Inhibition of hepatic and extrahepatic glutathione *S*-transferases by primary and secondary bile acids

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Glutathione *S*-transferases are a complex family of dimeric proteins that play a dual role in cellular detoxification; they catalyse the first step in the synthesis of mercapturic acids, and they bind potentially harmful non-substrate ligands. Bile acids are quantitatively the major group of ligands encountered by the glutathione *S*-transferases. The enzymes from rat liver comprise Yk (M_r 25 000), Ya (M_r 25 500), Yn (M_r 26 500), Yb₁, Yb₂ (both M_r 27 000) and Yc (M_r 28 500) monomers. Although bile acids inhibited the catalytic activity of all transferases studied, the concentration of a particular bile acid required to produce 50% inhibition (I_{50}) varies considerably. A comparison of the I_{50} values obtained with lithocholate (mono-hydroxylated), chenodeoxycholate (dihydroxylated) and cholate (trihydroxylated) showed that, in contrast with all other transferase monomers, the Ya subunit possesses a relatively hydrophobic bile-acid-binding site. The I_{50} values obtained with lithocholate and lithocholate 3-sulphate showed that only the Ya subunit is inhibited more effectively by lithocholate than by its sulphate ester. Other subunits (Yk, Yn, Yb₁ and Yb₂) were inhibited more by lithocholate 3-sulphate than by lithocholate, indicating the existence of a significant ionic interaction, in the bile-acid-binding domain, between (an) amino acid residue(s) and the steroid ring A. By contrast, increasing the assay pH from 6.0 to 7.5 decreased the inhibitory effect of all bile acids studied, suggesting that there is little significant ionic interaction between transferase subunits and the carboxy group of bile acids. Under alkaline conditions, low concentrations (sub-micellar) of non-sulphated bile acids activated Yb₁, Yb₂ and Yc subunits but not Yk, Ya and Yn subunits. The diverse effects of the various bile acids studied on transferase activity enables these ligands to be used to help establish the quaternary structure of individual enzymes. Since these inhibitors can discriminate between transferases that appear to be immunochemically identical (e.g. transferases F and L), bile acids can provide information about the subunit composition of forms that cannot otherwise be distinguished.

INTRODUCTION

A major function of the liver is removal of organic anions, such as bilirubin and bile salts, from the blood and their excretion into bile. Several groups of organic anion-binding proteins have been described in rat liver cytosol. These have been termed Y (M_r 49 000–59 000), Y' (M_r 30 000–40 000) and Z (M_r 12 000–16 000) (Levi *et al.*, 1969; Sugiyama *et al.*, 1983). Each of these fractions is heterogeneous and contains multiple binding species. The Y proteins, collectively referred to as 'ligandins', represent quantitatively the most important group of organic-anion-binding proteins (Jakoby & Habig, 1980; Wolkoff, 1980). The 'ligandins' also possess glutathione *S*-transferase activity, and an extensive literature exists describing these enzymes (Jakoby & Habig, 1980; Mannervik, 1985).

The glutathione *S*-transferases are members of a complex enzyme family that, in addition to binding a broad spectrum of non-substrate ligands (Ketley *et al.*, 1975), catalyse the conjugation of GSH with a wide range of potentially harmful alkylating agents (Chasseaud, 1979). This dual role of catalyst and binding protein has been discussed before (Jakoby, 1978).

The glutathione *S*-transferases are a large 'multi-gene' family of dimeric proteins. The elucidation of the quaternary structure of individual forms, as well as the structural, functional and immunochemical relationships between various subunits, currently represent active areas of research. To date, the hepatic transferases have been shown to comprise at least five types of monomer, designated Ya (M_r 25 500), Yn (M_r 26 500), Yb₁ (M_r 27 000), Yb₂ (M_r 27 000) and Yc (M_r 28 500). These enzymes can be divided into two groups according to their subunit compositions. Group I is made up of enzymes (transferases F, L, B and AA) that are composed of Ya and/or Yc monomers (Hayes *et al.*, 1979, 1980; Scully & Mantle, 1980; Sheehan & Mantle, 1984); group II comprises enzymes (transferases A, C, D, P and S) that contain binary combinations of Yb₁, Yb₂ and Yn monomers (Hayes, 1984).

The subunits appear to function independently (Jakobson *et al.*, 1977) and can be identified on the basis of their specific activity for a number of commonly used substrates (Mannervik & Jensson, 1982; Hayes, 1984). Yalçin *et al.* (1983) extended the catalytic characterization of subunits to include the inhibitory effect of various non-physiological ligands on the 1-chloro-2,4-dinitro-

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benzene-GSH-conjugating activity of a limited number of transferases. These workers used I_{50} values to identify certain rat subunits. Mannervik and his colleagues have also used I_{50} values to discriminate between the various classes (i.e. basic, neutral and acid) of human glutathione *S*-transferase (Tahir *et al.*, 1985).

Data describing the binding of ligands by the transferases are far from complete. Little is known about the features of non-substrate ligands that make them effective inhibitors, largely owing to the absence of any systematic study that compares the binding of structurally related ligands to a representative spectrum of transferases. Although the older literature contains detailed information about the binding activity of 'ligandin' (i.e. Ya-containing forms), much of this work has not withstood critical re-appraisal. More recent work has shown that the 'ligandin' preparations used in the early investigations contained several enzymes, and that different 'ligandin' preparations contained different mixtures of transferases (Carne *et al.*, 1979).

Bile acids are the major physiological group of ligands encountered by the glutathione *S*-transferases. The toxic effects of lithocholate have been described by many workers (Holsti, 1960; Palmer, 1966; Javitt & Emerman, 1968; Fischer *et al.*, 1974; Kakis & Yousef, 1978); lithocholate toxicity is most pronounced in those species that cannot sulphate the hydroxy group at the 3-position. A systematic study of the interaction of lithocholate or related bile acids with glutathione *S*-transferases has not been reported.

In the present study, the effects of lithocholate on the activity of 16 distinct transferases are described and related to individual subunits. The inhibitory effects of lithocholate 3-sulphate are compared with those of lithocholate to establish the effect of sulphation on inhibition. Inhibition by chenodeoxycholate and cholate have also been compared with inhibition by lithocholate, to establish the effect of decreasing the ligand's hydrophobic nature. Further, since organic anions were the first group of non-substrate ligands identified for the transferases, inhibition was studied at various pH values (6.0–7.5) to determine whether the ionization of the bile acid carboxy group resulted in an increase in inhibition. Below their critical micellar concentration (approx. 2 mM), the pK_a values of bile acids are about 5.0; above this value their pK_a is about 6.0. This aspect was also investigated to facilitate comparisons with earlier, more limited, studies (Vessey & Zakim, 1981; Boyer *et al.*, 1984).

The present study shows that bile acids can be used to distinguish between transferase subunits that cannot be discriminated immunochemically. We also describe the effects of bile acids on a novel hepatic transferase, transferase K, that is immunochemically distinct from other described forms (Hayes, 1986; Hayes & Mantle, 1986). Under acidic conditions, this transferase exhibits the lowest I_{50} value for cholate of any transferase studied.

MATERIALS AND METHODS

Animals

Tissues from male Wistar rats (300–350 g) were used throughout the study. For preparative purposes liver, kidney and testis were stored at -70°C before use.

Buffers

The buffers used in protein purification were prepared at 4°C . The pH values of the buffers used in the enzyme assays were determined at room temperature.

Enzyme assays

These were all performed in sodium phosphate buffer at 37°C by using manual methods (Habig & Jakoby, 1981) or a centrifugal analyser (Hayes & Clarkson, 1982).

Typically, the amount of enzyme added to the assay system (10 μl , 0.2–15 μg) was adjusted to provide an absorbance change, in the absence of any inhibitor, of $0.8 \Delta A_{340}/\text{min}$; less than 5% of this value represented the blank, non-enzyme-catalysed, rate. At a level of activity of $0.8 \Delta A_{340}/\text{min}$ repeat analyses possessed an S.D. of $0.034 \Delta A_{340}/\text{min}$. Similar replica measurements at $0.4 \Delta A_{340}/\text{min}$ (i.e. the approximate I_{50} value) revealed an S.D. of $0.024 \Delta A_{340}/\text{min}$.

Isolation of glutathione *S*-transferases

All the purification steps were performed at 4°C . Because of the large number of glutathione *S*-transferases that exist, several distinct purification strategies had to be used for their isolation. These are described briefly below.

(a) Hepatic enzymes. The various methods used to purify the transferases in rat liver all employed an initial DEAE-cellulose step. The basic enzymes are eluted in the 'flow-through' fractions, and so use of this exchanger separated the basic enzymes from the various peaks of anionic transferase activity, which were then resolved by elution with a salt gradient into N1, N2 and N3 fractions (Hayes, 1983).

The basic forms, originally isolated by Habig *et al.* (1974, 1976), are transferases C (Yb_1Yb_2), B (YaYc), A (Yb_1Yb_1) and AA (YcYc). These were purified in the present study by following the initial DEAE-cellulose step with $(\text{NH}_4)_2\text{SO}_4$ precipitation, CM-cellulose and then hydroxyapatite chromatography, essentially as described by these workers.

The additional YaYa-containing basic form(s), not recovered by Habig *et al.* (1974, 1976), are eluted from CM-cellulose before transferase C on the salt gradient (Hayes & Clarkson, 1982). Sheehan & Mantle (1984) reported that two basic Ya dimers, transferases F and L, were resolved by using two sequential CM-cellulose steps. In the present study transferases F and L were resolved along with C, B, A and AA in a single step, on a $81 \text{ cm} \times 3.2 \text{ cm}$ CM-cellulose column equilibrated with 10 mM-sodium phosphate buffer, pH 6.7. This column was developed with a 0–110 mM-NaCl gradient, formed by using a 325 ml constant-volume mixing reservoir. Under these conditions transferases F and L are eluted, respectively, in the zone II α and zone II γ pools described previously (Hayes, 1983). The F and L enzymes were further purified by using hydroxyapatite columns ($19.0 \text{ cm} \times 1.6 \text{ cm}$) developed with a 10–250 mM-sodium phosphate gradient at pH 6.7, formed by using a 200 ml constant-volume mixing reservoir.

Transferases E and M are characterized by their activities towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane and menaphthyl sulphate respectively. These isoenzymes are retained by DEAE-cellulose; the major portions of E and M are eluted in the N1 and N2 pools respectively (Hayes, 1986). Since it was found that neither transferase

E nor transferase M bound to the *S*-hexylglutathione and glutathione affinity matrices, these enzymes were resolved from the contaminating transferases by affinity chromatography.

Transferases K, D, P, R and S were purified from liver as described elsewhere (Hayes & Chalmers, 1983; Hayes, 1984, 1986).

(b) Kidney enzyme. The renal enzyme studied, transferase J, was purified by using *S*-hexylglutathione affinity chromatography followed by chromatofocusing (pH 8.0–6.0). This enzyme is distinct because it contains monomers that, during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, migrate faster than the Ya subunit(s).

(c) Testicular enzymes. The anionic enzymes from rat testis were isolated by using *S*-hexylglutathione affinity chromatography followed by chromatofocusing (pH 8.0–5.0). The major anionic form, transferase N (YnYn), was eluted from the PBE 94 chromatofocusing gel at pH 6.6; this was then further purified by hydroxyapatite chromatography (Hayes, 1984). Transferase Y, which was purified from testis by using the method employed to isolate transferase N, was eluted from the chromatofocusing column at pH 6.3. Transferase Y was not subjected to hydroxyapatite chromatography.

Bile acid inhibition of transferase activity

The effects of bile acids on the 1-chloro-2,4-dinitrobenzene–GSH-conjugating activity expressed by purified

transferase preparations were measured at pH 6.0, 6.5, 7.0 or 7.5 with a Rotochem IIa parallel fast analyser (American Instrument Co., Silver Spring, MD, U.S.A.). Portions of each enzyme (10 μ l; 0.2–15.0 μ g) were preincubated with GSH (50 μ l; 20 mM), for about 15 min, in the absence of the inhibitor and the second substrate, before the reactions were initiated. The reactions were started by centrifugation, which resulted in the simultaneous addition of bile acid and 1-chloro-2,4-dinitrobenzene to the GSH/protein mixture. The final reaction mixtures (0.5 ml) each contained 1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH.

The interaction between an enzyme and a particular bile acid was measured with eight different concentrations of the inhibitor. Individual experimental points were measured in quadruplicate. Each I_{50} value was determined, therefore, from at least 32 experimental results; this estimate was achieved graphically. Comparisons between enzymes were facilitated by the preparation of stock bile acid solutions at 12 concentrations. The eight stock solutions used to study a particular enzyme were chosen, when solubility of the ligand permitted, to encompass the I_{50} value as closely as possible.

During the study of chenodeoxycholate inhibition, 4% (v/v) methanol was included in each assay mixture (for every concentration of chenodeoxycholate) because of the limited solubility of this bile acid. The very limited solubility of the monohydroxylated bile acid, lithocholate, only allowed its inhibitory effects to be studied at pH 7.5, and in the presence of 10% (v/v) methanol.

Inhibition by bile acids of the 1,2-epoxy-3-(*p*-nitrophenoxy)propane–GSH-conjugating activity of

Table 1. Inhibition of glutathione *S*-transferase activity by primary bile acids

The inhibition of the 1-chloro-2,4-dinitrobenzene–GSH-conjugating activity of the transferases by the primary bile acids was measured at pH 6.0, 6.5 and 7.5. In each instance the non-enzymic rates were subtracted from the observed rate before the determination of the I_{50} . The ratio of the I_{50} value calculated at pH 7.5 to that calculated at pH 6.0 is shown in parentheses. The results from the extrahepatic enzymes (N, J and Y) are grouped separately at the bottom of the Table. Note that the I_{50} values are expressed as mM. Abbreviation: n.d. not determined.

Transferase	Subunit type*	Group	I_{50} value (mM)							
			With cholate				With chenodeoxycholate			
			pH 6.0	pH 6.5	pH 7.5	Ratio	pH 6.0	pH 6.5	pH 7.5	Ratio
F	YaYa	I	0.88	1.03	2.34	(2.7)	0.016	0.030	0.072	(4.5)
L	YaYa	I	0.77	0.95	1.49	(1.9)	0.017	0.036	0.057	(3.4)
B	YaYc	I	~ 1.7	~ 2.3	~ 13.50	—	0.101	0.168	0.395	(3.9)
AA	YcYc	I	> 1.7	> 2.3	> 13.50	—	> 0.15§	> 0.250	> 1.0†	—
A	Yb ₁ Yb ₁	II	0.175	0.234	0.950	(5.4)	0.102	0.097	0.520	(5.1)
C	Yb ₁ Yb ₂	II	0.200	0.290	n.d.	—	0.078	0.080	0.435	(5.6)
D	Yb ₂ Yb ₂	II	0.185	0.390	2.700	(14.6)	0.037	0.065	0.615	(16.6)
P	Yb ₁ Yn	II	0.390	0.705	3.900	(10.0)	0.087	0.115	0.630	(7.2)
S	Yb ₂ Yn	II	0.324	0.800	4.900	(15.1)	0.056	0.077	0.490	(8.8)
R	YbYb	II	n.d.	0.288†	n.d.	—	n.d.	0.064†	n.d.	—
K	YkYk	I	0.122	0.198	1.440	(11.8)	0.042	0.060	0.315	(7.5)
N	YnYn	II	0.460	1.080	4.900	(10.7)	0.135	0.169	0.490	(3.6)
J	YkYk	I?	0.135	0.230	1.500	(11.1)	0.054	0.054	0.255	(4.7)
Y	YbYb	II	0.720	1.080	4.600	(6.4)	n.d.	0.160	n.d.	—

* The subunit type is defined by SDS/polyacrylamide-gel electrophoresis. Where known, the identity of individual polypeptides within a given subunit family is denoted by a subscript number following the subunit type.

† Values from Hayes & Chalmers (1983).

‡ See Fig. 1(a).

§ See Fig. 1(b).

Fig. 1. Activation of glutathione *S*-transferases by chenodeoxycholate

The activities of transferases AA ($\square$, YcYc), D ($\bullet$, Yb₂Yb₂), A ($\circ$, Yb₁Yb₁) and N ($\blacktriangle$, YnYn) were measured in the presence of various concentrations of chenodeoxycholate. Panels (a) and (b) show the results of assays performed at pH 7.5 and 6.0 respectively; the chenodeoxycholate concentrations differ in each instance. The effect of chenodeoxycholate on the activity of transferase K ($\blacksquare$) is shown in panel (b) only.

transferase E, and of the 1-menaphthyl sulphate-GSH-conjugating activity of transferase M, was investigated at pH 7.5.

RESULTS

Bile acid inhibition of glutathione *S*-transferase activity

The effect of bile acids on the 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity of hepatic and extrahepatic transferases is shown in Tables 1 and 2. Inhibition was studied under standard assay conditions (i.e. 1 mM-1-chloro-2,4-dinitrobenzene and 2 mM-GSH) at various pH values, as described in the Materials and methods section. The activities of both the hepatic and the extrahepatic enzymes were inhibited by all the bile acids investigated. Although the transferase most sensitive to inhibition varied according to the bile acid used, and with pH, transferase AA (YcYc) was found on all occasions to be significantly more resistant to bile acid inhibition than was any of the other enzymes studied.

(a) Effects of primary bile acids. The interaction between cholate and various transferases is described in Table 1. This shows that changes of pH profoundly altered the inhibition of transferase activity, an effect that varied considerably according to the enzyme under investigation. These differences can be expressed by the ratio between the I_{50} values at pH 7.5 and pH 6.0 (Table 1); this ratio varied from 1.9 (transferase L, YaYa) to 15.1 (transferase S, Yb₂Yn).

At pH 6.0 and 6.5, cholate exerted its greatest inhibitory effect on the activity of the newly isolated enzymes, transferases J and K. However, at pH 7.5, transferase A (Yb₁Yb₁) possessed the lowest I_{50} value.

The interaction of transferases F and L, both Ya dimers, with cholate is interesting for two reasons.

Firstly, significant differences between the inhibition of these two forms by cholate are apparent at pH 7.5. Secondly, whereas transferases F and L are more resistant to cholate inhibition at pH 6.0 and 6.5 than any of the group II enzymes, this is not the case at pH 7.5.

The effects of chenodeoxycholate on the transferases were different from those observed for cholate. In general, the inhibitory effects of chenodeoxycholate were greater than those of cholate. However, major differences in the magnitude of this accentuated inhibition are apparent. A 30-fold difference exists in the I_{50} values of transferases F and L for these two bile acids, whereas transferase A only shows a 2-fold difference between the I_{50} values for these two inhibitors.

At pH 7.5 the 1-chloro-2,4-dinitrobenzene-GSH-conjugating activities of the Yb₁, Yb₂ and Yc monomers were significantly increased by chenodeoxycholate. Fig. 1 shows that, not only did the degree of this activation vary from enzyme to enzyme, but the concentration of chenodeoxycholate required to convert activation into inhibition differed markedly. With the exception of transferase AA (YcYc), this activation was not apparent at pH 6.0.

Transferases F and L were inhibited more by chenodeoxycholate at all pH values than was any of the other enzymes. As with cholate, transferase L possessed significantly lower I_{50} values than transferase F at pH 7.5.

(b) Effects of lithocholate. The interaction between lithocholate and various transferases is shown in Table 2. This monohydroxylated bile acid is highly insoluble and its inhibitory effects could only be studied at pH 7.5. Differences in the extent of inhibition were more varied with lithocholate than with any of the other bile acids investigated. Differences of at least two orders of magnitude exist in the I_{50} values of the various enzymes (Fig. 2).

Table 2. Inhibition of glutathione *S*-transferase activity by lithocholate and lithocholate 3-sulphate

The inhibition of transferase activity by lithocholate and its sulphate ester was measured as described in the text. The structure of this Table is the same as that of Table 1. Note that the I_{50} values shown below are expressed as μM .

Transferase	Subunit type*	Group	I ₅₀ value (μM)					Ratio
			With lithocholate	With lithocholate 3-sulphate				
			pH 7.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	
F	YaYa	I	1.0	0.50	0.63	n.d.	2.0	(4.0)
L	YaYa	I	1.1	0.70	0.68	n.d.	2.6	(3.7)
B	YaYc	I	7.0	25	11.7	n.d.	54	(2.2)
AA	YcYc	I	> 400‡	~ 50	> 135			
						n.d.	800	—
A	Yb ₁ Yb ₁	II	165	13	22	38	56	(4.2)
C	Yb ₁ Yb ₂	II	175	9.5	12	n.d.	45	(4.7)
D	Yb ₂ Yb ₂	II	~ 270‡	2.5	4.3	8.1	15	(6.0)
P	Yb ₁ Yn	II	~ 210‡	9.0	33	49	225	(25.0)
S	Yb ₂ Yn	II	~ 280‡	1.7	5.1	8.5	55	(32.3)
R	YbYb	II	n.d.	n.d.	11.7†	n.d.	n.d.	—
K	YkYk	I	118	3.5	7.1	10.8	32	(9.1)
N	YnYn	II	158	6.0	140	13.3	60	(10.0)
J	YkYk	I?	118	3.9	7.4	11.3	29	(7.4)
Y	YbYb	II	n.d.	5.2	18.4	18.9	67	(12.9)

* The subunit type is defined by SDS/polyacrylamide-gel electrophoresis. Where known, the identity of individual polypeptides within a given subunit family is denoted by a subscript number following the subunit type.

† Value from Hayes & Chalmers (1983).

‡ See Fig. 2.

Fig. 2. Interaction of lithocholate with glutathione *S*-transferases

The effect of lithocholate on transferase activity was studied at pH 7.5 as described in the text. The Ya-containing enzymes were more susceptible to inhibition by lithocholate than were other enzymes and the activity curves obtained from transferases L (▲) and B (□) are shown in panel (a). Lithocholate effects on the activity of transferases AA (□), D (●), S (■), P (△), A (○) and K (▲) are shown in panel (b). Significantly lower lithocholate concentrations were employed to study inhibition of the Ya-containing enzymes than were required to study the remaining forms, and hence the scales on the abscissae in the two panels differ.

Table 3. Calculation of the bile acid inhibitory ratios for transferase F and L and comparison with various homodimers

The ratio of I_{50} values for different inhibitors was calculated from assays performed at pH 7.5 to demonstrate the specificity of the Ya subunit(s) for hydrophobic bile acids. The values in parentheses were calculated from assays performed at pH 6.0 and are included because the Yn subunit exhibits a unique pH-dependence in its inhibition. No ratios could be calculated for the Yc subunit, since it is highly resistant to bile acid inhibition.

Transferase	Inhibitory ratio			
	Cholate/ chenodeoxycholate	Chenodeoxycholate/ lithocholate	Lithocholate/ lithocholate 3-sulphate	Chenodeoxycholate/ lithocholate 3-sulphate
F	32.5 (55.0)	72.0	0.5	36.0 (32.0)
L	26.1 (45.3)	51.8	0.4	21.9 (24.3)
K	4.6 (2.9)	2.7	3.7	9.8 (12.0)
A	1.8 (1.7)	3.2	2.9	9.3 (7.8)
D	4.4 (5.0)	~ 2.3	18.0	41.0 (14.8)
N	10.0 (3.4)	3.1	2.6	8.2 (22.5)

Fig. 3. Lithocholate 3-sulphate inhibition of glutathione S-transferase activity

At pH 7.5 the inhibition curves produced by lithocholate 3-sulphate were constructed by using stock solutions containing 0, 1.1, 2.2, 4.4, 9.0, 18, 27, 36, 45, 90, 180, 270, 360, 450 and 900 μM inhibitor. Each of the data points shown for transferases F ($\blacktriangle$), D ($\bullet$), A ($\circ$), N ($\triangle$), B ($\blacksquare$) and AA ($\square$) represents the mean of five determinations.

Lithocholate is a stronger inhibitor of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity than is chenodeoxycholate, but this increase in inhibition varies considerably. It is noteworthy that the forms that showed the largest decrease in I_{50} values when cholate (tri-hydroxylated) and chenodeoxycholate (dihydroxylated) inhibition were compared (i.e. transferases F, L and B) also showed the largest decreases in I_{50} value when chenodeoxycholate inhibition was compared with lithocholate inhibition (Table 3). This suggests that the bile-acid-binding site in the Ya monomer is predominantly hydrophobic. Although the order of sensitivity to chenodeoxycholate inhibition observed at pH 7.5 for the Yb₁, Yb₂ and Yn homodimers (i.e. transferase N followed by transferase A followed by transferase D) is retained for lithocholate, the marked difference in the hydrophobic nature of cholate, cheno-

deoxycholate and lithocholate is not reflected in the inhibition patterns of the group II enzymes for these bile acids. For example, the ratios of the I_{50} value for cholate to the I_{50} value for lithocholate were as follows: transferase F, 2340; transferase L, 1352; transferase A, 5.8; transferase D, 10.1; transferase N, 31 (Tables 1–3).

Fig. 2 shows that certain group II enzymes are activated by concentrations of lithocholate below 50 μM .

(c) Effects of lithocholate 3-sulphate. This bile acid is considerably more soluble than its non-sulphated counterpart. Inhibition by lithocholate 3-sulphate of 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity could therefore be studied at pH 6.0, 6.5, 7.0 and 7.5 (Fig. 3). Table 2 shows that at pH 7.5 lithocholate 3-sulphate inhibits both the group II enzymes and transferases J and K more than does lithocholate. This effect

Fig. 4. pH-dependence of the lithocholate 3-sulphate inhibition of group II glutathione *S*-transferases

The concentration of lithocholate 3-sulphate that produced 50% inhibition of transferases A (○, Yb₁Yb₁), P (△, Yb₁Yn), D (●, Yb₂Yb₂), S (■, Yb₂Yn) and N (▲, YnYn) is plotted against pH. Each data point was calculated from a minimum of 35 experimental results. Note that the scale on the ordinate is logarithmic.

was most apparent for transferase D (Yb₂Yb₂). However, the converse is true for the Ya-containing group I transferases, these forms being inhibited more by lithocholate 3-sulphate than by chenodeoxycholate (Table 3). The fact that lithocholate is a poorer inhibitor of the Yk-, Yb₁, Yb₂- and Yn-containing enzymes than is its sulphate ester strongly suggests that factors other than hydrophobicity influence the bile-acid-binding site on these polypeptides.

Transferase N (YnYn) showed no change in its *I*₅₀ for

lithocholate 3-sulphate between pH 6.5 and 7.0. This 'plateau' is also reflected in the *I*₅₀-versus-pH plots for the other Yn-containing enzymes. Fig. 4 demonstrates that the Yb₁Yn (transferase P) and Yb₂Yn (transferase S) hybrids all show much larger changes in their *I*₅₀ values for lithocholate 3-sulphate over the pH 6.0–7.5 range than would be predicted from their parental homodimers. For example, at pH 6.0 transferase S possesses a lower *I*₅₀ for lithocholate 3-sulphate than does either transferase D (Yb₂Yb₂) or transferase N (YnYn). Similarly, at pH 6.5–7.5 transferase P possesses a higher *I*₅₀ for lithocholate 3-sulphate than does either transferase A (Yb₁Yb₁) or transferase N (YnYn).

Effect of bile acids on the activity of transferases E and M

The hepatic transferases E and M both lack 1-chloro-2,4-dinitrobenzene-GSH-conjugating activity and fail to bind either to the *S*-hexylglutathione-Sepharose 6B or to the glutathione-Sepharose 6B affinity matrices. These two enzymes are therefore distinct from all the other forms studied.

The activities of transferases E and M were inhibited by bile acids (Table 4), lithocholate 3-sulphate being the most effective. Transferase E was more strongly inhibited by lithocholate 3-sulphate than was transferase AA (YcYc) or any of the Yn- and Yb₁-containing enzymes (Table 2).

DISCUSSION

The synthesis and repair of bile acids, and their removal from portal blood, are important aspects of liver function. In man, the liver transports as much as 20 g of bile acid each day from the portal blood into the biliary system. Much has still to be learnt about the handling of bile acids and their metabolites, both by the liver and by extrahepatic tissues. Although it has been recognized for several years that those transferases that were called 'ligandin', the Ya-containing forms, possess bile-acid-binding activity (Tipping *et al.*, 1976; Strange *et al.*, 1977), it is now known that other transferases also bind bile acids (Hayes *et al.*, 1980; Pattinson, 1981). Work by Vessey & Zakim (1981), Hayes & Chalmers (1983) and Boyer *et al.* (1984) led also to the recognition that bile acids inhibit glutathione *S*-transferase activity.

In the present paper the effects of bile acids have been studied to establish whether these inhibitors can distinguish between individual transferase subunits. The bile acids selected for investigation were chosen to

Table 4. Bile acid inhibition of transferase E and M

The activities of transferases E and M were measured with 1,2-epoxy-3-(*p*-nitrophenoxy)propane and 1-menaphthyl sulphate respectively as the second substrate; assays were all performed at pH 7.5. Where the *I*₅₀ value could not be calculated, the highest bile acid concentration employed is shown and the percentage of transferase activity remaining at this concentration is indicated in parentheses.

Transferase	<i>I</i> ₅₀ value (mM)			
	With cholate	With chenodeoxycholate	With lithocholate	With lithocholate 3-sulphate
E	5.4	> 0.78 (63%)	> 0.15 (87%)	0.040
M	> 8.0 (76%)	> 0.78 (93%)	> 0.15 (95%)	0.195

provide a related series, hydroxylated at the 3, 7 and 12 positions; 3 α ,7 α ,12 α -trihydroxycholanolic acid (cholate), 3 α ,7 α -dihydroxycholanolic acid (chenodeoxycholate) and 3 α -monohydroxycholanolic acid (lithocholate) were studied. This series provided a gradation in the hydrophobic nature of the ligand, but utilized the same carbon skeleton (Carey, 1982). In addition, inhibition by the sulphate ester of lithocholate was studied to establish the effect of introducing a negatively charged sulphate group attached to the steroid ring A.

Individual subunits exhibit characteristic patterns of inhibition. The Yk subunit is more sensitive to cholate inhibition, under acidic conditions, than is any other polypeptide. The Ya subunit(s) is not only more sensitive to di- and mono-hydroxylated bile acids than is any other monomer studied, but it is also the only monomer that is inhibited more by lithocholate than by its sulphate ester. The Yn subunit shows a characteristic pH-dependence of chenodeoxycholate and lithocholate 3-sulphate inhibition. The Yb₁ monomer is inhibited more by cholate, under alkaline conditions, than are other subunits. The Yb₂ monomer possesses a large I_{50} (lithocholate 3-sulphate)/ I_{50} (lithocholate) ratio and is markedly activated at pH 7.5 by both chenodeoxycholate and lithocholate. The Yc monomer is more resistant to bile acid inhibition than is any of the other transferase subunits studied. The I_{50} values calculated for various bile acids can thus help identify the quaternary structure of a particular transferase.

Both the cholate and the chenodeoxycholate inhibition data presented here support the hypothesis of Sheehan & Mantle (1984) that two basic Ya subunits exist in rat liver (i.e. Ya₁ and Ya₂). These workers reported that transferases F and L were structurally distinct, and proposed that one of these forms represented a homodimer and the other a heterodimer. If this hypothesis is correct, it is to be expected that these two enzymes might not be able to be distinguished immunochemically, and it is therefore very important to recognize that, at pH 7.5, the characteristic inhibition patterns of transferases F and L by tri- and di-hydroxylated bile acids contribute greatly to their identification. Transferase L (pI 9.2) was inhibited more by cholate and chenodeoxycholate than was transferase F (pI 9.0). It can be inferred, therefore, that the Ya₁ monomer has a higher affinity for primary bile acids, under alkaline conditions, than has Ya₂. Both Ya₁ and Ya₂ subunits are assumed to possess a high-affinity lithocholate-binding site.

The problems associated with determining the subunit compositions of individual glutathione S-transferases are considerable. The complex nature of this family of isoenzymes, and the close sequence homology between different gene products (Pickett *et al.*, 1984; Lai *et al.*, 1984), require the use of several criteria in order to identify particular subunits. As discontinuous SDS/polyacrylamide-gel electrophoresis is always used to help establish the purity of enzyme preparations, problems of distinguishing between the group I and the group II enzymes normally never occur. In practice, problems arise in discriminating between the various Yn/Yb-type subunits and between the various Yk/Ya-type subunits. Immunochemical techniques may not help to discriminate between the Yn/Yb-type subunits (Hayes & Mantle, 1986). However, discrimination can be effected with bile acid inhibition, since cholate and lithocholate are the best discriminators between Yk and Ya subunits, and cholate

and lithocholate 3-sulphate are the best discriminators between Yn, Yb₁ and Yb₂ subunits.

Bile acid inhibition of transferases E and M has not been reported before. This result emphasizes the common non-substrate-binding capability of all transferases. It was, however, somewhat unexpected, since both enzymes are catalytically distinct from the remaining transferases (groups I and II) and neither transferase E nor transferase M binds to S-hexylglutathione-agarose or glutathione-agarose affinity matrices. This result suggests that transferases E and M, both of which have proved difficult to purify, might be isolated by using bile acid affinity chromatography.

In obstructive liver disease there are high concentrations of serum bile acids in plasma, but the activity of the Yc subunit is likely to be diminished little by this group of inhibitors (Table 1). The resistance of transferase AA (YcYc) to bile acid inhibition is interesting, since it has been shown that the Yc monomer possesses significant cholate-binding activity (Hayes *et al.*, 1980; Pattinson, 1981). Both equilibrium gel-filtration chromatography (Hayes *et al.*, 1980) and affinity chromatography (Pattinson, 1981) showed that the Yc monomer bound cholate more tightly than did the Yk, Ya, Yn and Yb₂ subunits. Recent work, with the cholate-derived photo-affinity probe 3-azidocholelyl[¹²⁵I]iodohistamine (Henderson, 1985), has confirmed that transferase AA possesses significant bile acid-binding activity (Henderson *et al.*, 1986). As this is not in accord with the kinetics of inhibition, it appears that the Yc subunit can simultaneously serve the dual functions of enzyme and of bile-acid-carrier protein, indicating that multiple binding sites exist on transferase AA and that some of these would appear to play no catalytic role.

In the present paper we have shown that bile acids act as effective inhibitors of transferase activity, and that this property can be used to help identify particular subunits. Bile acids may be important modulators *in vivo* of the detoxication functions of these enzymes. The extent of the inhibition varies considerably according to the subunits represented in a particular organ, the hydrophobic nature of the bile acid and whether or not it is conjugated with sulphate, and the pH.

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