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Glutathione *S*-transferases in elasmobranch liver

Molecular heterogeneity, catalytic and binding properties, and purification

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In order to gain insight into the phylogeny and physiological significance of organic-anion-binding proteins in the liver, the hepatic glutathione *S*-transferases of rat and a typical elasmobranch, the thorny-back shark (*Platyrhinoides triseriata*), were compared with respect to both glutathione *S*-transferase activites and organic-anion-binding properties. On gel filtration (Sephadex G-75, Superfine grade) of rat cytosol, the elution volumes of enzyme activities with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates were identical (rat Y-fractions; M_r 45000). In contrast, two peaks of enzyme activity for 1-chloro-2,4-dinitrobenzene with elution volumes corresponding to M_r 52000 (PLAT Y_1) and M_r 45000 (PLAT Y_2) were detected on gel filtration of *P. triseriata* cytosol. Only fraction PLAT Y_2 had enzyme activity with *p*-nitrobenzyl chloride. Enzyme kinetic studies showed that rat Y-fraction had higher affinities for both 1-chloro-2,4-dinitrobenzene and glutathione than PLAT Y_1- and PLAT Y_2-fractions. The two forms of *P. triseriata* glutathione *S*-transferases differed greatly in affinity for glutathione. At a glutathione concentration that we found to be physiological in *P. triseriata*, PLAT Y_2 accounted for approx. 70% of the total glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene. Binding studies revealed that PLAT Y_1 and PLAT Y_2 fractions had much lower affinities for sulphobromophthalein and bilirubin than rat Y-fraction. In contrast, binding affinities of PLAT Y_1 and PLAT Y_2 for Rose Bengal and 1-anilino-8-naphthalenesulphonate were comparable with that of rat Y-fraction. Inhibitory kinetics suggested that sulphobromophthalein and Rose Bengal were non-competitive inhibitors of glutathione *S*-transferase activities when 1-chloro-2,4-dinitrobenzene was used as substrate for both PLAT Y_1 and PLAT Y_2. The major glutathione *S*-transferase from the PLAT Y_2 fraction was purified 81-fold by sequential chromatography on Sephadex G-75, DEAE-Sephadex and hydroxyapatite, and consisted of two identical subunits with p*I* 7.7. The highly enriched Y_2-fraction retained high affinity binding of Rose Bengal and 1-anilino-8-naphthalenesulphonate.

The glutathione *S*-transferases (EC 2.5.1.18) are a family of enzymes that are present in the cytosol of most cells (Habig *et al.*, 1974*b*; Jakoby *et al.*, 1976*a*,*b*). In rat liver cytosol there are at least seven glutathione *S*-transferases with an overlapping complex pattern of substrate specificities (Habig *et al.*, 1974*b*; Jakoby *et al.*, 1976*a*). Glutathione *S*-transferases have been implicated in organic-anion transport in the liver (Levi *et al.*, 1969; Reyes *et al.*, 1972; Litwack *et al.*, 1971; Habig *et al.*, 1974*a*; Ketley *et al.*, 1975). Binding to these proteins in cytoplasm may play a role in the net transport of organic anions from plasma into liver cells by minimizing back-diffusion (Wolkoff *et al.*, 1979).

The phylogeny of the role of organic-anion-binding proteins and biotransformation in the hepatic uptake and biliary excretion of organic anions has been studied in marine elasmobranchs. Levine *et al.* (1971) suggested that the appearance of Y-protein (ligandin), identified by sulphobromo-

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phthalein binding, coincided with the transition of life from water to land, and corresponded with the development of mechanisms for the selective removal of sulphobromophthalein by the liver. Boyer *et al.* (1976*a*,*b*), on the contrary, found that sulphobromophthalein undergoes selective hepatic uptake and biliary excretion in elasmobranchs without intracellular binding protein or biotransformation to a glutathione conjugate. Both groups agreed, however, on the absence of ligand (sulphobromophthalein binding protein) in elasmobranchs. On the other hand, the presence of glutathione *S*-transferase activity in the liver of dogfish (*Squalus acanthias*) was demonstrated by using 1,2-dichloro-4-nitrobenzene as substrate (Bend & Fouts, 1973). The fact that marine elasmobranchs had cytosolic glutathione *S*-transferase activity but lacked sulphobromophthalein-binding activity suggested a unique dissociation between these two functions of the transferases.

With the hope of gaining further insight into the phylogeny and physiological significance of organic-anion-binding proteins in the liver, we purified over 80-fold hepatic glutathione *S*-transferase from a typical elasmobranch, the thorny-back shark (*Platyrhinoides triseriata*), and compared it with rat hepatic glutathione *S*-transferases with respect to both enzyme kinetics and organic-anion-binding properties.

Materials and methods

Animals and preparation of liver cytosol

The livers of diethyl ether-anaesthetized male Sprague–Dawley rats weighing approx. 260g were perfused *in situ* with 0.01M-phosphate buffer (pH 7.4)/0.25M sucrose. Liver homogenates (33%, w/v) were prepared in the same buffer and the supernatant fraction was harvested after centrifugation at 100000*g* for 60min. Thorny-back sharks (*Platyrhinoides triseriata*), supplied by Pacific Marine Biologicals, Culver City, CA, U.S.A. were allowed to swim free in tanks for 1–2 days before being killed by a blow to the head. The liver was removed and a 33% homogenate was prepared and treated as described above for rat liver. Reduced glutathione in the fresh *P. triseriata* liver homogenate was measured by using reversed-phase high-pressure liquid chromatography (Reeve *et al.*, 1980). Specimens were stored at −15°C.

Enzyme kinetic studies

Eight glutathione *S*-transferase activities were determined by previously described methods (Habig *et al.*, 1974*b*) with liver cytosol preparations from rat and *P. triseriata*. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The following substrates were used in 3 ml reaction volumes: 0.1mM-1-chloro-2,4-dinitrobenzene (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); 1.0mM-1,2-dichloro-4-nitrobenzene (Aldrich); 0.5mM-*p*-nitrobenzyl chloride (Aldrich); 0.03mM-sulphobromophthalein (J. T. Baker Chemical Co., Philipsburg, NJ, U.S.A.); 0.5mM-1,2-epoxy-3-(*p*-nitrophenoxy)propane (Eastman Kodak Co., Rochester, NY, U.S.A.); 0.2mM-ethacrynic acid (a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.); 0.05mM-*trans*-4-phenylbut-3-en-2-one (Aldrich); 0.2mM-4-nitropyridine *N*-oxide (Aldrich). Excess glutathione (6·67mM; Sigma) was used for reactions with each substrate, with the exception of ethacrynic acid, *trans*-4-phenylbut-3-en-2-one and 4-nitropyridine *N*-oxide, for which glutathione concentrations of 0.25mM were employed. The initial rates of reactions at 37°C were determined by measurement of the production of glutathione conjugates by using a Beckman ACTA MVI spectrophotometer. Non-enzymic reaction rates of substrates were subtracted from the enzymic rates.

Gel filtration was performed on a column (2.5cm × 108cm) of Sephadex G-75 (superfine grade; Pharmacia, Uppsala, Sweden) at 4°C with 0.01M-sodium phosphate, pH 7.4, as elution buffer, and 2.5ml fraction volumes were collected. A 6ml portion of cytosol was charged on the column and eluted at a flow rate of 12–16ml/h. The column was standardized with Blue Dextran (void volume), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000) and chymotrypsinogen (M_r 25000) as markers.

Protein concentrations of eluate fractions were measured and glutathione *S*-transferase activities were determined. Portions (20μl for rat, 50μl for *P. triseriata*) of eluate fractions were used as source of enzyme. In the other gel-filtration experiments with *P. triseriata* cytosol (Fig. 5 below), high (6.67mM) and low (0.067mM) concentrations of glutathione were used to determine the glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene.

The protein factions that eluted between fractions 105 and 110 (rat Y), 99 and 103 (PLAT Y_1) and 106 and 111 (PLAT Y_2) were combined separately and used in the enzyme and binding kinetic studies after adjustment of protein concentrations. Each activity in the Y-fractions was determined over a range of substrate concentrations (0.025–0.30mM-1-chloro-2,4-dinitrobenzene; 0.067–1.02mM-glutathione). Data were expressed by the method of Lineweaver & Burk (1934). The Michaelis constant (K_m) was calculated by the non-linear least-squares method. Inhibitory kinetics were investigated by using three or four substrate concentrations and a range of inhibitor concentrations (0.16–6.4μM-

sulphobromophthalein; 0.03–0.90 μM-Rose Bengal). The data were expressed by the method of Dixon (1953). The inhibitor constants were calculated by non-linear least-squares method.

Binding studies

Sulphobromophthalein binding to Y-protein fraction was measured by equilibrium dialysis as previously described (Sugiyama *et al.*, 1979).

Rose Bengal and bilirubin binding to Y-protein fraction was determined spectophotometrically as described by Sugiyama *et al.* (1978) and Tipping *et al.* (1976). Rat Y and PLAT Y_1 and Y_2 fractions produced almost the same difference spectrum with Rose Bengal, and the absorption coefficients for bound Rose Bengal were the same with rat Y, PLAT Y_1 and PLAT Y_2 fractions. Results were expressed as described by Scatchard (1949), and the dissociation constant for the first binding site was obtained from the initial linear portion of the plot calculated by non-linear least-squares method.

1-Anilino-8-naphthalenesulphonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) binding was determined by a fluorescence method previously described (Sugiyama *et al.*, 1979, 1980). The dissociation constant was calculated from a plot of the reciprocal of the change in fluorescence against the reciprocal of the total 1-anilino-8-naphthalenesulphonate concentration.

Purification of glutathione S-transferase from P. triseriata

P. triseriata liver cytosol (30 ml) was chromatographed on a column (5 cm × 97 cm) of Sephadex G-75 (superfine grade) at 4°C, with 0.01 M-sodium phosphate, pH 7.4, for elution, and 4.5 ml fractions were collected. Glutathione *S*-transferase activity in the fractions was measured by using both high (6.67 mM) and low (0.067 mM) glutathione concentrations and 1-chloro-2,4-dinitrobenzene as substrate (see the Results section). Two peaks of activity were obtained (PLAT Y_1 and PLAT Y_2) and pooled separately. The PLAT Y_2 fraction (62 ml) was dialysed at 4°C against two changes of 2000 ml of 0.01 M-Tris/HCl, pH 8.0, and batch-adsorbed with 25 ml of DEAE-Sephadex (A-25; Pharmacia) swollen with the same buffer.

The unadsorbed fraction contained 90% of initial 1-chloro-2,4-dinitrobenzene activity. This fraction (78 ml) was dialysed at 4°C against two changes of 2000 ml of 0.01 M-potassium phosphate, pH 6.7, containing 30% glycerol and 0.1 mM-EDTA, and subsequently applied to a column (1.0 cm × 12 cm) of hydroxyapatite equilibrated with the same buffer. After being washed with 25 ml of buffer, the column was eluted with a 100 ml linear gradient of 10–200 mM-potassium phosphate, pH 6.7, in a solution containing 30% glycerol and 0.1 mM-EDTA. Fractions (1.15 ml) were collected and assayed for glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene (see Fig. 10 below). The fractions that contained the enzyme activity were pooled and stored at −40°C.

Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was performed in vertical slab gels by using the method of Laemmli (1970). The stacking gels and resolving gels contained 3 and 12.5% acrylamide respectively. Calibration proteins included ovalbumin (M_r 45000), chymotrypsinogen (M_r 25000) and ribonuclease A (M_r 13700).

Isoelectric focusing was performed in a DESAGA TLE double chamber (DESAGA/Brinkmann, Westbury, NY, U.S.A.) as described by Radola (1969), with Sephadex G-75 (superfine grade) in pH 3.5–10 Ampholine (LKB Instruments, Rockville, MD, U.S.A.) for resolution.

Purified enzyme (150 μg in 1.8 ml) was dialysed overnight against 1000 ml of double-distilled water. The sample was hydrolysed in 6 M-HCl at 110°C for 24 h and amino acid analysis was performed on the hydrolysate by using a Beckman model 118 CL amino acid analyser (Beckman Instruments, Fullerton, CA, U.S.A.). Tryptophan was determined spectrophotometrically (Edelhoch, 1967).

Results

Substrate specificities of glutathione S-transferase activity in the liver cytosol of rat and P. triseriata

Glutathione *S*-transferase activities, towards eight substrates, in the liver cytosol of rat and *P. triseriata* are presented in Table 1. In each instance, *P. triseriata* cytosol had lower enzyme activities than rat cytosol. However, *P. triseriata* cytosol had enzyme activities approaching those of rat cytosol when *p*-nitrobenzyl chloride, ethacrynic acid and 1-chloro-2,4-dinitrobenzene were used as substrates.

Gel-filtration pattern of liver cytosol of rat and P. triseriata

Gel filtration was performed with the liver cytosol of rat and *P. triseriata*. Each eluted fraction was assayed for protein concentration and glutathione *S*-transferase activity by using 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride as substrates (Fig. 1). Glutathione *S*-transferase activities of rat cytosol with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride had superimposable elution patterns with a peak corresponding to M_r 45000. In contrast, glutathione *S*-transferase activities towards both substrates in *P. triseriata* did not coincide, although the peak position was the same. The enzyme activities of the peak fraction with 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzyl chloride

Table 1. *Substrate specificities of glutathione S-transferase activity in rat and P. triseriata liver*

Cytosol preparation from five rats and five *P. triseriata* were pooled separately and used to determine enzyme activities. Enzyme activities were expressed as nmol/min per mg of soluble proteins. The ratio of enzyme activities in *P. triseriata* and rat cytosol are expressed as a fraction in the last column. For details, see the Materials and methods section.

Substrate	Enzyme activity		Enzyme activity ratio
	P. triseriata	Rat	(*P. triseriata*/rat)
p-Nitrobenzyl chloride	315	852	0.370
Ethacrynic acid	16.9	53.2	0.318
1-Chloro-2,4-dinitrobenzene	269	1350	0.199
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	10.8	145	0.075
4-Nitropyridine *N*-oxide	2.02	33.7	0.060
trans-4-Phenylbut-3-en-2-one	2.13	50.1	0.043
1,2-Dichloro-4-nitrobenzene	1.99	85.7	0.023
Bromosulphophthalein	0.13	19.6	0.007

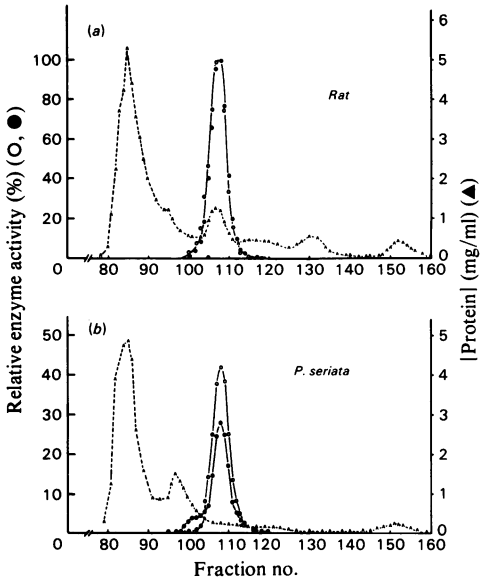

Fig. 1. *Comparison of the gel-filtration patterns of glutathione S-transferase activities in liver cytosol of rat (a) and P. triseriata (b)*

Liver cytosol (6 ml) was charged on a column (2.5 cm × 108 cm) of Sephadex G-75 (superfine grade). Fractions (2.5 ml) were collected, and protein concentrations were determined by the method of Lowry *et al.* (1951). Activities towards 1-chloro-2,4-dinitrobenzene (●) and *p*-nitrobenzyl chloride (O) were measured at concentrations of 0.1 mM and 0.5 mM respectively, with 6.7 mM-glutathione. The results for *P. triseriata* and rat cytosol are expressed as a proportion of maximum activity (observed in fraction 108 for both).

were 28 and 42% of those of rat respectively. A shoulder of enzyme activity with 1-chloro-2,4-dinitrobenzene preceded the main peak. The M_r of this shoulder was estimated to be 52000. This result suggested molecular heterogeneity of *P. triseriata* glutathione *S*-transferases. Tube fractions from 105 to 110 in rat, from 99 to 103 and from 107 to 111 in *P. triseriata* were combined separately and designated as 'rat Y', 'PLAT Y_1' and 'PLAT Y_2' respectively. These fractions were used for further kinetic studies.

With the addition of tracer glutathione, a peak of radioactivity bound to the Y_1 fraction was found in the gel-filtration profile of *P. triseriata* cytosol (Fig. 2). Glutathione is known to bind to rat Y-protein fraction in a similar fashion (Kaplowitz *et al.*, 1973).

Enzyme kinetics

A comparison of enzyme kinetics with respect to 1-chloro-2,4-dinitrobenzene or glutathione is summarized in Table 2. PLAT Y_1 and Y_2 had markedly lower affinity for both substrates than rat Y. Comparison of PLAT Y_1 and Y_2 revealed a 7-fold difference in K_m for glutathione. The higher affinity of Y_1 for glutathione is reflected in the finding of tracer glutathione predominantly bound to Y_1 in Fig. 2.

Utilizing the difference in affinity for glutathione, the chromatographic separation of PLAT Y_1 and Y_2 was more clearly delineated as shown in Fig. 3(*a*). High GSH concentration optimized the detection of PLAT Y_2, whereas low GSH concentration optimized the detection of Y_1. This approach demonstrated two activity peaks corresponding to different M_r values. In order to determine the relative physiological significance of PLAT Y_2 and PLAT Y_1, we compared the activity ratios of the two enzymes at increasing concentration of glutathione as shown in Fig. 3(*b*). As GSH concentration increased, the ratio PLAT Y_2/PLAT Y_1 increased. Endogenous glutathione concentrations in freshly killed *P. triseriata* livers was 1.31 ± 0.21 mM (mean ± S.E.M., n = 6). Therefore, at physiological glu-

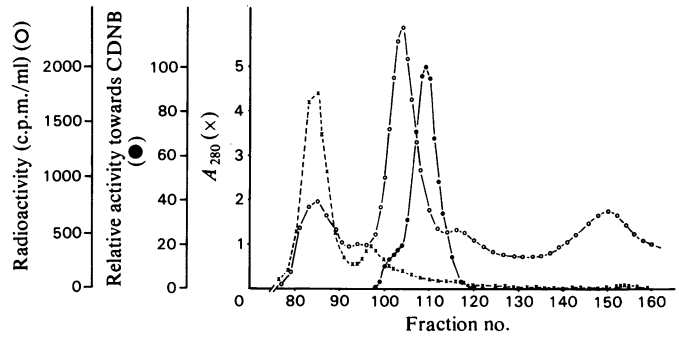

Fig. 2. *Binding of [glycine-^{3}H]glutathione to cytosol proteins of P. triseriata liver*

[*glycine*-^{3}H]Glutathione (1.9 μmol; specific radioactivity 1.6 Ci/mmol) was added to 6 ml of *P. triseriata* liver cytosol and chromatographed on Sephadex G-75 (superfine grade). A_{280}, radioactivity, and activity towards 1-chloro-2,4-dinitrobenzene (1-chloro-2,4-dinitrobenzene, 0.1 mM; glutathione, 6.67 mM) were measured in each fraction. The conditions of gel filtration are the same as those described in Fig. 1. Abbreviation used: CDNB, 1-chloro-2,4-dinitrobenzene.

Table 2. *Comparison of kinetic and binding parameters*

	K_m (mM)		K_d (μM)‡				K_i (μM)§	
	1-Chloro-2,4-dinitrobenzene*	Glutathione†	Sulphobromophthalein	Bilirubin	Rose Bengal	1-Anilino-8-naphthalenesulphonate	Sulphobromophthalein	Rose Bengal
Rat Y	0.039	0.067	0.13	0.30	0.059	36	ND‖	ND‖
P. triseriata								
Y_1	0.38	0.22	6.7	12.6	0.17	34	11.2	3.0
Y_2	0.30	2.08	2.5	9.1	0.12	53	1.7	0.2
Purified Y_2	0.28	4.3	ND‖	ND‖	0.26	25	ND§	ND§

* 1-Chloro-2,4-dinitrobenzene varied (0.025–0.3 mM) and glutathione fixed (6.67 mM).

† Glutathione varied (0.067–1.02 mM) and 1-chloro-2,4-dinitrobenzene fixed (0.1 mM).

‡ See the Materials and methods section for a description of binding kinetic studies.

§ Incubations were performed at three concentrations of 1-chloro-2,4-dinitrobenzene (0.0375, 0.075 and 0.2 mM) and a single concentration of glutathione (6.67 mM) in the presence of various concentrations of sulphobromophthalein (0.16–6.4 μM) or Rose Bengal (0.03–0.9 μM). Since the plots were curvilinear, the linear portions were used for calculation of K_i at low inhibitor concentrations (sulphobromophthalein < 1.29 μM; Rose Bengal < 0.178 μM).

‖ Abbreviation used: ND, not done.

tathione concentration, Y_2 would be expected to account for approx. 70% of total glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene in *P. triseriata* liver.

Binding studies

The results of binding studies for organic anions (sulphobromophthalein, bilirubin, Rose Bengal and 1-anilino-8-naphthalenesulphonate) with rat Y, PLAT Y_1 and PLAT Y_2 fractions are summarized in Table 2. The dissociation constants (K_d) for the primary binding sites of sulphobromophthalein and bilirubin were one to two orders of magnitude greater in PLAT Y_1 and Y_2 compared with rat Y. With Rose Bengal the difference in binding affinity between rat and *P. triseriata* was smaller (3–4-fold) and with 1-anilino-8-naphthalenesulphonate there were no differences.

Inhibitory kinetics

Inhibitory kinetics were performed with sulphobromophthalein and Rose Bengal in order to confirm that organic-anion binding to PLAT Y_1 and PLAT Y_2 represented binding to glutathione *S*-transferases. Sulphobromophthalein and Rose Bengal were found to be non-competitive inhibitors of glutathione *S*-transferases with respect to 1-chloro-2,4-dinitrobenzene in both PLAT Y_1 and PLAT Y_2 fractions. Except for Rose Bengal binding to PLAT Y_1, K_i values are comparable with the corresponding K_d values (Table 2).

Purification of glutathione S-transferase from PLAT Y_2

Table 3 summarizes the results of the purification of glutathione *S*-transferase from PLAT Y_2. An 81-fold purification of the enzyme from *P. triseriata* liver cytosol was achieved. The final step of purification, hydroxyapatite column chromatography, is depicted in Fig. 4. Since the specific activity in the fractions identified by the horizontal bar vary by about 10%, the highly enriched final PLAT Y_2 fraction cannot be taken as completely pure. The final enriched enzyme had a specific activity with 1-chloro-2,4-dinitrobenzene as substrate of 21.7 μmol/min per mg, which is comparable with that observed with rat glutathione *S*-transferases (Habig *et al.*, 1974*b*; Jakoby *et al.*, 1976*a*,*b*): 14, 62, 11 and 10 μmol/min per mg for AA, A, B, and C respectively.

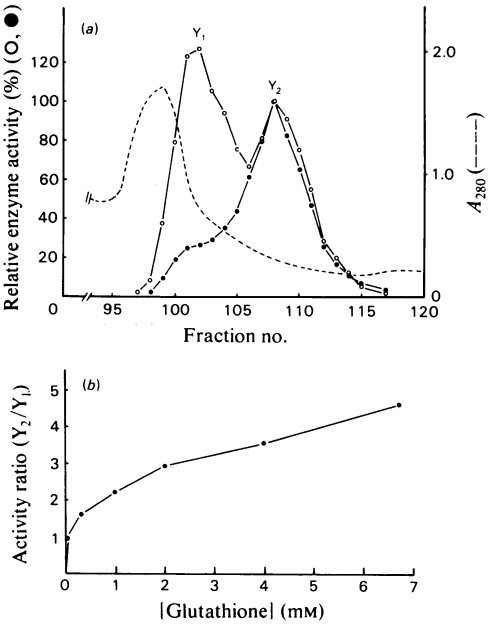

Fig. 3. *Gel-filtration pattern of 1-chloro-2,4-dinitrobenzene-conjugating activities with high and low glutathione concentrations*

The conditions of gel filtration are the same as those described for Fig. 1. Activities towards 1-chloro-2,4-dinitrobenzene were measured with high (6.67 mM, ●) and low (0.067 mM, O) glutathione concentrations. The 1-chloro-2,4-dinitrobenzene concentration was 0.1 mM. The results are expressed as a ratio of peak activity (fraction 108) under each condition (1.6 μmol/min per ml for high glutathione concentration and 0.13 μmol/min per ml for low glutathione concentration). (*a*) Gel-filtration profiles; (*b*) ratio (Y_2/Y_1) of activity towards 1-chloro-2,4-dinitrobenzene obtained by using fractions with peak activities from gel filtration (fractions 102 and 108) as a function of glutathione concentration.

Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis of the final enriched enzyme and other crude fractions is shown in Fig. 5. The enzyme purified from PLAT Y_2 migrated as a single band in the same position as rat Y_b band (M_r 24000) (Bass *et al.*, 1977; Hayes *et al.*, 1979, 1980). This suggests that purified PLAT Y_2 is composed of two subunits of the same size. Isoelectric focusing in the pH 3.5–10 range resulted in a single protein band focusing at pH 7.7.

Amino acid analysis of highly enriched enzyme is presented in Table 4. Comparison with published analyses of purified glutathione *S*-transferase A and B from rat showed major differences in the overall amino acid composition. The lower net charge of PLAT Y_2 is accounted for by the lower lysine and arginine contents. The results of binding and enzyme kinetic studies with the final Y_2 preparation are summarized in Table 2. The binding stoichiometry of 0.63 mol of Rose Bengal bound/mol of enzyme was obtained, which suggests loss of activity with purification, as described by Ketley *et al.* (1975), as well as the possibility of minor impurities.

Discussion

Because of their ability to conjugate, bind and possibly transport organic anions, the glutathione *S*-transferases are thought to play an important physiological role in hepatic organic-ion uptake. However, the reported absence of sulphobromophthalein-binding protein (ligandin) in livers of marine elasmobranchs (Levine *et al.*, 1971; Boyer *et*

Table 3. *Purification of P. triseriata Y_2 activity*

Purification step	Volume (ml)	Total protein (mg)	Total activity (μmol/min)*	Recovery (%)	Specific activity (μmol/min per mg)	Purification (fold)
1. Cytosol	30	510.0	138.0	100	0.27	1
2. Sephadex G-75	62	16.7	80.2	58	4.8	18
3. DEAE-Sephadex	78	5.2	68.1	49	13.1	49
4. Hydroxyapatite	11	1.7	36.9	27	21.7	81

* Assay with 1-chloro-2,4-dinitrobenzene (0.1 mM) and glutathione (6.67 mM) as substrates.

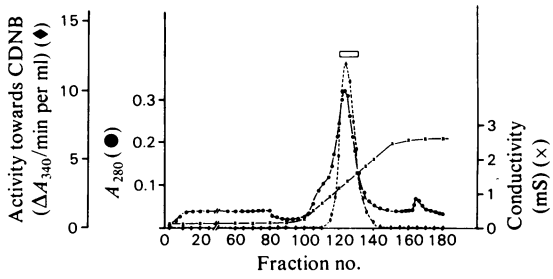

Fig. 4. *Chromatography of fraction PLAT Y_2 on hydroxyapatite*

Enzyme activity (♦) was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate; protein was measured by its A_{280} (●). The unadsorbed fraction after DEAE-Sephadex chromatography, containing PLAT Y_2, was applied to a column of hydroxyapatite (1.0cm × 12cm) and eluted with a linear gradient of 10–200mM-K_3PO_4, pH6.7 (×). □, fractions pooled as final highly enriched Y_2 enzyme.

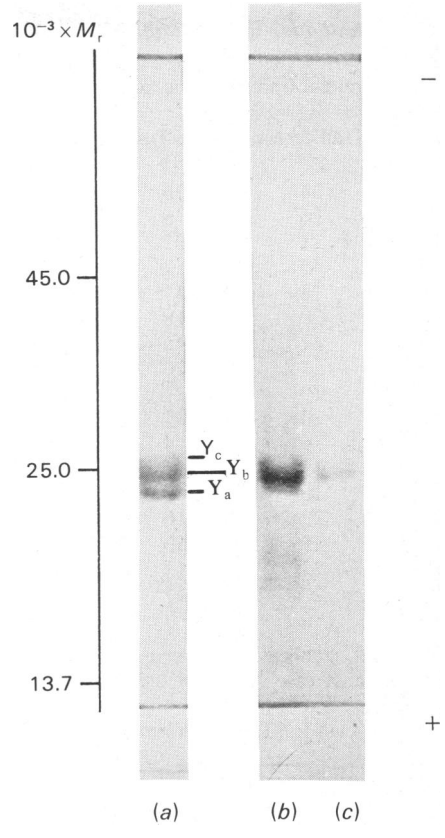

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis*

(*a*) Rat Y fraction. (*b*) PLAT Y_2 fraction. (*c*) Enzyme purified from PLAT Y_2 fraction. A 10μl portion of the pooled Y fraction and purified enzyme was subjected to electrophoresis in sodium dodecyl sulphate/12.5%-acrylamide-slab gel (see the text for details). The origin is at the top of the gel. The Y_a–Y_c bands are indicated in the rat Y-fraction. M_r markers included ovalbumin (45000), chymotrypsinogen (25000) and ribonuclease A (13700).

al., 1976*a*,*b*), coupled with the observation that net sulphobromophthalein uptake is relatively unimpaired in those animals (Boyer *et al.*, 1976*a*,*b*), has led to the conclusions that: (*a*) ligandin evolved when life moved from sea to land (Levine *et al.*, 1971; Boyer *et al.*, 1976*a*,*b*), and (*b*) ligandin is not essential for organic-anion transport (Boyer *et al.*, 1976*a*,*b*). However, in mammals, the glutathione *S*-transferases are heterogeneous, with complex overlapping substrate and binding specificities. Thus absent binding or enzyme activity with respect to one ligand (e.g. sulphobromophthalein) or substrate may not indicate complete absence of binding protein or enzyme.

We examined specific enzyme activities of *P. triseriata* cytosol towards a spectrum of substrates and found activities towards some substrates (1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride and ethacrynic acid) comparable with those of the rat, although very low activity was found when others (1,2-dichloro-4-nitrobenzene, sulphobromophthalein, *trans*-4-phenylbut-3-en-2-one, 4-nitropyridine *N*-oxide and epoxide) were used. With 1-chloro-2,4-dinitrobenzene as a substrate, elution of *P. triseriata* enzyme on gel filtration revealed a major peak (PLAT Y_2) and a preceding shoulder (PLAT Y_1). The principal difference between PLAT Y_1 and PLAT Y_2 was that the K_m with respect to glutathione was approximately one order of magnitude lower for PLAT Y_1. When 1-chloro-2,4-dinitrobenzene activity was measured in gel-filtration fractions at low and high glutathione concentrations, the distinct presence and separation of the two enzyme fractions was accentuated.

At the glutathione concentration normally present in *P. triseriata* liver, we determined that PLAT Y_2 accounts for most of the enzyme activity (70%). For this reason, and also because of the marked instability of PLAT Y_1 over time, we limited our attempts to purify *P. triseriata* liver glutathione *S*-transferases to PLAT Y_2. An enrichment of 81-fold was obtained of Y_2, which consisted of two identical subunits (M_r 24000), pI 7.7 and amino acid composition markedly different from rat enzymes.

P. triseriata glutathione *S*-transferases bound sulphobromophthalein and bilirubin with an affinity far lower than that observed in rats. However, the binding affinity of other organic anions, such as Rose Bengal and 1-anilino-8-naphthalenesulphonate, by PLAT Y_1 and Y_2 was similar to that observed with rat glutathione *S*-transferases. The specificity of binding to *P. triseriata* glutathione

Table 4. *Amino acid analysis of glutathione S-transferases*

Amino acid	Content (mol of amino acid/mol of protein) Transferase A*	Transferase B*	*P. triseriata* enzyme
Lysine	34	36	15
Histidine	6	6	8
Arginine	21	22	10
Aspartic acid	45	37	32
Threonine	13	11	12
Serine	20	18	31
Glutamic acid	42	46	46
Proline	22	20	16
Glycine	19	21	40
Alanine	20	31	23
Cysteine	6	4	ND†
Valine	11	25	17
Methionine	10	8	9
Isoleucine	22	18	10
Leucine	45	50	25
Tyrosine	23	13	8
Phenylalanine	20	17	12
Tryptophan	6	9	8

* Amino acid analysis of previously purified rat glutathione *S*-transferase A and B are shown for comparison (Habig *et al.*, 1974*b*).

† Abbreviation used: ND, not determined.

S-transferases was confirmed by inhibitory kinetics. Furthermore, highly enriched PLAT Y_2 exhibited similar dissociation constants for Rose Bengal and 1-anilino-8-naphthalenesulphonate as observed with crude PLAT Y_2.

In summary, we have shown, in contrast with previous beliefs, that marine elasmobranchs do indeed have hepatic organic-anion-binding proteins with glutathione *S*-transferase activity (ligandin), although their substrate and ligand specificities and affinities are somewhat different from those of rat glutathione *S*-transferases. The lower affinity of the elasmobranch enzyme for sulphobromophthalein which we found correlates with the slower hepatic uptake in this species (Levine *et al.*, 1971). However, the binding affinities for other organic anions, such as 1-anilino-8-naphthalenesulphonate, are comparable in elasmobranchs and rats. The remarkable phylogenetic preservation of the glutathione *S*-transferases (ligandin) may attest to their functional importance in Nature in both transport and detoxification.

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