*Biochem. J.* (1980) **185**, 83–87
*Printed in Great Britain*



# Cholic Acid Binding by Glutathione *S*-Transferases from Rat Liver Cytosol

John D. HAYES, Richard C. STRANGE and Iain W. PERCY-ROBB
*Department of Clinical Chemistry, University of Edinburgh, Royal Infirmary, Edinburgh EH3 9YW, Scotland, U.K.*

(*Received* 16 *July* 1979)

Cholic acid-binding activity in cytosol from rat livers appears to be mainly associated with enzymes having glutathione *S*-transferase activity; at least four of the enzymes in this group can bind the bile acid. Examination of the subunit compositions of different glutathione *S*-transferases indicated that cholic acid binding and the ability to conjugate reduced glutathione with 1,2-dichloro-4-nitrobenzene may be ascribed to different subunits.

The removal of bile acids from the blood and their secretion into bile is an important aspect of hepatic function. Transport across the hepatocyte from the sinusoidal membrane to the canalicular membrane may involve cytosolic bile acid-binding components whose molecular weight (about 45000) and lack of specificity towards different bile acids (Strange *et al.*, 1977*a*) suggested the involvement of ligandin, a non-specific anion-binding protein (Litwack *et al.*, 1971). Although the identity of ligandin may now be in doubt (Hayes *et al.*, 1979), it is one of at least seven enzymes found in rat liver cytosol which have glutathione *S*-transferase activity.

The glutathione *S*-transferases comprise a group of enzymes with overlapping substrate specificities, some of which can also bind non-substrate ligands (Jakoby, 1978). They have molecular weights of about 46000 and comprise two of three possible subunits: Ya (mol.wt. 22000), Yb (mol.wt. 23500) and Yc (mol.wt. 25000) (Bass *et al.*, 1977). Ligandin has been described as a YaYa dimer (Bass *et al.*, 1977; Hayes *et al.*, 1979), a YaYc dimer (Listowsky *et al.*, 1976) or as a mixture of two proteins comprising YaYa and YaYc dimers (Carne *et al.*, 1979). In the present study, we have defined ligandin (prepared by the method of Arias *et al.*, 1976) as a YaYa dimer.

We have purified two lithocholic acid-binding proteins from rat liver cytosol (Strange *et al.*, 1977*b*; Hayes *et al.*, 1979); both proteins possessed glutathione *S*-transferase activity, and they were identified as ligandin and glutathione *S*-transferase B (Hayes *et al.*, 1979). We now describe experiments firstly to determine if these and other glutathione *S*-transferases can bind the quantitatively more important primary bile acid, cholic acid, and secondly to compare the subunit composition of the enzymes that bind cholic acid with those that do not.

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

## Materials and Methods

### *Chemicals*

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was from Maybridge Chemical Co., Tintagel, Cornwall, U.K., and [2,4-$^3$H]cholic acid (14Ci/mmol) was from New England Nuclear, Dreieichenhain, West Germany. They were shown to be 99% pure by t.l.c. (Hamilton & Muldrey, 1961). Bio-Gel A-0.5m (200–400mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K.

### *Analytical methods*

Glutathione *S*-transferase activity was measured at 37°C by following the conjugation of glutathione with either 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene at 340nm (Habig *et al.*, 1974). Radioactivity counting, determination of $Na^+$ and protein concentrations were performed as described by Strange *et al.* (1977*b*).

### *Buffers*

The compositions of the buffers used, and the temperatures at which they were prepared, were: buffer A, 10mM-sodium phosphate, pH7.4 (20°C); buffer B, 20mM-sodium phosphate/100mM-NaCl, pH7.4 (20°C); and buffer C, 20mM-Tris/HCl, pH8.6 (4°C).

## Experimental and Results

### *Separation of glutathione S-transferases in cytosol and examination of their cholic acid-binding activity*

Cytosol prepared from two rat livers (Strange *et al.*, 1977*a*) was dialysed (4°C, 9h) against two changes, each of 2 litres, of buffer A and eluted from a CM-



0306-3275/80/010083-05 $1.50/1

Table 1. *Elution characteristics, subunit compositions and cholic acid binding by glutathione S-transferases from rat liver cytosol*
The glutathione *S*-transferases in rat liver cytosol were separated by a combination of CM-Sephadex and DEAE-Sephadex chromatography. Their monomer composition was determined by SDS/polyacrylamide-gel electrophoresis and their ability to bind cholic acid by an equilibrium chromatographic method.

| Peak | Elution vol. from CM-Sephadex (ml) | Elution vol. from DEAE-Sephadex (ml) | Percentage subunit composition Ya | Yb | Yc | Cholic acid binding |
|---|---|---|---|---|---|---|
| 1 | 15–40 | | 5 | 25 | 70 | Yes |
| 2 | 43–85 | | 5 | 95 | 0 | No |
| 3 | 91–115 | | 90 | 5 | 5 | Yes |
| 4 | 130–180 | | | | | |
| 4(i) | 130–154 | 20–53 | 50 | 0 | 50 | Yes |
| 4(ii) | 155–180 | 127–153 | 0 | 100 | 0 | No |
| 5 | 197–241 | | 0 | 0 | 100 | Yes |

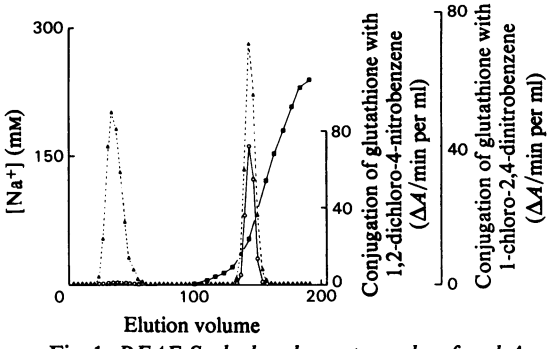


Fig. 1. *DEAE-Sephadex chromatography of peak* 4
Cytosol was eluted from a CM-Sephadex column and peak 4 (elution vol. 130–180ml) was combined and concentrated. After dialysis against buffer C, peak 4 was eluted from a DEAE-Sephadex column. Fractions (3.4ml) were collected and the glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) and 1,2-dichloro-4-nitrobenzene (○) and the $Na^+$ concentration (■) were measured.

Sephadex column (2.2cm×15cm) (Hayes *et al.*, 1979). Five peaks with GSH–1-chloro-2,4-dinitrobenzene-conjugating activity were eluted and designated 1–5 by their elution volumes (Table 1) (Hayes *et al.*, 1979). They were each combined and peaks 2–5 were concentrated, to about 5ml, by dialysis at 4°C against poly(ethylene glycol). Peaks 1, 2, 3 and 5 were dialysed against two changes, each of 2 litres, of buffer B (4°C, 15h).

After concentration the glutathione *S*-transferases in peak 4 were dialysed against two changes, each of 2 litres, of buffer C. This solution was applied to a DEAE-Sephadex A-50 column (2.2cm×15cm), which was equilibrated and eluted (20ml/h, 4°C) with buffer C. A NaCl gradient (0–500mM) in buffer C was established after 95ml of the buffer had passed through (Strange *et al.*, 1977*b*). Fractions (3.3ml) were collected and analysed for glutathione *S*-transferase activity by measuring the conjugation of GSH with either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene. Peak 4 was resolved into two peaks of enzyme activity (Fig. 1); these were designated peak 4(i) and peak 4(ii) (Table 1). Both peaks were concentrated to about 5ml by dialysis at 4°C against poly(ethylene glycol) and then dialysed against two changes, each of 2 litres, of buffer B (4°C, 15h).

The glutathione *S*-transferases have previously been classified by their order of elution from CM-cellulose (Habig *et al.*, 1974; Jakoby *et al.*, 1976), which suggests that transferases D, E and M are eluted in peak 1, transferase C is eluted in peak 2, ligandin and glutathione *S*-transferase B are eluted in peaks 3 and 4(i) respectively (Hayes *et al.*, 1979), transferase A is eluted in peak 4(ii) and transferase AA in peak 5. The presence of transferase C in peak 2 and transferase A in peak 4(ii) is supported by the substantial GSH–1,2-dichloro-4-nitrobenzene-conjugating activity found in these two peaks (Habig *et al.*, 1974).

The binding of cholic acid by whole cytosol and the separate transferase peaks was studied by using an equilibrium chromatographic method (Wood & Cooper, 1970). A Bio-Gel A-0.5m column (2.5cm×38cm) was equilibrated and eluted at 4°C with buffer B containing [$^3$H]cholic acid (10nM; 150d.p.s./ml). The flow rate was 20.5ml/h and the fraction volume was 3.4ml. The elution volume of Blue Dextran was 75ml and of [$^3$H]cholic acid 200ml. Portions (3ml) of cytosol and glutathione *S*-transferase peaks 1, 2, 3, 4(i), 4(ii) or 5, containing approx. 80, 100, 15, 15, 15 or 15mg of protein respectively, were diluted with an equal volume of buffer B containing [$^3$H]cholic acid (20nM; 300d.p.s./ml). After incubation (60min, 4°C), this mixture was eluted from the Bio-Gel column with buffer B containing [$^3$H]cholic acid (10nM; 150d.p.s./ml). The elution of cytosol from the Bio-Gel column showed that the cholic acid-

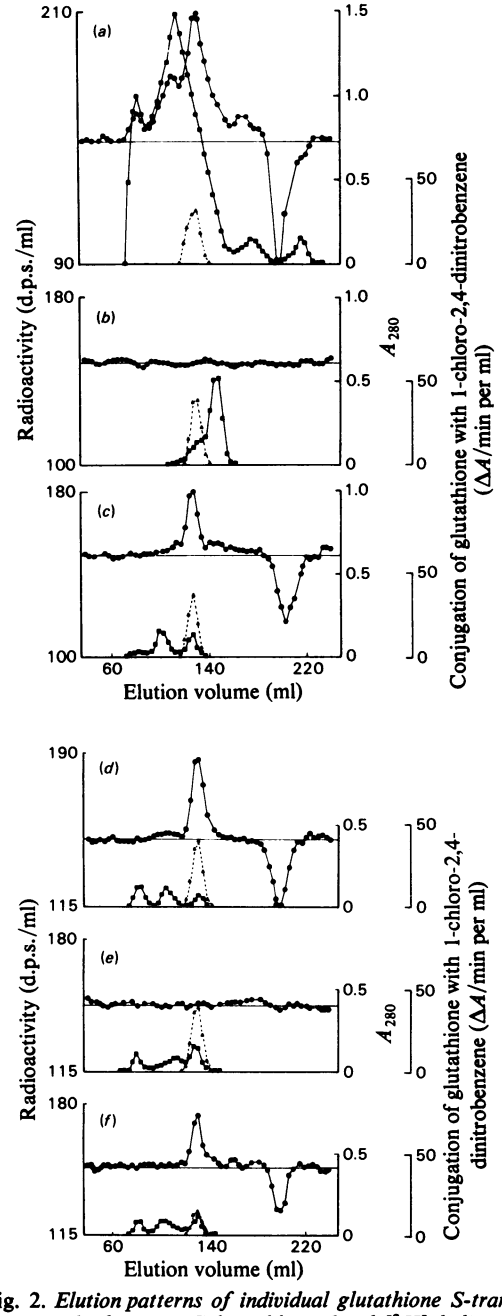


Fig. 2. *Elution patterns of individual glutathione S-transferase peaks from Bio-Gel equilibrated with [$^3$H]cholic acid*

The transferase peaks [1, 2, 3, 4(i), 4(ii) and 5] obtained after ion-exchange chromatography were concentrated and dialysed against buffer B. These were eluted from a Bio-Gel A-0.5m column equilibrated with [$^3$H]cholic acid as follows: peak 1, (*a*); peak 2, (*b*); peak 3, (*c*); peak 4(i), (*d*); peak 4(ii), (*e*); and peak 5, (*f*). Fractions (3.4ml) were collected and the $A_{280}$ (■), the radioactivity (●) and glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (▲) were measured. The solid horizontal line represents the radioactivity in the elution buffer.

binding activity was eluted as a single peak, which was associated with glutathione *S*-transferase activity. The elution of peaks 1–5 from the Bio-Gel column also showed that cholic acid binding by peaks 1, 3, 4(i) and 5 was associated with enzyme activity (Fig. 2). Cholic acid binding by peak 1 was not restricted to enzyme-containing fractions, but was also found in fractions with the same elution volume as rat albumin (110ml). Peak 1 also contained a component of mol.wt. about 15000 which bound cholic acid. This may be the lithocholic acid-binding protein previously described by Strange *et al.* (1977*a*), which was shown to be eluted from CM-Sephadex in peak 1 (J. D. Hayes, R. C. Strange & I. W. Percy-Robb, unpublished work). Ketterer *et al.* (1976) have also described a binding component of mol.wt. 14000 which binds cholic acid. No cholic acid binding was demonstrated in peaks 2 or 4(ii), despite the recovery of substantial enzyme activity.

*Subunit composition of the glutathione S-transferases*

Before discontinuous SDS/polyacrylamide-gel electrophoresis, the glutathione *S*-transferases were partially purified. Two portions of cytosol (each 5ml; approx. 180mg of protein) were eluted (22ml/h) with buffer B from the Bio-Gel column (2.5cm× 38cm). The enzyme-containing solutions, eluted between 120 and 145ml, were combined. Examination of a small portion of this mixture by SDS/polyacrylamide-gel electrophoresis showed that about 85% of the protein in this mixture migrated with the Ya, Yb or Yc monomer bands (Bass *et al.*, 1977; Hayes *et al.*, 1979). $(NH_4)_2SO_4$ was added to the combined enzyme-containing eluate, and the precipitate formed between 55 and 85% saturation was collected, redissolved in 12ml of buffer A and dialysed against two changes, each of 2 litres, of the same buffer (4°C, 15h) (Dixon & Webb, 1964; Strange *et al.*, 1977*b*). The dialysed solution was eluted from CM-Sephadex and five peaks of glutathione *S*-transferase activity were obtained. Peak 4 was rechromatographed on DEAE-Sephadex as described above. Portions (10μg of protein) of each peak were examined by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970; Hayes *et al.*, 1979). The gels were scanned at 580nm as previously described (Hayes *et al.*, 1979) and the percentage monomer composition of each peak was determined (Table 1). Peak 1 probably comprises a mixture of transferases, since it contains mainly unequal amounts of the Yb and Yc monomers. Peak 2 appears to comprise a YbYb dimer. Peaks 3 and 4(i) have previously been

purified and shown to comprise YaYa and YaYc dimers respectively (Hayes *et al.*, 1979). Peak 4(ii) comprised a YbYb dimer and peak 5 a YcYc dimer.

## Discussion

We have previously described two lithocholic acid-binding proteins in rat liver cytosol which possessed glutathione *S*-transferase activity (Strange *et al.*, 1977*b*). These two enzymes were eluted in peaks 3 and 4(i) and have been identified as ligandin (prepared by the method of Arias *et al.*, 1976) and glutathione *S*-transferase B respectively (Hayes *et al.*, 1979). Ligandin has variously been described as a YaYa dimer (Bass *et al.*, 1977; Hayes *et al.*, 1979), a YaYc dimer (Listowsky *et al.*, 1976) or as a mixture of two proteins comprising YaYa and YaYc dimers (Carne *et al.*, 1979). Since the subunit composition of the original ligandin preparations (Litwack *et al.*, 1971) is unknown, the term ligandin should perhaps be abandoned and the proteins redefined according to their subunit composition as YaYa or YaYc protein.

The experiments now described show that the YaYa protein and YaYc protein as well as glutathione *S*-transferase AA and one or more of the transferases in peak 1 can bind cholic acid. SDS/polyacrylamide-gel electrophoresis indicated that cholic acid binding was associated with either the Ya or the Yc monomer. The proteins that catalysed the conjugation of GSH with 1,2-dichloro-4-nitrobenzene [in peaks 1, 2 and 4(ii)] all possessed the Yb band.

Although at least seven ionically distinct glutathione *S*-transferases exist in rat liver, SDS/polyacrylamide-gel electrophoresis has demonstrated that they each comprise two of three known monomers (Ya, Yb, Yc). Habig *et al.* (1974, 1976) have shown that transferases C and A, and B and AA, have similar amino acid compositions: significant differences exist between the alanine and valine contents of these two groups of transferases. The compositions suggest that transferases B and AA may be the product of a single gene, and transferases C and A may also be coded for by a single but separate gene. Habig *et al.* (1974) have reported that both transferases C and A cross-react with antisera raised against each other, which supports the hypothesis that these two enzymes are the product of a single gene. The elution position and substrate specificity of peaks 2 and 4(ii) suggested that they contained transferase C and transferase A respectively. Both of these enzymes comprise YbYb dimers and therefore they may be formed by post-synthetic modification involving minor structural changes. Peak 4(i) has been shown to contain transferase B (YaYc protein) (Hayes *et al.*, 1979), and the elution position and substrate specificity of peak 5 suggested that it contained transferase AA. Both of these transferases possess a Yc monomer. We have previously shown (Hayes *et al.*, 1979) that ligandin (YaYa protein) and glutathione *S*-transferase B (YaYc protein) are separate proteins, and we postulated that they may be synthesized as a YcYc protein, which is subsequently converted into YaYc or YaYa protein by the specific removal of 26 terminal amino acids. Our subunit data show that transferase AA comprises a YcYc dimer. This may represent the putative precursor protein.

Listowsky *et al.* (1976) have described a preparation of glutathione *S*-transferase A which had the same monomer composition (YaYc) as glutathione *S*-transferase B. Our results do not agree with this finding, since only peak 4(i) has this composition and this peak has been identified as glutathione *S*-transferase B (Hayes *et al.*, 1979). Further, this peak possesses no GSH–1,2-dichloro-4-nitrobenzene-conjugating activity, which indicates that it does not contain transferase A (Fig. 1).

The physiological importance of bile acid binding by the transferases is not clear. It appears unlikely that the bile acids are transported across the hepatocyte bound to glutathione *S*-transferases, since the observed bile acid transit times across the liver appear to be too rapid for the bile acids to diffuse across the hepatocyte bound to protein (Strange *et al.*, 1979). The transferases may keep bile acids in cytosol, and so restrict their partitioning into membrane lipid, thereby promoting their rapid clearance into bile.

J. D. H. thanks the University of Edinburgh for a Faculty of Medicine Scholarship.

## References

Arias, I. M., Fleischner, G., Kirsch, R., Mishkin, S. & Gatmaitan, Z. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 175–188, Raven Press, New York

Bass, N. M., Kirsch, R. E., Tuff, S. A., Marks, I. & Saunders, S. J. (1977) *Biochim. Biophys. Acta* **492**, 163–175

Carne, T., Tipping, E. & Ketterer, B. (1979) *Biochem. J.* **177**, 433–439

Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 39–41, Longmans Green, London

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139

Habig, W. H., Pabst, M. J. & Jacoby. W. B. (1976) *Arch. Biochem. Biophys.* **175**, 710–716

Hamilton, J. G. & Muldrey, J. E. (1961) *J. Am. Oil Chem. Soc.* **38**, 582–585

Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1979) *Biochem. J.* **181**, 699–708

Jakoby, W. B. (1978) *Adv. Enzymol.* **46**, 383–414

Jakoby, W. B., Habig, W. H., Keen, J. H., Ketley, J. N. & Pabst, M. J. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 189–202, Raven Press, New York

Ketterer, B., Tipping, E., Hackney, J. F. & Beale, D. (1976) *Biochem. J.* **155**, 511–521

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Listowsky, I., Kamisaka, K., Ishitami, K. & Arias, I. M. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, E. B., eds.), pp. 233–239, Raven Press, New York

Litwack, G., Ketterer, B. & Arias, I. M. (1971) *Nature (London)* **234**, 466–467

Strange, R. C., Nimmo, I. A. & Percy-Robb, I. W. (1977*a*) *Biochem. J.* **162**, 659–664

Strange, R. C., Cramb, R., Hayes, J. D. & Percy-Robb, I. W. (1977*b*) *Biochem. J.* **165**, 425–429

Strange, R. C., Chapman, B. J., Johnston, J. D., Nimmo, I. A. & Percy-Robb, I. W. (1979) *Biochim. Biophys. Acta* **573**, 535–545

Wood, G. C. & Cooper, P. F. (1970) *Chromatogr. Rev.* **12**, 88–107