Partial Purification of Two Lithocholic Acid-Binding Proteins from Rat Liver 100000g Supernatants

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1. The partial purification of two lithocholic acid-binding proteins from liver 100000g supernatants is described. 2. Gel-filtration, $(\text{NH}_4)_2\text{SO}_4$ fractionation, $\text{Ca}_3(\text{PO}_4)_2$ fractionation and ion-exchange chromatography were used. 3. Both proteins exhibited glutathione *S*-transferase activity; one may be the non-specific anion-binding protein ligandin. 4. Glutathione *S*-transferase activity of one of the binding proteins was inhibited by lithocholic acid.

The removal of anions from plasma and their excretion into bile is an important function of the liver. Ligandin, a non-specific anion-binding protein present in hepatic 100000g supernatants, may be an important component of the uptake of many different anions from plasma (Levi *et al.*, 1969; Litwack *et al.*, 1971). Kaplowitz *et al.* (1973) showed that ligandin has glutathione *S*-transferase activity (EC 2.5.1.18), and Habig *et al.* (1974*a,b*) showed that purified ligandin had the same amino acid composition and enzymic activities as glutathione *S*-transferase B.

Bile acid transport is one aspect of liver function which, although quantitatively important, is poorly understood. In the rat (total bile acid pool about 40 μmol) approx. 400 μmol of bile acid is removed from the portal vein and is transported across the liver each day. The uptake of sodium taurocholate from the extracellular fluid into the liver follows Michaelis-Menten kinetics both in the intact dog liver (Glasinovic *et al.*, 1975) and in isolated rat hepatocytes (Schwarz *et al.*, 1975). This suggests that bile acid transport is a carrier-mediated process. Proteins having binding sites for cholic acid, glycocholic acid, chenodeoxycholic acid and lithocholic acid were demonstrated in 100000g supernatants from rat livers by an equilibrium-dialysis technique (Strange *et al.*, 1976, 1977). The large values for both the dissociation constants of binding of these bile acids (approx. 1 μM) and the concentration of binding sites (approx. 1 $\mu\text{mol/g}$ of supernatant protein) suggested that these binding proteins might be acting as carrier proteins in the transport of bile acids across the hepatocyte.

We now describe (1) the partial purification of two lithocholic acid-binding components, (2) their examination for glutathione *S*-transferase activity and (3) the effect of bile acids on the glutathione *S*-transferase activity of the two lithocholic acid-binding components.

Materials and Methods

Chemicals

Lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid), chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid), deoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid), cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) and the glycine conjugate of cholic acid were purchased from Maybridge Chemical Co., Tintagel, Cornwall, U.K., and were purified as described by Strange *et al.* (1977). [24- ^{14}C]Lithocholic acid (59 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and shown to be 99% pure by t.l.c. Calcium phosphate gel was obtained from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-75, DEAE-Sephadex A-50 and CM-Sephadex C-50 were from Pharmacia Fine Chemicals Ltd., London W5 5SS, U.K. Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid] was generously given by Merck, Sharp and Dohme, Hoddesdon, Herts., U.K.

Analytical methods

Enzyme assays were performed as described by Habig *et al.* (1974*b*) except that a temperature of 37°C was used. The conjugation of glutathione with 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene was studied at 340 nm at pH 7.5 and 6.5 respectively. Conjugation with ethacrynic acid was studied at 270 nm at pH 7.5. Reaction rates were corrected for small rates (less than 5% of total rate) of non-enzyme-catalysed conjugation of glutathione with 1-chloro-2,4-dinitrobenzene and ethacrynic acid.

To identify the lithocholic acid-binding components radioactive lithocholic acid (16 nmol; 1 μCi) was added to portions (2 ml) of the partially purified

preparations described below. The mixtures were eluted from a column (30cm×2cm; void volume 50ml) of Sephadex G-75 at 4°C by using sodium/potassium phosphate buffer (20mm; pH7.4) containing NaCl (100mm) (Strange *et al.*, 1976, 1977). This buffer is referred to as phosphate/NaCl buffer. The flow rate was 22ml/h and the fraction volume was 3.5ml. Radioactive lithocholic acid was eluted with proteins of mol.wt. approx. 40000 and the [¹⁴C]lithocholic acid-binding activity was expressed as the ratio between the peak radioactivity (d.p.s./ml) and the protein concentration (mg/ml) in the fraction.

All buffers were prepared at 20°C. Radioactive counting and protein assays were performed as described by Strange *et al.* (1976). Agarose-gel electrophoresis was performed in sodium barbiturate/barbituric acid buffer (50mm; pH8.6) on Universal agarose plates obtained from Corning-Eel (Evans Electroselenium Ltd., Halstead, Essex, U.K.). Polyacrylamide-gel electrophoresis was performed at room temperature (20°C) on discs of 10% gel prepared from acrylamide and *NN'*-methylenebisacrylamide (30:1, w/w) with *NNN'*-tetramethylethylenediamine as catalyst. Na⁺ concentrations in column fractions were measured by flame photometry with an IL 343 photometer [Instrumentation Laboratory (U.K.) Ltd., Altrincham, Cheshire, U.K.].

Purification of lithocholic acid-binding components

Solution 1. Male Wistar rats (300–350g) were anaesthetized with diethyl ether and their livers were perfused *in situ* with the phosphate/NaCl buffer to which sucrose was added to a final concentration of 250mm. The livers were homogenized and 100000g supernatants were prepared as described by Strange *et al.* (1976). Portions (40ml; approx. 40mg of protein/ml) of supernatant were transferred to a column (5.5cm×24cm) of Sephadex G-75 which was equilibrated and eluted with the phosphate/NaCl buffer at 4°C. The flow rate was 60ml/h, the fraction volume was 10ml and the void volume was 160ml. Fractions eluted between 250 and 350ml contained proteins of mol.wt. 30000–60000. They were collected and combined (solution 1).

Solution 2. Solid (NH₄)₂SO₄ was added at room temperature with stirring to solution 1 until 55% saturation was obtained (Dixon & Webb, 1964). The solution was left without stirring for 30min then centrifuged (2500g, 15min, 20°C) and the precipitate discarded. More (NH₄)₂SO₄ was added to the supernatant until 85% saturation was reached, and the solution was left for 30min then centrifuged (50000g, 25min, 10°C) and the supernatant discarded. The precipitate was redissolved in sodium/potassium phosphate buffer (20mm; pH7.4; 10ml) and the solution was dialysed at 4°C for 15h against four changes (each of 2 litres) of the same buffer, giving solution 2.

Solution 3. Calcium phosphate gel was washed with sodium/potassium phosphate buffer (20mm; pH7.4) and added to solution 2 (1mg of gel/mg of protein) at room temperature. The solution was stirred for 1h, centrifuged (2500g, 15min, 20°C) and the precipitate discarded. The supernatant was dialysed against four changes (each of 2 litres) of Tris/HCl buffer (20mm; pH8.1) at 4°C for 15h, giving solution 3.

Solution 4. Solution 3 was added to a column (0.9cm×15cm) of DEAE-Sephadex A-50 equilibrated and eluted at 4°C with Tris/HCl buffer (20mm; pH8.1). The flow rate was 6.5ml/h and the fraction volume was 1.5ml. A NaCl gradient was established after 30ml. To obtain the gradient a solution of NaCl (500mm) in Tris/HCl buffer (20mm; pH8.1) was used. This solution was added (6.5ml/h), with mixing, to 40ml of Tris/HCl buffer (20mm; pH8.1) contained in the eluent reservoir. Eluent was pumped (6.5ml/h) from this reservoir to the DEAE-Sephadex column. Fractions eluted between 3.0 and 18.0ml were combined and dialysed against four changes (each of 2 litres) of sodium/potassium phosphate buffer (10mm; pH7.4) at 4°C for 20h giving solution 4. Fractions eluted between 37.5 and 45.0ml were also combined and dialysed against sodium/potassium phosphate buffer (10mm; pH7.4).

Solution 4 was mixed with 50μl of [¹⁴C]lithocholic acid (16nmol; 1μCi) and was added to a column (0.9cm×15cm) of CM-Sephadex C-50, equilibrated and eluted with sodium/potassium phosphate buffer (10mm; pH7.4). After 27ml had been collected a NaCl gradient was established. NaCl was added to phosphate buffer (10mm; pH7.4) to give a final concentration of 50mm. This solution was added (6.5ml/h), with mixing, to 50ml of phosphate buffer (10mm; pH7.4) in the eluent reservoir. Eluent was pumped (6.5ml/h) from the reservoir to the CM-Sephadex column. The fraction volume was 1.5ml. The fractions eluted between 9.0 and 30.0ml, 31.5 and 39ml (peak 1) and 45.0 and 58.5ml (peak 2) were separately combined and the three solutions were examined for the presence of lithocholic acid-binding components.

Peak 1 and peak 2 were concentrated by dialysis at 1°C against poly(ethylene glycol) (mol.wt. 25000) and were run in agarose-gel and polyacrylamide-gel electrophoresis systems.

Bile acid inhibition of glutathione S-transferase activity

The effects of cholic acid, glycocholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid on glutathione *S*-transferase activity in peak 1 and peak 2 from the CM-Sephadex column were studied by using reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates. Portions (5μl; 1.5μg of protein) of peak 1 and peak 2 were added to

reaction mixtures containing sodium/potassium phosphate buffer (1.3 ml; 100 mM; pH 7.45), the bile acid (0–300 μ M) under study and 1-chloro-2,4-dinitrobenzene (1 mM). Since lithocholic acid is only slightly soluble in aqueous solution concentrations up to 40 μ M were used. The reaction was initiated by adding either 0.5 or 1.0 mM-glutathione. Since binding of lithocholic acid to supernatant proteins had been studied only at pH 7.4 the effects of bile acids on glutathione *S*-transferase activity were studied at this pH. Initial experiments confirmed that the reaction rate was not altered by changing the pH of the reaction mixture from 6.5 to 7.45. The non-enzymic rate, however, was increased to 20% of the total reaction rate in the absence of lithocholic acid. It was subtracted from the total measured reaction rate in all cases. Assays were performed in quadruplicate.

Results

Purification of lithocholic acid-binding components

The protein contents, [14 C]lithocholic acid-binding activities and specific enzyme activities for 100 000g supernatant from rat liver and for each of the partially purified preparations from it are shown in Table 1.

At each stage of the purification procedure the discarded solutions were also tested for [14 C]lithocholic acid-binding activity and glutathione *S*-transferase activity. Except for low [14 C]lithocholic acid-binding activity (less than 350 d.p.s./mg of protein) and glutathione *S*-transferase activity (less than 10 A_{340} units change/min per mg of protein) found in both the redissolved precipitate formed by adding $(\text{NH}_4)_2\text{SO}_4$ to 55% saturation to solution 1 and in a sodium/potassium phosphate buffer (500 mM; pH 7.4) wash of the discarded calcium phosphate gel, none was found.

When solution 3 was added to a DEAE-Sephadex A-50 column, a broad peak of protein was eluted at 3.0–18.0 ml which was partly resolved into two peaks of glutathione *S*-transferase activity (Fig. 1). These fractions were combined and shown to contain lithocholic acid-binding activity. A second group of protein-containing fractions was eluted with the NaCl gradient at 36.0–60.0 ml. Some of these showed glutathione *S*-transferase activity, but combined fractions 37.5–45.0 ml did not contain [14 C]-lithocholic acid-binding activity.

Solution 4 was applied to a CM-Sephadex C-50 column (Fig. 2). Some protein was eluted between 9.0 and 30.0 ml before the start of the NaCl gradient. These fractions contained little glutathione *S*-transferase activity or [14 C]lithocholic acid-binding activity. Two peaks of protein (peak 1 and peak 2) were eluted with the NaCl gradient; both contained [14 C]lithocholic acid-binding activity and glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene and ethacrynic acid as substrates, but were unable to catalyse the conjugation of glutathione with 1,2-dichloro-4-nitrobenzene. A similar elution pattern was obtained when solution 4 was added without lithocholic acid to the CM-Sephadex column.

On agarose-gel and polyacrylamide-gel electrophoresis, samples of both peak 1 and peak 2 showed single bands of protein.

Bile acid inhibition of glutathione S-transferase activity

Cholic acid, glycocholic acid, chenodeoxycholic acid and deoxycholic acid had no effect on glutathione *S*-transferase activity in either peak 1 or peak 2 from the CM-Sephadex column. Lithocholic acid had no effect on enzyme activity in peak 2, but did inhibit activity in peak 1. Straight lines were obtained by plotting the reciprocal of the maximum velocity

Table 1. *Purification of the lithocholic acid-binding proteins*

Supernatant fractions (100 000g) were prepared from liver homogenates and two lithocholic acid-binding proteins were isolated by using $(\text{NH}_4)_2\text{SO}_4$ and $\text{Ca}_3(\text{PO}_4)_2$ -gel fractionation and ion-exchange chromatography (see the Materials and Methods section). At each stage of the purification procedure the preparations were examined for lithocholic acid-binding activity and glutathione *S*-transferase activity.

	Protein (mg)	Lithocholic acid binding (d.p.s./mg)	Conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (change in A_{340} /min per mg of protein)
100 000g supernatant	1600	1200	27
Sephadex G-75	500	2000	50
$(\text{NH}_4)_2\text{SO}_4$ fractionation	120	4000	70
Calcium phosphate gel	50	6000	100
DEAE-Sephadex	10	10 000	200
CM-Sephadex			
Peak 1	5.5	13 000	250
Peak 2	3.5	8000	230

Fig. 1. Elution pattern of solution 3 of the partially purified lithocholic acid-binding proteins from DEAE-Sephadex. Solution 3 of the partially purified lithocholic acid-binding proteins, isolated from rat liver 100000g supernatants by gel-exclusion chromatography, $(\text{NH}_4)_2\text{SO}_4$ and $\text{Ca}_3(\text{PO}_4)_2$ gel fractionation (see the Materials and Methods section) was eluted from a DEAE-Sephadex A-50 column with Tris/HCl buffer (20mM; pH8.1). Fractions (1.5ml) were collected and the A_{280} (●), Na^+ concentration (■) and glutathione *S*-transferase activity with ethacrynic acid (Δ) and 1-chloro-2,4-dinitrobenzene (○) as substrates were measured.

against the concentration of lithocholic acid. At glutathione concentrations of 0.5 and 1.0mM the intercepts ($\pm$ S.E., $n = 5$) were $18.5 \pm 3.5 \mu\text{M}$ and $20.4 \pm 3.8 \mu\text{M}$ respectively. The confidence interval for the difference between the two estimates of the intercept is -1.03 to $+1.41$. Since this interval includes 0, the intercepts are not significantly different, suggesting that the inhibition is non-competitive.

Discussion

The experiments described show the presence of at least two proteins in hepatic 100000g supernatants able to bind lithocholic acid. Although exhaustive criteria of protein purity were not applied, agarose- and polyacrylamide-gel electrophoresis of the two protein-containing solutions obtained from the CM-Sephadex column demonstrated the presence of only a single protein band in each solution. Both these lithocholic acid-binding components had glutathione *S*-transferase activity. The system used to detect lithocholic acid binding is a non-equilibrium one, and was used in preference to an equilibrium system (such as equilibrium dialysis), since it was known to be less likely to detect non-specific binding of lithocholic acid (Strange *et al.*, 1977).

Ligandin is one of a group of several enzymes that have glutathione *S*-transferase activity (Habig *et al.*, 1974b). These workers have determined the relative

Fig. 2. Elution pattern of solution 4 of the partially purified lithocholic acid-binding proteins from CM-Sephadex. Fractions eluted between 3.0 and 18.0ml from the DEAE-Sephadex column were collected, combined and after dialysis against sodium/potassium phosphate buffer (10mM; pH7.4) were mixed with [^{14}C]lithocholic acid (16nmol; $1 \mu\text{Ci}$) and eluted from a column of CM-Sephadex C-50. Fractions (1.5ml) were collected and the A_{280} (●), [^{14}C]lithocholic acid (□), Na^+ concentration (■) and glutathione *S*-transferase activity with ethacrynic acid (Δ) and 1-chloro-2,4-dinitrobenzene (○) as substrates were measured.

specific activities of purified glutathione *S*-transferases A, B, C and E for various substrates, but, since other glutathione *S*-transferases are known to exist although not yet purified, identification of the protein solutions from the CM-Sephadex column is difficult. Habig *et al.* (1974b) showed that the ratio of glutathione-1-chloro-2,4-dinitrobenzene-conjugating activity to glutathione-ethacrynic acid-conjugating activity of ligandin was 45:1. Peak 1 and peak 2 from the CM-Sephadex column have ratios of 1000:1 and 250:1 respectively. Since peak 2 exhibited the highest activity for conjugating ethacrynic acid and glutathione, but had virtually no activity for conjugating glutathione and 1,2-dichloro-4-nitrobenzene, it is suggested that it is ligandin. The difference between the ratios obtained in these experiments and those of Habig *et al.* (1974a,b) may be a result of the use of lithocholic acid binding as a primary marker during the purification procedure; no attempt was made to ensure retention of enzyme activity. Glutathione *S*-transferase activity of the two lithocholic acid-binding proteins was not affected by cholic acid, glycocholic acid, chenodeoxycholic acid or deoxycholic acid in concentrations up to 300 μ M. Okishio & Nair (1966) have shown that the concentration of cholic acid, the predominant bile acid in rat liver 100000g supernatants, is approx. 100 μ M. Although lithocholic acid in concentrations up to 60 μ M had no effect on enzyme activity in the protein provisionally identified as ligandin (peak 2), inhibition of glutathione *S*-transferase activity of peak 1 did occur. Since the concentration of lithocholic acid in the liver is unknown the relevance of this finding is not clear.

Both of the lithocholic acid-binding proteins described here have glutathione *S*-transferase activity and one of these may be the non-specific anion-binding protein ligandin. Although purified ligandin can bind cholic acid and its taurine and glycine conjugates (Tipping *et al.*, 1976) it is not known whether hepatic transport of bile acids is mediated by a mechanism similar to that of the transport of bromosulphthalein and bilirubin. O'Maille *et al.* (1966) showed two effects when they studied the excretion of sodium taurocholate and bromosulphthalein into bile. Although infusions of sodium taurocholate to anaesthetized dogs increased the maximum rate of

bromosulphthalein excretion into bile the maximum excretion rate of sodium taurocholate was decreased by bromosulphthalein infusion. They concluded that the increased excretion of bromosulphthalein was secondary to the increased bile flow stimulated by infusion of sodium taurocholate, and further that the inhibition of sodium taurocholate excretion suggested a common pathway for the hepatic transport of bile acids and bromosulphthalein. Since lithocholic acid binding to proteins in 100000g supernatants is inhibited by other bile acids (Strange *et al.*, 1977), ligandin (or other glutathione *S*-transferase enzymes) may act as a non-specific bile acid-binding protein involved in the transport of bile acids as well as other anions.

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