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SHORT COMMUNICATIONS

The influence of phenobarbital pretreatment on the subcellular distribution in liver and transport rate in isolated hepatocytes of dibromosulfophthalein

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The mechanism by which the hepatic clearance of non-metabolized organic anions is increased by phenobarbital pretreatment is rather complex. Probably, influences of an increased functional liver mass and bile flow [1], increased hepatic content of Y proteins [2], functional changes in hepatocyte membranes [3] and also an increased hepatic blood flow [4, 5], are involved. Our previous study using the isolated perfused liver technique, under flow standardized conditions, showed, in contrast to *in vivo* studies, an unchanged hepatic uptake of dibromosulfophthalein (DBSP). This might indicate that the increased uptake rate *in vivo* is due to increased hepatic blood flow instead of functional changes in hepatocyte membranes [6]. In addition, the rate of release of DBSP from liver into plasma was decreased by phenobarbital pretreatment, while the transport rate across the canalicular membrane was increased. The former effect was suggested to be due to changes in hepatic content of Y proteins and increased intracellular binding while the latter effect was suggested to be the consequence of an increased bile flow (reduced bile to liver back transport and/or enhanced canalicular transport rate). To clarify these points, we decided to study the subcellular distribution of DBSP in control rats and in rats pretreated with phenobarbital. Since isolated hepatocytes lack a closed canalicular lumen, the possible influence of biliary dilution can be excluded in this preparation. Therefore the secretion of DBSP from hepatocytes isolated from phenobarbital pretreated rats was also studied.

Materials and methods

Materials. DBSP was obtained from Société d'Etudes et de Recherches Biologiques (S.E.R.B.), Paris, France; albumin (demineralized bovine albumin) from Poviet (Oss, Holland); collagenase (type I) from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from E. Merck A.G. (Darmstadt, F.R.G.).

Analysis of DBSP. DBSP in plasma and bile samples was measured spectrophotometrically at 575 nm at an alkaline pH [6]. DBSP in liver homogenates and in the particulate fractions was measured after methanol extraction. DBSP in the isolated cells was measured as described before [8].

In vivo *experiments.* Male Wistar rats (290–310 g), which had free access to food and water, were used. The animals were anaesthetized intraperitoneally with sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, IL), (60 mg/kg body wt). The common bile duct was cannulated and the animals were kept at 38° ± 0.5. The renal pedicles were ligated prior to injection of DBSP. DBSP was injected i.v. into the femoral vein in two doses: 3.0 and 30 μmole, respectively. Livers were removed after 10 min at which time the liver content appeared to be maximal, immediately cooled by perfusion of saline via the hepatic veins and thereafter homogenized as described in detail elsewhere [7, 24]. Supernatant and particulate fractions were subsequently prepared [7, 24].

Pretreatment of rats with phenobarbital. Rats were pretreated with phenobarbital (90 mg/kg i.p. for 5 days) as described previously [6]. The animals were used 24 hr after the last dose.

Protein binding. Unbound concentrations of DBSP in the supernatant fractions and in the plasma were determined in ultrafiltrates [8]. The amount of DBSP bound to the cytosolic Y (ligandin) and Z proteins was quantified after applying the 165,000 *g* supernatant to a Sephadex G-75 column [6]. The concentrations of ligandin and Z protein in the cytosol of livers from phenobarbital pretreated and control rats were estimated by dye binding as described in detail in previous papers [6, 8].

Isolated hepatocytes. Isolation of liver cells, incubation conditions and measurement of uptake and release from the cells and also the assessment of viability are described elsewhere [8]. Rate constants for uptake and release velocity were calculated both by determining the slope of the initial part of the individual uptake and release curves as well as analysing these data according to a closed two-compartment pharmacokinetic model comprising a cellular and medium compartment, using the initial uptake rates and the ratio of the amounts in the cells and medium at the time that equilibrium between uptake and release is reached [9, 10]. Uptake and release experiments were performed in media containing 2 and 1% albumin, respectively.

Statistical analysis. The values of all calculated parameters were obtained from at least four separate experiments and expressed as mean ± S.E.M., unless otherwise indicated in the text. Student's *t*-test was utilized to determine a significant difference between control and experimental group.

Results

Distribution studies. The subcellular distribution of DBSP in the liver was studied at two doses administered to rats *in vivo*. The results after a relatively low dose of 3 μmole of the substrate are summarized in Table 1. Forty-four per cent of the amount of DBSP present in the liver 10 min after injection, was found in the particulate fraction and 56% in the cytosol. The amount of DBSP in the cytosol was bound mainly to Y and Z proteins. A small fraction of 1.5% of the liver content (2.7% of the total concentration in the cytosol) was found unbound in the cytosol. After phenobarbital pretreatment the particle/cytosol ratio appeared to be unchanged. The relative amount of DBSP bound to the Y protein was increased significantly by about 40%, whereas binding to the Z protein decreased by about 70%. However, the total amount of substrate which was bound to Y and Z proteins, did not change. Finally, the unbound fraction in the cytosol decreased significantly by about 65%. After the relatively high dose of 30 μmole, over 70% of the liver content was found to be bound to the particulate fraction (Table 2), with less than 30% found in the supernatant fraction. Only about 7% of the liver content was bound to the cytosolic Y protein; a comparable fraction appeared to be bound to Z protein. 1.5% of the liver content (5.6% of the total concentration in the cytosol) of DBSP was found unbound in the cytosol. After phenobarbital the particle/cytosol ratio appeared to be unchanged. The unbound fraction in the cytosol decreased by about 30%.

Table 1. The influence of phenobarbital pretreatment on the hepatic distribution of DBSP in rat livers after a dose of 3 μmole per animal

	Controls	Phenobarbital
Liver weight (g)	10.1 ± 0.5	13.1 ± 0.6*
Fraction in particles (% of liver content)	43.6 ± 3.5	46.3 ± 2.0
Fraction in cytosol (% of liver content)	56.4 ± 3.5	53.7 ± 2.0
Fraction bound to Y protein (% of liver content)	39.1 ± 1.9	55.5 ± 3.0†
Fraction bound to Z protein (% of liver content)	21.5 ± 1.6	6.9 ± 0.8‡
Fraction unbound in cytosol (% of liver content)	1.5 ± 0.1	0.5 ± 0.1*
Fraction unbound in cytosol (% of total concentration in the cytosol)	2.7 ± 0.3	1.0 ± 0.2‡

DBSP was injected into the femoral vein of anaesthetized rats. Livers were taken 10 min after the dose was given. Homogenates were separated in supernatant and particulate fractions. Unbound concentrations of DBSP in plasma and supernatants were determined in ultrafiltrates. Amounts of the substrate bound to Y and Z proteins were quantified using a Sephadex filtration technique.

Significantly different from controls *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, Student's t-test.

Hepatocyte transport studies. The viability of hepatocytes isolated from phenobarbital-pretreated rats, was found to be comparable with cells isolated from untreated rats (controls). The Y protein binding capacity for DBSP in the cytosol of isolated hepatocytes was 0.122 ± 0.027 μmole/g wet liver weight ($n = 4$, ± S.E.M.) after phenobarbital pretreatment of the donor rats, whereas that of control cells was 0.062 ± 0.004 μmole DBSP/g ($n = 4$). For the Z protein values of 0.044 ± 0.008 ($n = 3$) and 0.048 ± 0.011 μmole/g were found in phenobarbital cells and control cells [8] respectively. The uptake of DBSP in hepatocytes isolated after phenobarbital pretreatment was studied in time. The initial uptake rate appeared to be unchanged (Table 3); the rate constant for uptake into the isolated cells, calculated according to [9], was 0.028 ± 0.002 min^{-1} after phenobarbital pretreatment while in control cells this value was 0.027 ± 0.002 min^{-1}. The release of DBSP from preloaded cells enabled calculation of the rate constant for release from the cells, according to a closed two-compartment model [9, 10]. No statistically significant change in the rate constant of release from the cells after phenobarbital pretreatment could be established, an observation which was confirmed by an almost identical initial rate of transport out of the cells (Table 3).

Discussion

From the present results it can be concluded that the distribution of DBSP between cytosol and particulate fractions of rat liver is clearly dose dependent. At a relatively low dose (3 μmole) binding to ligandin and Z protein amounted to more than 50% of the total liver content. At the high dose (30 μmole), the cytosolic concentration, calculated from the directly determined liver supernatant concentration after applying corrections for dilution, exceeded 600 μM and hepatic storage of DBSP is largely dependent on binding to cell organelles. Binding to high affinity sites of ligandin and Z protein, as determined by gel filtration of liver cytosol becomes saturated at this dose [6]: multiple

Table 2. The influence of phenobarbital pretreatment on the hepatic distribution of DBSP in rat livers after a dose of 30 μmole per animal

	Controls	Phenobarbital
Liver weight (g)	10.1 ± 0.5	13.8 ± 1.2
Fraction in particles (% of liver content)	72.8 ± 1.8	70.6 ± 1.5
Fraction in cytosol (% of liver content)	27.2 ± 1.8	29.4 ± 1.5
Fraction bound to Y protein (% of liver content)	7.2 ± 0.6	17.4 ± 1.8†
Fraction bound to Z protein (% of liver content)	6.8 ± 0.8	6.2 ± 1.1
Fraction unbound in cytosol (% of liver content)	1.5 ± 0.1	1.0 ± 0.1*
Fraction unbound in cytosol (% of total concentration in the cytosol)	5.6 ± 0.4	3.5 ± 0.1†

Experimental details as in Table 1.

Significantly different from controls *$P < 0.05$, †$P < 0.01$, Student's t-test.

Table 3. Phenobarbital pretreatment and transport of DBSP in isolated hepatocytes

	Controls		Phenobarbital pretreatment	
Rate constant for uptake*	0.027 ± 0.002	(n = 17)	0.028 ± 0.002	(n = 6)
Rate constant for release*	0.149 ± 0.010	(n = 17)	0.162 ± 0.005	(n = 6)
Initial rate of uptake†	0.67 ± 0.05	(n = 23)	0.73 ± 0.01	(n = 6)
Initial rate of release†	0.44 ± 0.04	(n = 7)	0.42 ± 0.04	(n = 4)
Ligandin content‡	0.062 ± 0.027	(n = 4)	0.122 ± 0.027*	(n = 4)

* Calculated from the entire cell content–time curves according to two-compartment analysis, expressed in fraction per min.

† Calculated from the initial slopes of the cell content–time curves for the uptake and release studies, expressed in nmole/min/10^6 cells.

‡ Expressed as μmole DBSP bound to ligandin per g wet liver weight in the presence of an excess of DBSP.

The number of experiments (separate cell isolations) are indicated in parentheses.

binding sites for DBSP at ligandin and Z protein were detected with dissociation constants in the order of magnitude of 10 μM for the combined proteins in accordance with studies on purified ligandin [11] (which is identical to glutathione *S*-transferase B) and purified Z protein [12]. The moderately increased unbound concentration of DBSP and the different intracellular distribution pattern at the high dose imply low affinity binding sites with a high capacity associated with cell organelles.

Phenobarbital pretreatment which results in a roughly two-fold increase in cytosolic Y protein concentration was expected to decrease the unbound concentration of organic anions and consequently increase hepatic distribution volume [6]. Indeed, the present results show a decrease in unbound DBSP concentration in cytosol at both the low and high doses of DBSP. However the partition between the cytosol and particulate fractions remained unchanged (Tables 1 and 2). We conclude, therefore, that phenobarbital pretreatment probably resulted in an increase in binding both to the cytosolic proteins and the cell organelles. At the dose of 3 μmoles the total hepatic fraction of DBSP bound to Y and Z proteins was not affected by phenobarbital pretreatment but there was a shift from Z protein to ligandin. Such a shift was not seen at the 30 μmoles dose, probably because an excess of DBSP kept binding to Z protein saturated. In any event the increase of both high affinity and low affinity binding sites in the liver resulted in a reduced free concentration of DBSP. This concentration very likely represents the driving force for transport of DBSP out of the cells. The decreased free concentration explains the reduced liver to plasma transport rate observed for organic anions in isolated perfused liver [6, 15]. If we assume that at the liver content resulting from the low dose of DBSP, the cell to medium transport operates at first order conditions, the 60–70% decrease of the free concentration of DBSP would be expected to a result in a reduction of release velocity by a comparable factor. The present release experiments with isolated hepatocytes from phenobarbital pretreated rats did not show an effect of this magnitude compared with controls, although the intracellular concentrations were in the same range as obtained at the low dose of DBSP *in vivo*. In fact no significant change was observed, in spite of a normal viability and clearly increased ligandin content of the hepatocytes from the phenobarbital pretreated rats. This result may indicate that the supposed effect on transport, due to a decreased unbound concentration, is compensated for by another effect of phenobarbital on transport out of the cells. Since a closed canalicular lumen is lacking in isolated hepatocytes, biliary dilution of DBSP resulting in an increased net secretion rate cannot occur and an effect of phenobarbital via an increase in bile salt independent bile flow as reported for the intact organ [1, 3, 6, 22–24] is therefore excluded. Also increased conjugation with GSH as reported for BSP [3, 21] is not a likely explanation [1, 3, 6, 20]. It is possible however that phenobarbital treatment resulted in an increased membrane transport rate by changes in affinity and/or number of membrane carrier sites. Such an effect of phenobarbital has been reported for liver plasma membrane Na^+, K^+-ATPase [13, 14].

Our studies with isolated hepatocytes showing no effect of phenobarbital pretreatment on the rate of uptake confirm the idea from *in vivo* [3–5] and perfused rat liver [6, 15] studies that the increased initial plasma disappearance due to phenobarbital treatment observed for various anions is due to increased hepatic blood flow rather than to increased membrane transport. They also imply that ligandin is not involved in the membrane transport process responsible for organic anion-uptake in the liver as was originally suggested for BSP [2] and more recently for bilirubin [16]. This is in accordance with several other studies showing that variations in hepatic content of ligandin are not correlated with changes in initial hepatic uptake rate of organic anions [2, 6, 15, 17–19].

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Conjugation of 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) with glutathione via a sulphate ester in hepatic cytosol

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7-Hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA), a carcinogenic major metabolite of 7,12-dimethylbenz[*a*]anthracene (DMBA) [1–4], has been demonstrated to be activated to a potent frame shift type of mutagen, 7-HMBA sulphate, by rat liver cytosolic sulphotransferase [5]. The mutagenic activity of 7-HMBA which was exerted by the hepatic 105,000 *g* supernatant fraction (S105) in the presence of a PAPS-generating system (ATP, sulphate and magnesium ions) was much higher than that exerted by the hepatic 9000 *g* supernatant fraction (S9) in the presence of an NADPH-generating system [5]. This strongly suggests that in rat liver sulphate conjugation may play an important role in the metabolic activation of 7-HMBA rather than epoxidation. In addition, the mutagenicity of DMBA was exerted to a greater extent by the addition of the PAPS-generating system to S9 fortified with the NADPH-generating system. The active metabolite, 7-HMBA sulphate, has been isolated as a crystalline sodium salt both from the S9-NADPH-PAPS system containing DMBA and from the S105–PAPS system containing 7-HMBA [5].

During the course of our investigation of the metabolic activation of DMBA and 7-HMBA, we found that the addition of glutathione (GSH) to the S105–PAPS system potentially inhibited the mutagenicity of the carcinogens towards *Salmonella typhimurium* TA 98. The present communication deals with (1) the metabolic inactivation of the intrinsic mutagenicity of 7-HMBA sulphate by GSH *S*-transferase in rat liver S105 and (2) the isolation and identification of a GSH *S*-conjugate formed from 7-HMBA via 7-HMBA sulphate by rat liver S105 fortified with a PAPS-generating system and GSH as well as from 7-HMBA sulphate by S105 in the presence of GSH.

The hepatic soluble supernatant fraction, S105, obtained from untreated male Wistar rats, weighing 180–200 g, was dialysed at 0–2° for 20 hr against 0.1 M phosphate buffer (2500 vol.), pH 7,4, and preincubated at 37° for 20 min with 7-HMBA in the presence of an overnight culture of *S. typhimurium* TA 98 and the PAPS-generating system. His^+ reverse mutation of TA 98 was determined by the method of Ames *et al.* [6] after the mixture was diluted with soft agar, poured onto a hard agar plate, and incubated at 37° for 48 hr. GSH (4 mM) added to the preincubation mixtures containing various amounts of S105 completely inhibited the mutagenicity exerted by 7-HMBA (Fig. 1). In the absence of GSH, 7-HMBA induced 921 His^+ revertant colonies/S105 from 50 mg liver/plate as the maximal activity. Under these conditions using S105 from 50 mg liver, the rate of biological formation of 7-HMBA sulphate determined by the previous method [5] was 53.5 nmole/mg protein/min in the absence of GSH. 7-HMBA, however, neither showed any degree of mutagenicity nor yielded the sulphate when any one of the ingredients of the PAPS-generating system, sodium sulphate, ATP and S105, was omitted from the preincubation medium. The formation of 7-HMBA sulphate was also not observed when GSH was added to the preincubation medium, suggesting that the metabolically formed mutagen was completely scavenged by GSH and GSH *S*-transferase under these conditions.

Synthetic 7-HMBA sulphate (Na) induced 1965 His^+ revertant colonies/0.1 μmole/plate as the maximal activity after 20 min preincubation with TA 98 in the presence and absence of S105 (from 50 mg rat liver). In the presence of S105, the intrinsic mutagenicity of the sulphate ester was potentially inhibited by the addition of GSH (4 mM) to the preincubation medium (Fig. 2).

Isolation of a glutathione conjugate formed from 7-