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INITIAL RATE OF SODIUM TAUROCHOLATE UPTAKE IN ISOLATED ELUTRIATED HEPATOCYTES FROM UNTREATED AND PHENOBARBITAL-TREATED RATS*

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Abstract—The initial rate of sodium taurocholate uptake was measured in rat hepatocytes separated by centrifugal elutriation into five cell fractions whose difference in size was verified by flow cytometry. The hepatocytes were prepared from untreated and phenobarbital-treated rats. For untreated animals, the initial rate of taurocholate uptake at concentrations of 5 or 50 μM was the same for hepatocytes prior to fractionation and for each of the five elutriated fractions. Treatment of the animals with phenobarbital was associated with a significant increase in hepatocyte size in all fractions and caused a significant increase in the initial uptake rate. The extent of the rate increase in hepatocytes prior to fractionation was similar to that observed for each of the five hepatocyte subpopulations. Our observation indicates that phenobarbital causes a significant increase in the initial rate of sodium taurocholate uptake and suggests that large and small hepatocytes possess no inherent differences controlling the initial uptake process.

A gradient of bile salt uptake is observed along the hepatic sinusoids from the periportal to the centrolobular regions [3]. Such a gradient may be affected by a decreasing sinusoidal bile salt concentration, and/or differences in the density of bile salt receptors on the sinusoidal plasma membranes [4]. Current evidence suggests that all hepatocytes along the sinusoidal gradient are capable of bile salt transport [3–7] but whether the rate of this transport differs between hepatocytes from centrolobular or periportal regions remains unknown. Cells separated by centrifugal elutriation, largely on the basis of size difference, retain excellent viability as assessed by both structural and functional criteria [8–10]. The contribution to a given functional process by hepatocytes separated by centrifugal elutriation into large and small cells may therefore be examined. In a previous study, we used hepatocytes from phenobarbital-pretreated rats, separated by this technique, to show the heterogeneity in the distribution of the total cytochrome P-450 content in cells of different size [10]. In the present study, hepatocytes from untreated and phenobarbital-treated rats were separated into five cell size fractions by centrifugal elutriation, and the initial rate of sodium taurocholate uptake was measured for each of the subfractions. For the untreated rat, our findings indicate that the initial rate of sodium taurocholate uptake over a physiologic concentration range was comparable for both freshly isolated hepatocytes and elutriated hepatocyte subpopulations. For the phenobarbital-treated rat a significant enhancement was observed in the initial rate of sodium taurocholate uptake, and this increase in uptake was equal for freshly isolated and the elutriated hepatocytes. Thus, unlike the heterogeneous hepatocellular distribution of the phenobarbital-induced total cytochrome P-450 content, the phenobarbital-induced enhancement in sodium taurocholate initial uptake is uniform.

MATERIALS AND METHODS

Chemicals and isotopes. Radiolabeled taurocholate ([24-^{14}C]taurocholate, 52 mCi/mole) was purchased from New England Nuclear (Boston, MA). Purity of the label was established by thin-layer chromatography in three systems [11, 12]. Impurities were less than 3%. Unlabeled taurocholate was obtained from the Calbiochem-Behring Corp. (La Jolla, CA), and its purity was confirmed by thin-layer chromatography. All other chemicals were of reagent grade quality.

Animal preparation, isolation of hepatocytes, centrifugal elutriation, flow cytometry and microscopy. Male Sprague–Dawley rats that weighed 250–300 g and were 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., Indianopolis, IN, 80 mg/kg/day in 0.9% saline) by i.p. injection for 4 days. Their hepatocytes were isolated

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on the morning of day 5, after nembutal anesthesia (45 mg/kg, i.p.). The hepatocytes were obtained by the two-step procedure of *in situ* perfusion described by Seglen [13]. The details of this procedure and the preparation of the cells for centrifugal elutriation, flow cytometry, and light and electron microscopy are given in previous publications [10, 14].

Following purification and filtration of the isolated hepatocytes, the single cells were resuspended in carbogenated (5% CO_2 and 95% O_2) Krebs–Henseleit buffer. 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 100 *g* (840 rpm). 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 within each of the five elutriated fractions were separately concentrated by centrifugation at 1000 rpm for 3 min at 4°. The supernatant fraction was removed by aspiration and the cells were resuspended in cold carbogenated Krebs–Henseleit buffer. Viability was measured by the degree of trypan blue exclusion and by release of lactate dehydrogenase in freshly isolated hepatocytes, hepatocytes prior to elutriation, and in each elutriated fraction. The fractions were assessed by inverted microscopy for cell size distribution and by Coulter counter for quantity. Resuspended cell fractions were then prepared for flow cytometry.

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 the hepatocyte subpopulations prepared by centrifugal elutriation. All samples were suspended in carbogenated Krebs–Henseleit buffer at a concentration of 1–3 $\times$ 10^6 cells/ml. Immediately prior to analysis, samples were filtered through a 100 μm nylon mesh to remove clumps 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 cytofluorograf has been published [16]. 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 from analysis. The light scatter histogram recorded for untreated freshly isolated hepatocytes was arbitrarily divided into four cell size regions as follows:

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

These divisions were used for analysis for all subsequent experiments (Fig. 1). The computer-determined mean intensity light scatter produced by cells falling within each region was expressed as mean channel number. Because the cytofluorograf utilized was not equipped with a volume sensing device generating data on cell size, the mean channel number of forward light scatter for each region had to be used as an indicator of relative cell size. Five to ten thousand cells from each elutriated hepatocyte subpopulation were analyzed by cytofluorography and the number of cells falling in each of the four regions on the light scatter histogram was recorded. Fixed calf thymocyte nuclei were used for instrument standardization. Calibration was performed immediately prior to each analysis with polystyrene beads (5, 10 and 20 μm size) by determining the mean channel numbers. Mean channel number plotted against bead size was linear over each of the size range analyzed.

The cell distribution in the elutriated fractions was estimated by inverted microscopy prior to analysis by flow cytometry. In some experiments following elutriation, cell aliquots were fixed for light and electron microscopy.

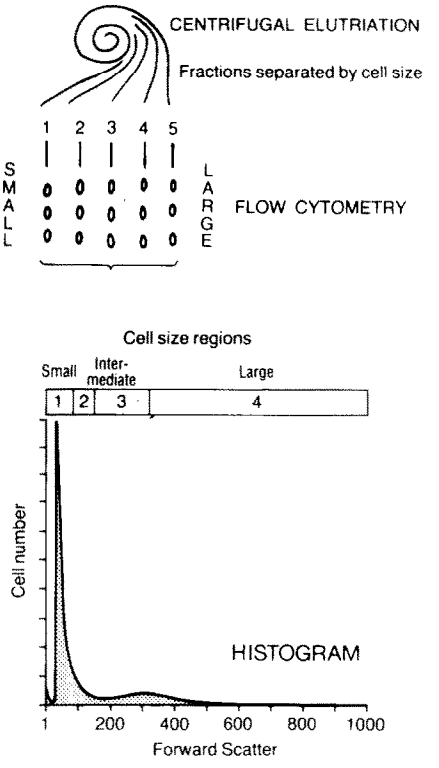

Fig. 1. Upper part of the figure: a schematic representation of the separation of hepatocytes into five subpopulation fractions (centrifugal elutriation) followed by cytofluorographic analysis for characterization of cell size distribution within these five fractions. Lower part of the figure: a representative size distribution histogram from flow cytometry analysis of freshly isolated hepatocytes from an untreated animal. On this histogram the horizontal axis represents forward narrow angle light scatter (cell size). The vertical axis represents frequency (cell number). The four light scatter cell size regions are depicted at the top of the histogram.

Hepatocyte incubation. Both freshly isolated hepatocytes and elutriated cell subfractions were prepared for the taurocholate uptake studies by suspending them in Krebs–Henseleit buffer pre-equilibrated with carbogen. The buffer contained 3 g/dl of bovine serum albumin. The final volume of each incubate was 1 ml, and the final concentration of cellular protein was 2–4 mg/ml. The incubation procedure protocol was a combination of those described by Schwartz *et al.* [17], Seglen [13] and Kimmich [18]. Hepatocytes were preincubated for 5 min at 37° in an oscillating (120 oscillations/min) water bath. The incubation was started by addition of 50 nCi [^{14}C]taurocholate/ml incubate to two concentrations (5 and 50 μM) of unlabeled taurocholate. Duplicate incubations were carried out for periods of 15, 30, 45 and 60 sec at 37°. At the termination of the study, the incubates were rapidly diluted with iced buffer and centrifuged at 290 *g* for 45 sec in a refrigerated centrifuge. The supernatant fraction was decanted and the cell pellet was resuspended in 5 ml of ice-cold 0.9% NaCl. The cells were washed in iced physiologic saline and centrifuged, and the final cell pellet was lysed in 0.5 ml of distilled water, transferred sequentially to protosol (New England Nuclear) and to scintillation vials containing aqueous scintillant, ASC II (Amersham-Searle, Chicago, IL), and counted by scintillation spectrometry (Tracor Analytical Counter, model 6892). Aliquots of the supernatant fractions from the three centrifugations were similarly processed. Recovery of the combined radioactive material was 97.4 ± 1.1% (N = 65). Conversion to dpm was by external standardization. Cellular protein was determined by a modification [19] of the method of Lowry *et al.* [20], using bovine serum albumin as a standard. Initial sodium taurocholate uptake is expressed as nmoles/min/mg cell protein. Cell size and number distribution are expressed as mean channel number and percent of counted cells respectively. Statistical indices were calculated by standard methods and are expressed as mean ± S.E.M. Student's *t*-test of differences between two sample means was used to assess the significance between parameters [21].

RESULTS

Elutriation, flow cytometry and microscopy. The isolated hepatocytes were separated into five cellular subfractions. Excellent cell viability was substantiated by low lactate dehydrogenase leakage (<15%), trypan blue exclusion (>90%) and normal structure by electron microscopy as previously reported [10]. The recovery of hepatocytes following elutriation was calculated for each experiment and was 89 ± 3.3% (N = 21). Each cellular subfraction was analyzed by flow cytometry to estimate the distribution of cells of different sizes (mean channel number) within this hepatocyte subpopulation. The hepatocytes of animals treated with phenobarbital showed a significant increase in cell size within fraction 1 (small cells) and fraction 5 (large cells) when compared with the corresponding hepatocyte fractions from untreated animals (Table 1). As reported previously, this increase in cell size was equivalent for freshly isolated hepatocytes and in all elutriated fractions [14]. Analysis of the distribution by cytofluorography of hepatocytes from phenobarbital-treated animals in fractions 1 and 5 indicated that all cells were significantly ($P < 0.01$) and equally enlarged (previously observed [14]). Morphologic assessment indicated that fractions 1 through 5 contained only hepatocytes and rarely a double hepatocyte. Morphometric measurement of mitochondrial volume indicated that the small cells (fraction 1) had significantly larger mitochondrial volume than the large cells (fraction 5). This was observed in both untreated and phenobarbital-treated hepatocytes [10].

Taurocholate incubation studies. We assessed the rate of the initial uptake of sodium taurocholate over a 1-min period at 37° at two different bile acid concentrations. The initial uptake rates in this study were determined from the slope of a linear regression analysis of the time points between 15 and 60 sec. A positive time 0 intercept was obtained, and was similar to those reported in the literature [22]. This intercept was taken to represent nonspecific binding rather than uptake. Phenobarbital treatment did not change significantly the position of the 0 time inter-

Table 1. Effect of phenobarbital treatment on size distribution of freshly isolated and elutriated subpopulations of rat hepatocytes by flow cytometry

Experimental group	Hepatocyte size (mean channel number)	
	Region 1 (small cells)	Region 4 (large cells)
Freshly isolated hepatocytes		
Untreated	47.2 ± 2.0 (5)	555.4 ± 24.4 (5)
Phenobarbital-treated	58.2 ± 2.6*(5)	706.6 ± 44.3*(5)
Elutriated hepatocytes		
Fraction 1		
Untreated	48.2 ± 1.5 (5)	536.4 ± 34.4 (5)
Phenobarbital-treated	58.2 ± 2.1*(5)	647.2 ± 39.2*(5)
Fraction 5		
Untreated	47.4 ± 1.3 (5)	724.6 ± 40.0 (5)
Phenobarbital-treated	59.2 ± 3.1*(5)	847.2 ± 42.1*(5)

Values are the mean ± S.E.M. with the number of observations in parentheses.
* $P < 0.01$, as compared to untreated.

Table 2. Rate of initial uptake of sodium taurocholate (NaTC) by freshly isolated and elutriated subpopulations of rat hepatocytes

Experimental group		Initial uptake (nmoles/min/mg protein)	
		5 μM NaTC*	50 μM NaTC*
Freshly isolated		0.126 ± 0.002 (9)	0.750 ± 0.027 (12)
Elutriated fraction	1	0.127 ± 0.001 (6)	0.782 ± 0.027 (12)
	2	0.128 ± 0.001 (6)	0.739 ± 0.029 (12)
	3	0.122 ± 0.004 (6)	0.773 ± 0.011 (12)
	4	0.126 ± 0.001 (6)	0.756 ± 0.035 (12)
	5	0.125 ± 0.002 (6)	0.745 ± 0.026 (12)

Values are the mean ± S.E.M. with the number of observations in parentheses.
* Bile salt concentration.

cept. When using a final taurocholate concentration of 50 μM, the initial bile salt uptake linear over the 1-min period, the respective values of uptake at 15, 30, 45 and 60 sec being 0.401 ± 0.023, 0.537 ± 0.023, 0.633 ± 0.028 and 0.754 ± 0.026 nmole/mg protein. The influence of temperature on the uptake process was determined by incubation at 4°. The rate of uptake at 4° was only 5% of that of 37°.

As depicted in Table 2, the initial rates of sodium taurocholate uptake at 50 and 5 μM were not significantly different between freshly isolated hepatocytes and any of the elutriated subfractions. Following treatment of the animals with phenobarbital for 4 days, the initial rates of sodium taurocholate uptake at 5 μM were greatly ($P < 0.001$) increased, and still equivalent for all cell populations (Table 3).

DISCUSSION

Our results on the initial rate of sodium taurocholate uptake indicate that no difference exists in uptake by small versus large hepatocytes. These results are based on the protein content per cell, and it is possible that uptake rates might be different if they were based on cell surface area, a measure which could not be obtained by the technique available to us. Our observation is in accord with the results of others, suggesting that all hepatocytes along the sinusoidal gradient transport bile salts [4–7]. The present results also support the hypothesis that the sinusoidal gradients of bile salts is determined by the bile salt concentration rather than by a graduated number of the receptor(s) on the sinusoidal membrane. We studied only two taurocholate concentrations and, thus, cannot calculate kinetic parameters. Yet, the two concentrations employed fall within the physiologic range for taurocholate concentrations encountered in the portal vein of the rat [23, 24]. Finding no apparent difference in initial uptake rates of taurocholate according to cell size suggests that there is probably also no difference in K_m and V_{max} in small and large hepatocytes. Over the concentration range studied, 90% of the initial uptake of taurocholate has been reported to be sodium coupled [25], making it unlikely that sodium-independent mechanisms might explain our findings. We did not specifically measure sodium-dependent and sodium-independent uptake so it does remain possible that reciprocal changes occur in these parameters in accordance to cell size.

Our observations suggest that taurocholate uptake is uniform along the sinusoidal gradient irrespective of cell size distribution within the liver lobule. Results of morphometric studies from the intact liver

Table 3. Effect of phenobarbital pretreatment on the rate of initial uptake of sodium taurocholate (NaTC) by freshly isolated and elutriated subpopulations of rat hepatocytes

Experimental group	Initial uptake (nmoles/min/mg protein)*	
	5 μM NaTC*	
	Untreated	Phenobarbital-treated
Freshly isolated	0.126 ± 0.002 (9)	0.240 ± 0.009‡(12)
Elutriated fraction		
1	0.126 ± 0.002 (9)	0.242 ± 0.010‡(4)
5	0.125 ± 0.002 (6)	0.247 ± 0.007‡(4)

Values are the mean ± S.E.M. with the number of observations in parentheses.
* Bile salt concentration
‡ $P < 0.001$, as compared with untreated.

provide conflicting evidence on cell size differences between centrolobular and periportal regions [26–28]. In a previous publication, we provide morphometric evidence for the difference in mitochondrial size between small cells and large cells [10] that corresponds with the difference in mitochondrial size in periportal cells when compared with centrolobular cells in the intact liver [26] and in isolated subpopulations [29, 30]. Conflicting data on this morphometric parameter were reported [27]. Our observations, like those of others, still cannot provide the assignment of distinct characteristics of the hepatocyte according to lobular region.

Our data must be interpreted with caution. First, interpretation of data on plasma membrane function neglects the effect digestive enzymes exert during hepatocyte isolation on plasma membrane receptor(s) and enzyme markers [31–33], including the putative bile salt receptor(s) [34]. Regeneration of the functions of these receptors and enzymes occurs [35], but it is unknown how rapid and complete the regeneration is. Our finding of no difference in taurocholate uptake among freshly isolated and any of the elutriated hepatocyte subpopulations could reflect a uniform effect of collagenase (plus impurities) on surface membrane enzymes as well as on the putative bile salt receptor. Evidence against this argument is the fact that the rate of bile salt uptake for hepatocytes from phenobarbital-treated animals was greater than that from untreated animals, which, in turn, suggests that the bile salt receptor(s) is intact in both freshly and elutriated hepatocyte subpopulations (*vide infra*).

Second, interpretation of our findings is complicated by the fact that the elutriated fractions do not represent homogeneous cell size populations. Because of the overlap of cells of different sizes within the elutriated fractions, small differences in initial taurocholate uptake between the small cells could have been missed. Our results differ from those reported in one study in which the initial rate of sodium taurocholate uptake was found to be greater in large hepatocytes than in small hepatocytes [36]. The results of this study [36] were interpreted to indicate that the receptor affinities for the two types of cells were similar, whereas, the number of receptors on the large hepatocytes was increased. The reason for the difference in findings in these two studies is unknown. The experimental design of Stacey and Klaassen [36] differed from ours in that hepatocytes in their study were separated by a density gradient technique, and incubations were carried out in albumin-free medium. We separated hepatocytes by centrifugal elutriation and bathed them in a physiologic concentration of albumin (3 g/dl). The presence of albumin may have nullified differences in the initial rate of uptake between small and large cells, as recently reported for bromosulfophthalein transport in the intact rat liver [37] and rose bengal transport by isolated rate hepatocytes [38]. Because our taurocholate uptake studies were carried out in the presence of 3 g/dl bovine albumin, our results are not directly comparable with those of several other authors. In the taurocholate uptake studies by Anwer *et al.* [22], a similar concentration of bovine serum albumin was used. The rates predicted from the kinetic data for the initial rate of taurocholate uptake reported by Anwer *et al.* [22] correspond closely to the initial uptake rates at concentrations of 50 μM in our experiments (Table 2).

It is unlikely that our findings of an enhanced rate of initial uptake of sodium taurocholate by hepatocytes of phenobarbital-treated animals is related only to the recognized enlargement in cell size induced by phenobarbital [30, 39], as we found no difference in bile salt uptake between the large and small cells of untreated animals. The effects of phenobarbital upon the liver are multiple. Our findings may be related to other phenobarbital-induced changes in structure and function of the hepatocyte plasma membrane. Phenobarbital may induce the putative bile salt receptor(s) of the plasma membrane as shown for isolated hepatocyte plasma membranes [40]. The receptor(s) is also induced following expansion of the bile salt pool by taurocholate feeding [41].

The enzyme, sodium-potassium activated adenosite triphosphatase (Na^+, K^+-ATPase), which is localized within the hepatocyte sinusoidal membrane [42, 43], is currently considered to be an important regulator of bile salt uptake by the sinusoidal plasma membrane of the hepatocyte [44–46]. Our results fall in line with those of several reports on the phenobarbital-mediated enhancement of the activity of this enzyme [47–49]. Inasmuch as all reports in the literature are not consistent as regards this effect of phenobarbital [50–52], it remains possible that there is no direct relationship between Na^+, K^+-ATPase activity, bile acid transport and the administration of phenobarbital.

Yet another consideration deserves discussion. Since our taurocholate uptakes were carried out in the presence of 3 g/dl serum bovine albumin, an influence of phenobarbital on the plasma membrane albumin receptor [53–55] may be operative. An earlier study in which albumin was not used showed no influence of phenobarbital on the rate of taurocholate initial uptake [56]. A more recent study, which did not address the effect of phenobarbital treatment, substantiates that the initial rate of sodium taurocholate uptake by isolated hepatocytes is saturable in the presence or absence of albumin [57]. In this study, the presence of albumin (3 g/dl) was found to increase markedly the K_m for taurocholate but to have essentially no effect on the V_{max}. The results were interpreted to be compatible with the expected lowering influence of albumin on the free concentration of taurocholate and to suggest that the initial rate of sodium taurocholate uptake of isolated hepatocytes occurs by qualitatively similar mechanisms whether or not albumin is present. At the present time, the question as to the existence and/or physiologic role of the albumin receptor has not been resolved [58–60].

In conclusion, our findings suggest that subpopulations of hepatocytes separated by centrifugal elutriation show no difference in the rates of initial uptake of sodium taurocholate and that this rate of uptake is uniformly increased by phenobarbital treatment. Therefore, our observations support the hypothesis that the bile salt sinusoidal gradient is determined by the bile salt concentration gradient

within the hepatic microcirculation, and that the phenobarbital-induced enhancement of taurocholate uptake may be related to an increase in activity of sinusoidal plasma membrane Na^+, K^+-ATPase and/or the induction of a (the) taurocholate specific plasma membrane receptor(s) phenobarbital.

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