on cytochrome P-450 content. After AIA treatment, the total cytochrome P-450 content of the hepatocytes was decreased markedly in all elutriated fractions. The relative change in cytochrome P-450 content was 32, 36, 15, 13 and 18% of control in fractions 1 through 5, respectively (Fig. 4), and reveals that the total cytochrome P-450 content had decreased proportionately more in fractions 3 through 5 as compared to fractions 1 and 2. The cytochrome P-450 content was decreased

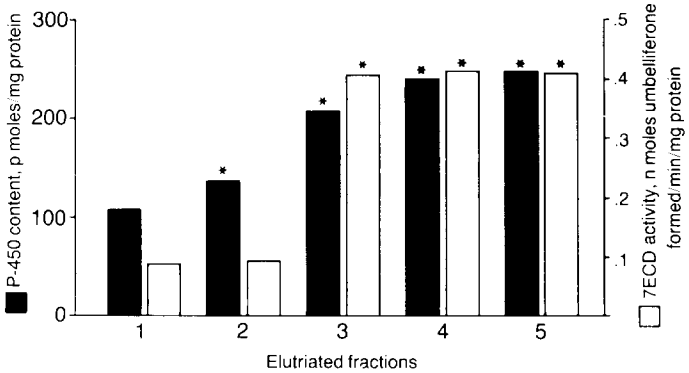


Fig. 3. Cytochrome P-450 content and 7-ethoxycoumarin *O*-deethylase (7-ECD) activity in elutriated hepatocyte fractions from phenobarbital-treated rats. The bars (open, 7-ECD activity; closed, cytochrome P-450 content) represent the mean of three to five experiments for each measure. Fraction 1 is enriched in small cells, whereas fraction 5 is enriched in large cells. An asterisk (*) indicates $P < 0.01$, comparing the difference between each respective fraction and fraction 1.

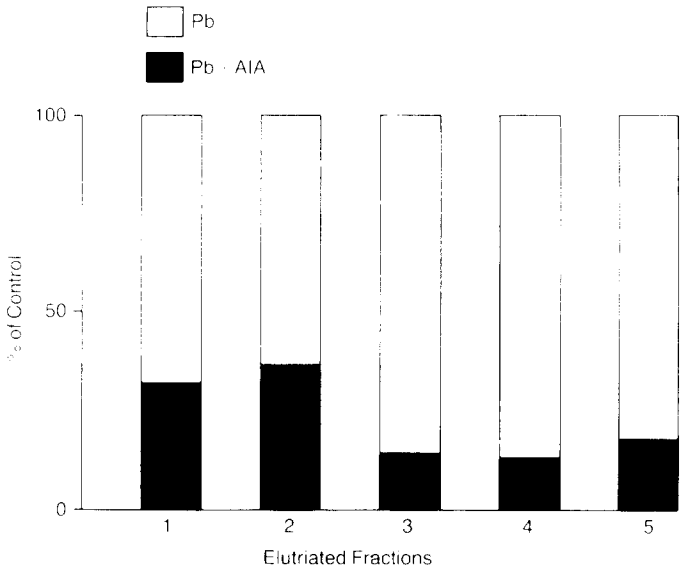


Fig. 4. Effect of allylisopropylacetamide (AIA) on the cytochrome P-450 content (% of control) in elutriated hepatocyte fractions from phenobarbital (Pb) treated rats. The bars represent the mean (N = 2–5) cytochrome P-450 content in Pb (control group) and Pb-plus-AIA (experimental group) treated animals. Fraction 1 is enriched in small cells and fraction 5 is enriched in large cells.

significantly ($P < 0.05$) in fraction 5 ($17.8 \pm 4.2\%$) as compared with fraction 1 ($31.7 \pm 3.2\%$, N = 4).

Fluorescein diacetate staining. In an attempt to provide evidence for the anatomic origin of the elutriated hepatocytes, we examined the uptake of FDA according to the protocol of Gumucio *et al.* [24]. In experiments *in vitro*, after 5 min of incubation with 1×10^{-5} M FDA, all hepatocytes stained equally with the dye. Thus, no preferential uptake of FDA by any hepatocyte subpopulation could be demonstrated. Following perfusion *in situ* of buffer containing 1×10^{-5} M FDA, we also could not discern any difference in the lobular distribution of the fluorescent stain within the four lobes. Hepatocytes were isolated following *in situ* FDA perfusion, and elutriated, and their fluorescence was measured by flow cytometry; again, small hepatocytes showed no increase in fluorescence over that in the large cells.

DISCUSSION

Several methods separate hepatocytes into fractions containing cells which differ in size and/or density [4, 8, 25–27]. Recently, centrifugal elutriation has been employed to separate different cell types, but this technique has been used to sort parenchymal liver cells into subpopulations in only a few studies. We selected centrifugal elutriation to separate hepatocytes of different sizes for three reasons: (1) because it allows substitution of a soluble physiologic medium for density colloidal matrices, (2) because it is associated with enhanced cell viability as compared to nonelutriated cells, and (3) because it provides a higher cell yield sufficient for cell incubation and culture studies. Excellent structural preservation of the separated hepatocyte populations was shown by Wanson and coworkers [8–10] utilizing this technique. We extended these observations, and at the same time determined some metabolic characteristics of these separated hepatocytes.

The functional performance of the hepatocyte subpopulations of our study suggest that the phenobarbital-inducible forms of cytochrome P-450 predominant in the large hepatocytes. This finding is in agreement with studies in which hepatocytes have been separated into different subpopulations by density gradient [4] and sedimentation velocity [27]. A recent report on elutriation data indicates similar observations [28]. In the density gradient studies, a 1.5- to 2.0-fold difference in cytochrome P-450 level was found between large and small cells in both untreated [4] and phenobarbital-treated animals [4, 29]. The induction of specific isoenzymes may not be reflected in the measurement of total cytochrome P-450 content [30]. Thus, the increment in total cytochrome P-450 content may only be 2-fold following phenobarbital treatment, but certain isoenzymes may be induced to a much larger extent [30, 31], and the synthesis of some may even be reduced [32].

In addition to the total P-450 content, we also studied the metabolism of 7-ethoxycoumarin which is catalyzed by an *O*-deethylase activity partly attributed to by a phenobarbital-inducible cytochrome P-450 isoenzyme [33–36]. Our study indicates that the distribution of the 7-ethoxycoumarin *O*-deethylase activity between small and large cells paralleled, in general, that of the total cytochrome P-450 content. This finding is in agreement with a study in which hepatocytes have been separated into different subpopulations by density gradient [36]. This finding also corresponds with a recent report indicating that 7-ethoxycoumarin *O*-deethylase activity shows a heterogeneous distribution within the hepatic lobule

[6]. In contrast to that report, which indicates a 2-fold concentration gradient between periportal and centrolobular regions, our results suggest a 5-fold concentration gradient in the activity of this enzyme between small cells and large cells. Results of immunocytochemical investigations have suggested that a gradual increase in the cytochrome P-450 content occurs between the periportal and the centrolobular regions [2]. Our findings of the distribution of the total cytochrome P-450 content in elutriated hepatocytes (Fig. 1B) support this observation in the intact liver. However, the abrupt increase in the activity of 7-ethoxycoumarin *O*-deethylase between fractions 2 and 3 suggests that a distinct change in the content of cytochrome P-450 isoenzymes may occur between large and small cells. In fact, this distinct difference in function may be underestimated because of the overlap in cell size within the fractions, and thus the differences may be more pronounced than our data would indicate.

It is presumed that the small cells are of periportal origin and the large cells of centrolobular origin. However, results of morphometric studies from the intact liver provide conflicting observations on cell size differences between centrolobular and periportal regions [7, 37, 38]. Studies from the intact liver do suggest morphometric evidence of an increased size of mitochondria in periportal cells when compared with centrolobular cells [7]. In addition, morphometric evidence from isolated subpopulations of hepatocytes suggests that small cells contain larger mitochondria than large cells [25, 27]. Our observations obtained by electron microscopy of differences in size of mitochondria between hepatocyte subpopulations is in accord with this morphometric evidence, and they provide additional support to the presumed anatomic origin of the small and large cells. Nevertheless, like other studies, the present investigation does not allow a definite assignment of the separated hepatocytes to specific lobular regions. Neither did the *in vitro* nor the *in vivo* studies with FDA demonstrate a selective uptake of this dye by a particular subpopulation of hepatocytes. These findings are at variance with data recently reported [24].

Our finding of a larger decrease in total cytochrome P-450 content in fraction 5 as compared to that in fraction 1 following AIA administration supports the greater cytochrome P-450 content level in the large over the small cells. Drug-mediated decrease in cytochrome P-450 level has been studied extensively in rats treated with AIA, an agent causing a dose-dependent destruction of hepatic cytochrome P-450 content that is most pronounced for the phenobarbital-inducible isoenzyme(s) of cytochrome P-450 [39–41]. The destructive process involves covalent binding of an activated metabolite of AIA to the heme moiety [39, 42, 43]. Our observations by light and electron microscopy demonstrate the absence of cellular necrosis, and our histologic findings were indistinguishable from those in the phenobarbital-treated animals. Because of sample loss, the 7-ethoxycoumarin *O*-deethylase activities in the AIA experiments could not be determined.

It has been reported that phenobarbital pretreatment influences the density of isolated hepatocytes [25, 27], and such a change in density might conceivably influence the separation of hepatocytes into subpopulations. However, following centrifugal elutriation, we did not detect any shift in hepatocyte number from one fraction to another following pretreatment with phenobarbital when compared with elutriated hepatocytes from untreated animals. Current evidence implies that cell separation by means of centrifugal elutriation is almost exclusively by cell size if the density difference between cells is less than 0.05 g/cm^3 (personal communication, D. M. Griffith, Beckman Instruments, Palo Alto, CA). Therefore, the reported changes in hepatocyte density following pretreatment with phenobarbital are not of the order to influence cell separation by centrifugal elutriation.

We utilized flow cytometry to assess cell size and number distribution for these hepatocyte subpopulations [44]. Our results are in accord with results of experiments in which other cell sizing techniques were employed to measure hepatocyte size differences in untreated and phenobarbital-treated animals [24, 26, 27, 45]. At the time of these studies, our Ortho-flow cytometry did not have the capability for giving cell measurements as either volume or diameter, and there is no formula available for conversion of the data into these parameters. In cytofluorographic analysis, the light scatter intensity, as measured from the forward narrow angle direction, connotes whole cell size, whereas the light scatter intensity, as measured at larger angles, contains information about internal structure of the cell [46–48]. Inasmuch as we utilized the forward narrow angle direction in our cell sizing analysis, the effect on the hepatocyte of phenobarbital pretreatment which we would most likely measure would be whole cell size, and not an effect that phenobarbital pretreatment might have on the internal structure of the cell. The major application of flow cytometry has been to measure cellular DNA content for estimating cell cycle distribution and ploidy [49, 50]. The functional significance of ploidy remains unclear. The cell size appears directly related to the cell ploidy in all mammalian tissue [51]. The degree of ploidy may be an important determinant of the heterogeneity of cell size distribution in normal rat parenchymal liver cells [52]. A recent study, employing centrifugal elutriation, indicated that synthesis of albumin in rat hepatocytes seems to be related to the degree of ploidy [53]. Other studies implied that hepatocytes which differ in size do not appear to have differences in function except when they are very large or very small [52]. We did not systematically evaluate ploidy, but our finding of a significantly increased number of double nucleated hepatocytes in fraction 5 as compared with fraction 1 would support the observation that cell size is determined by cell ploidy.

In conclusion, our *in vitro* investigation established quantitative differences in metabolic function and structure for cells of different sizes. Additional investigations will be needed for a more complete understanding of the functional importance of the *in vitro* metabolic heterogeneity in hepatocyte subpopulations described in this report.

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