Use of immuno-blot techniques to discriminate between the glutathione *S*-transferase Yf, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissues

Evidence for three immunochemically distinct groups of transferase in the rat

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The glutathione *S*-transferases are dimeric enzymes whose subunits can be defined by their mobility during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as Yf (M_r 24 500), Yk (M_r 25 000), 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) [Hayes (1986) *Biochem. J.* 233, 789–798]. Antisera were raised against each of these subunits and their specificities assessed by immuno-blotting. The transferases in extrahepatic tissues were purified by using, sequentially, *S*-hexylglutathione and glutathione affinity chromatography. Immune-blotting was employed to identify individual transferase polypeptides in the enzyme pools from various organs. The immuno-blots showed marked tissue-specific expression of transferase subunits. In contrast with other subunits, the Yk subunit showed poor affinity for *S*-hexylglutathione–Sepharose 6B in all tissues examined, and subsequent use of glutathione and glutathione affinity chromatography. Immuno-blotting was employed to identify a new cytosolic polypeptide, or polypeptides, immunochemically related to the Yk subunit but with an electrophoretic mobility similar to that of the Yc subunit; high concentrations of the new polypeptide(s) are present in colon, an organ that lacks Yc.

INTRODUCTION

The glutathione *S*-transferases are multifunctional proteins, found in a variety of species and organs (Jakoby, 1978). The transferases are dimeric. A large number of isoenzymes exist in both man and the rat, but these arise from binary combinations of only a limited number of subunits (Hayes *et al.*, 1981; Mannervik & Jensson, 1982; Stockman *et al.*, 1985). Individual transferases are best described by their quaternary structure. To date, Yf (M_r 24 500), Yk (M_r 25 000), 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) subunits have been described in the rat (Bass *et al.*, 1977; Beale *et al.*, 1983; Hayes, 1983, 1986; Hayes & Chalmers, 1983).

Marked tissue-specific expression of transferase activity has been reported. Extrahepatic tissues, with the exception of kidney, contain predominantly acidic enzymes, but the molecular basis, or even the polypeptide basis, of these differences is not clear (Hales *et al.*, 1978, 1980; Dierickx & De Beer, 1981; Koskelo, 1983; Tu *et al.*, 1983; Ishikawa & Sies, 1984; Singh & Awasthi, 1984).

In the accompanying paper a novel hepatic enzyme, transferase K (YkYk), is described that possesses low affinity for *S*-hexylglutathione–Sepharose 6B but binds to glutathione–Sepharose 6B (Hayes, 1986). The immunochemical relationship between Yk and the other hepatic subunits Ya, Yb, Yn and Yc is not known.

In the present study, antisera were raised separately against Yf, Yk, Ya, Yn, Yb₁, Yb₂ and Yc subunits: (1)

to determine the immunological relationship between Yk and other subunits; (2) to determine the organ distribution of Yk, as well as of other monomers; (3) to investigate the possibility that Yk is expressed coordinately with another transferase polypeptide; (4) to establish whether extrahepatic forms of transferase K display differential chromatographic behaviour on *S*-hexylglutathione and glutathione affinity columns.

MATERIALS AND METHODS

Chemicals

These were of analytical grade. The nitrocellulose paper and immuno-blot assay kit were purchased from Bio-Rad Laboratories, Watford, Herts., U.K.

Animals

Tissues from male Wistar rats (250–400 g) fed *ad libitum* were used as enzyme source. Liver, kidney, spleen, heart, lung, testis and thymus were stored at –85 °C for 1–20 days before use. The transferases in the small-intestinal and colonic mucosa were prepared immediately after removal from the rat.

Enzyme assays

Assays with 1-chloro-2,4-dinitrobenzene as electrophile were carried out at 37 °C with a centrifugal analyser (Hayes & Clarkson, 1982). Assays with ethacrynic acid, also carried out at 37 °C, were performed manually (Habig & Jakoby, 1981).

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Enzyme purification

The methods used to purify the transferases have been described before (Hayes, 1983, 1984, 1986). All the enzymes studied, with the exception of transferase K, bind to *S*-hexylglutathione-Sepharose 6B. Transferase K is isolated by affinity chromatography on glutathione-Sepharose 6B (Hayes, 1986).

Preparation of antisera

Portions (0.2 mg/ml) of purified glutathione *S*-transferases N (YnYn), D (Yb₂Yb₂), A (Yb₁Yb₁), F (YaYa), L (YaYa), AA (YcYc), K (YkYk) and λ (YfYf) were separately emulsified with an equal volume of Freund's complete adjuvant. Approx. 200 μ g of each of the resulting solutions was injected subcutaneously at eight separate sites on the back of female New Zealand White rabbits. After 6 weeks each rabbit was re-inoculated with 200 μ g of the original immunogen of incomplete Freund's adjuvant, and 2 weeks later the animals were killed and bled. Antisera against each immunogen were raised in two or three rabbits.

Immuno-blotting: determination of cross-reactive and organ survey

The immunological relationship between the hepatic transferases and their existence in extrahepatic tissues were established by using the 'Western' blotting technique (Towbin *et al.*, 1979). The transferase subunits were resolved by discontinuous SDS/polyacrylamide-gel electrophoresis, as described previously (Hayes & Clarkson, 1982).

After electrophoresis, the polyacrylamide-gel slabs were equilibrated with a solution containing 25 mM-Tris/192 mM-glycine, pH 8.3, for 30 min at room temperature. The polypeptides were then electrophoretically transferred to nitrocellulose, in the same equilibration buffer, by using a Bio-Rad Trans-blot cell and a Bio-Rad model 250/2.5 power supply (Bio-Rad Laboratories). Transfer to nitrocellulose paper was performed for 3.5 h at 10 °C with 0.25–0.35 A (18 W). Free binding sites on the paper were blocked (for 16 h, at 20 °C) by using a 3% (w/v) solution of gelatin in buffer A (20 mM-Tris/HCl buffer, pH 7.5, containing 500 mM-NaCl and 0.05% Tween-20).

After blocking with gelatin, the nitrocellulose paper (which contained adsorbed transferase polypeptides) was incubated (3 h, 20 °C) with the primary antibody (20–200 μ l) in 150 ml of buffer A containing 1% (w/v) gelatin. The enzymes that cross-reacted with the antisera were detected with the Bio-Rad goat anti-(rabbit IgG) antibody-horseradish-peroxidase conjugate immuno-blot assay kit.

During the cross-reactivity studies an identical portion of the immunogen was run in parallel with the test proteins. The polyacrylamide gel was stained to check that transfer was equally effective for all polypeptides.

Immunochemical identification of glutathione *S*-transferases in extrahepatic tissues

Extracts of kidney, spleen, heart, lung, testis and thymus (either 33% or 15%, w/v) were prepared in 50 mM-Tris/HCl buffer, pH 7.5, and the 105000 *g* supernatant (cytosol) was filtered through a plug of glass-wool to remove lipid. The cytosol from various tissues was dialysed for 18 h against two changes, each

of 2 litres, of 50 mM-tris/HCl buffer, pH 7.5, before being applied to 1.6 cm $\times$ 29 cm columns of *S*-hexylglutathione-Sepharose 6B (Mannervik & Guthenberg, 1981).

The material that did not bind to the *S*-hexylglutathione affinity matrix was subjected to glutathione affinity chromatography (Simons & Vander Jagt, 1977). The transferases in the cytosol of mucosa obtained from small intestine and colon were purified by using only glutathione affinity chromatography in an attempt to minimize proteolysis. The proteins that were specifically isolated by using these two sequential affinity steps were dialysed against 20 mM-Tris/HCl buffer, pH 7.4, and analysed by SDS/polyacrylamide-gel electrophoresis followed by the immuno-blot technique.

RESULTS

Large numbers of glutathione *S*-transferases were included in the present immunochemical study. Most of these were isolated from rat liver and included Ya, Yb₁, Yb₂, Yc and Yk homodimers. However, the Yn homodimer was from rat testis and the Yf homodimer from human lung.

Table 1 lists the enzymes studied. During this study particular emphasis was placed on the new hepatic enzyme, transferase K (YkYk). In contrast with transferase K, transferase J, a renal enzyme that possesses catalytic and binding properties similar to those of transferase K (Hayes & Mantle, 1986), was isolated from the fraction that specifically bound to *S*-hexylglutathione-Sepharose 6B (Table 1).

Cross-reactivities

Immunological relationships between the purified enzymes were investigated to provide an assessment of their structural relationships. These data were obtained with the antisera raised against transferases F (YaYa), L (YaYa), AA (YcYc), A (Yb₁Yb₁), D (Yb₂Yb₂), N (YnYn), K (YkYk) and λ (YfYf).

'Western' blots show that transferases J and K are immunochemically distinguishable from other transferases (Fig. 1 and Table 2). When excess anti-(transferase K) IgG is used in the blotting procedure, a small amount of cross-reactivity (less than 15%) is observed with Ya and

Table 1. Electrophoretic properties of glutathione *S*-transferases

For full experimental details see the text.

Transferase	Subunits	10 ⁻³ $\times$ Subunit M_r	Tissue source
K	YkYk	25.0	Rat liver
J	YkYk	25.0	Rat kidney
F	YaYa	25.5	Rat liver
L	YaYa	25.5	Rat liver
B	YaYc	25.5 and 28.5	Rat liver
AA	YcYc	28.5	Rat liver
A	Yb ₁ Yb ₁	27.0	Rat liver
C	Yb ₁ Yb ₂	27.0	Rat liver
D	Yb ₂ Yb ₂	27.0	Rat liver
P	Yb ₂ Yn	26.5 and 27.0	Rat liver
S	Yb ₂ Yn	26.5 and 27.0	Rat liver
N	YnYn	26.5	Rat testis
R	Yb ₂ Yb ₃	27.0	Rat liver
λ	YfYf	24.5	Human lung

Fig. 1. Immuno-blot analysis of purified glutathione *S*-transferase

Portions (20 μ g) of various transferases, which had been applied to SDS/polyacrylamide gels in the same order, were resolved by electrophoresis and transferred to nitrocellulose paper. The nitrocellulose papers were washed with (a) anti-(transferase K), (b) anti-(transferase L), (c) anti-(transferase AA), (d) anti-(transferase N) and (e) anti-(transferase D) antisera. The polypeptides that cross-reacted were detected with the Bio-Rad goat anti-(rabbit IgG) antibody-horseradish-peroxidase conjugate immuno-blot assay kit. In each panel the same protein samples were blotted. The order of samples, their loadings and the orientation of the paper are identical. The lanes on the immune replicas were obtained from polyacrylamide gels loaded as follows: lane 1, transferase B; lane 2, transferase J; lane 3, transferase K; lane 4, transferase F; lane 5, transferase L; lane 6, transferase B; lane 7, transferase AA; lane 8, transferase N; lane 9, transferase A; lane 10, transferase D. The top of the nitrocellulose paper represents the origin of the SDS/polyacrylamide gel.

Table 2. Cross-reactivity of glutathione *S*-transferases with 'Western'-blot analysis

Equal portions (10 μ g) of glutathione *S*-transferases were subjected to SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper by the method of Towbin *et al.* (1979). Immunochemical analysis was performed as described in the Materials and Methods section. Normally a 75–200 μ l portion of the primary antiserum (1/900–1/200 dilution) was sufficient to provide a strong peroxidase stain when the immunogen was examined in the assay system. Faint positive results, which were only observed when a 5- or 10-fold excess of a particular primary antibody was employed, are reported as $\pm$.

			Intensity of peroxidase staining on 'Western' blot							
Transferase	Group	Antiserum . . .	Anti-K	Anti-F	Anti-L	Anti-AA	Anti-A	Anti-D*	Anti-N	Anti- λ †
K	I		+++++	$\pm$	$\pm$	—	—	—	—	—
J	I		+++++	—	—	—	—	—	—	—
F	I		$\pm$	+++++	+++++	—	—	—	—	—
L	I		$\pm$	+++++	+++++	—	—	—	—	—
B	I		$\pm$	+++++	+++++	+++++	—	—	—	—
AA	I		$\pm$	$\pm$	$\pm$	+++++	—	—	—	—
A	II		—	—	—	—	+++++	$\pm$	+++++	—
C	II		—	—	—	—	+++	+++	+++++	—
D	II		—	—	—	—	$\pm$	+++++	+++	—
P	II		—	—	—	—	+++++	+	+++++	—
S	II		—	—	—	—	++	+++++	+++++	—
N	II		—	—	—	—	+++	+	+++++	—
R	II		—	—	—	—	++	+++++	+++++	—
λ †	III		—	—	—	—	—	—	—	+++++

* Results for anti-(transferase D) IgG have been published previously (Hayes, 1984).

† Though transferase λ was purified from human lung, antisera raised against this enzyme display strong cross-reactivity with Yf subunits isolated from rat tissues (Figs 2c and 3c). Homogeneous YfYf protein from the rat was not available and therefore is not included in this Table.

Yc subunits, but no cross-reactivity with Yb₁, Yb₂ or Yn subunits. A small amount of cross-reactivity of transferases F and L (both are YaYa proteins) with anti-[transferase AA (YcYc)] serum is apparent in Fig. 1(c). However, the blot shows that this is due to small amounts of contaminating Yc monomer. A Coomassie Blue stain of the SDS/polyacrylamide gel after electrophoresis showed that this contaminant represented less than 2% of the protein sample.

Considerable difficulty was experienced in obtaining specific antisera against transferase A (Yb₁Yb₁); a total of nine rabbits were immunized with Yb₁Yb₁, and from these only two sets of antisera showed no cross-reactivity with Yb₂Yb₂. Although anti-[transferase D (Yb₂Yb₂)] serum showed very little cross-reactivity with Yb₁, and only about 15% cross-reactivity with Yn, antiserum raised to YnYn or Yb₁Yb₁ did not display similar selectivity (Table 2). The fact that Yn-type and Yb-type subunits are resolved during SDS/polyacrylamide-gel electrophoresis allows them to be discriminated after 'Western' blotting.

The anti-[transferase λ (YfYf)] IgG did not cross-react with the Yk-, Ya- or Yc-containing enzymes (group I transferases) nor the Yn-, Yb₁- or Yb₂-containing enzymes (group II transferases). However, the anti-transferase λ IgG cross-reacted strongly with a polypeptide in the rat that specifically binds *S*-hexylglutathione-Sepharose 6B. Moreover, this polypeptide from the rat co-migrates during SDS/polyacrylamide-gel electrophoresis with transferase λ from human lung (Hayes, 1986). Hence the immunochemical data presented allow these enzymes to be divided into three distinct types.

It was found that, over a period of 16 months' storage

[in 10 mM-sodium phosphate buffer, pH 6.7, containing 1% SDS and 2% (v/v) 2-mercaptoethanol], transferases A, N and λ yielded lower-*M_r* fragments, which were detected following immuno-blotting. Conversely, during the same period, transferases B and AA produced higher-*M_r* aggregates. The appearance of these additional polypeptide bands was time-dependent, but the rate of production was reduced by storage at -20°C .

Organ distribution of transferases

As the transferases represent only a small proportion of the total protein in certain extrahepatic tissues, it was necessary to concentrate the transferase fraction by affinity chromatography to avoid false negative results. The portion of protein in different tissues that specifically bound to the *S*-hexylglutathione-Sepharose 6B gel varied markedly. A significant portion of the transferase activity in each organ that failed to bind to the *S*-hexylglutathione-Sepharose 6B (and was eluted in the flow-through fractions) was subsequently purified by glutathione affinity chromatography. The amount of transferase purified by the second affinity step varied from organ to organ.

Considerable differences exist in the transferase activity obtained from each organ. Table 3 shows the specific activities of the cytosols and the various pooled fractions from the affinity columns. It is noticeable that the specific activity of the *S*-hexylglutathione-affinity-purified enzymes is significantly higher for 1-chloro-2,4-dinitrobenzene than that of the glutathione-affinity-purified transferases, whereas smaller differences were observed with ethacrynic acid as substrate. The high ratio of the specific activity for ethacrynic acid to the

Table 3. Glutathione *S*-transferase activity in extrahepatic tissues and analysis of fractions purified by affinity chromatography

The cytosolic transferases present in various rat organs were purified by sequential *S*-hexylglutathione and glutathione affinity chromatography. Dialysed cytosol was applied to *S*-hexylglutathione-Sepharose 6B, and the breakthrough material from this matrix was directly applied to glutathione-Sepharose 6B as described in the text. The transferase activity in the fractions specifically eluted from these affinity columns was measured with 1-chloro-2, 4-dinitrobenzene (CDNB) and ethacrynic acid (EA) as substrates. These electrophiles were chosen to help establish whether transferase K, which has a high activity for ethacrynic acid and relatively low activity for 1-chloro-2,4-dinitrobenzene (Hayes, 1986), is preferentially recovered in the pools obtained from glutathione-Sepharose 6B. Specific activities are quoted as μmol of product formed/min per mg of protein.

Tissue	Pool . . . Cytosol			<i>S</i> -Hexylglutathione-affinity-purified			Glutathione-affinity-purified		
	Protein* (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)		Protein (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)		Protein (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	
		With CDNB	With EA		With CDNB	With EA		With CDNB	With EA
Liver	—	3.56	0.026	—	—	—	—	—	—
Kidney	1004	0.35	0.037	9.1	25.7	5.14	4.5	2.11	2.13
Lung	1168	0.20	0.028	6.3	37.9	5.82	4.1	4.91	5.41
Heart	944	0.09	0.012	2.4	42.2	2.90	3.9	0.87	1.24
Spleen	1370	0.08	0.010	6.0	18.0	4.50	4.4	3.97	2.03
Testis	620	2.93	0.062	25.5	66.1	3.41	2.0	5.00	1.50
Thymus	392	0.09	0.016	1.4	24.0	5.00	3.9	2.40	0.78
Small intestine	232	0.25	0.011	—	—	—	4.3	5.10	1.01
Colon	189	0.17	0.009	—	—	—	4.4	0.60	0.71

* The protein value given in the cytosol pool is the amount of kidney, lung, heart, spleen, testis or thymus extracts applied to *S*-hexylglutathione-Sepharose 6B. In the case of small intestine and colon the cytosol was applied directly to glutathione-Sepharose 6B.

specific activity for 1-chloro-2,4-dinitrobenzene in the glutathione affinity pool suggests that this fraction is enriched for transferase K [see Hayes (1986) for substrate specificity of transferase K]. However, it should be noted that considerably more ethacrynic acid-GSH-conjugating activity was recovered from the pools of purified enzyme than was predicted from the cytosolic activities (Table 3). The reason for this apparent discrepancy is not clear.

The polypeptides isolated by the two affinity steps were resolved by SDS/polyacrylamide-gel electrophoresis. Immuno-blots of the SDS/polyacrylamide gels show that, although transferase K can be detected in all the organs examined, it is present in highest concentration in lung and in modest amounts in kidney, heart and spleen (Figs 2*b* and 3*b*). The transferase pool obtained by glutathione affinity chromatography was enriched for the Yk monomer, but smaller amounts were found in the *S*-hexylglutathione-affinity-purified pools from kidney tissue, and only trace amounts in the *S*-hexylglutathione-affinity-purified pools from other organs. Thus the selective nature of the two affinity matrices appears to operate with transferase K from all tissues. Immuno-blots obtained with the anti-[transferase λ (YfYf)] IgG showed that Yf, unlike Yk, was preferentially recovered in the *S*-hexylglutathione-Sepharose-6B-purified pool. Moreover, anti-(transferase λ) gave a strikingly different organ distribution pattern from that observed for Yk, Ya or Yc (Figs 2*c* and 3*c*). Marked differences were observed in the Ya content of different organs. Large amounts of Ya-type subunits were recovered from kidney and small intestine, whereas only traces were detected in lung, heart, spleen, testis, thymus and colon (Figs 2*d* and 3*d*). The

organ differences in Yc content (Figs 2*e* and 3*e*) were less extreme than noted for Ya, but a tissue-specific expression was nevertheless observed.

Similar experiments with antisera against Yb₁Yb₁ and Yb₂Yb₂ (group II enzymes) are shown in Fig. 4. These immuno-blots show that significantly more Yb₁ than Yb₂ is expressed in spleen and thymus. Conversely, marginally more Yb₂ than Yb₁ is found in both kidney and small intestine (Table 4).

Cross-reactivity of anti-(transferase K) IgG with cytosolic proteins

The cytosols from liver, kidney, small intestine, colon, spleen, lung, heart, thymus and testis were probed with anti-(transferase K) serum, after SDS/polyacrylamide-gel electrophoresis and transfer to nitrocellulose paper, to determine whether additional proteins existed that were immunochemically related to transferase K. Fig. 5 shows the presence of major cytosolic polypeptide(s) (M_r 28000–30000) in all the tissues examined except small intestine, which displays a highly significant cross-reactivity with the anti-(transferase K) serum. This polypeptide species was distinct from Yk and had a similar electrophoretic mobility to the basic Yc monomer.

Immuno-blotting the same cytosols with anti-(transferase YcYc) serum showed a different pattern of cross-reactivity. Likewise, similar immuno-blots with anti-(transferase YaYa), anti-(transferase Yb₁Yb₁) or anti-(transferase Yb₂Yb₂) sera showed distinct patterns (results not shown), suggesting that the species that cross-reacts with anti-(transferase YkYk) is a new polypeptide. The high degree of cross-reactivity and size

Fig. 2. Immuno-blot analysis of the glutathione *S*-transferase pools purified by affinity chromatography from kidney, lung, heart, small intestine and colon

The cytosolic transferases from a variety of extrahepatic tissues were purified by two sequential affinity steps as described in the text. Panel (a) shows the SDS/polyacrylamide gel stained with Coomassie Blue R250 for protein. Gels to which similar amounts of protein had been applied were blotted to nitrocellulose papers and washed with: (b) anti-[transferase K (YkYk)] IgG; (c) anti-[transferase λ (YfYf)] IgG; (d) anti-[transferase L (YaYa)] IgG; (e) anti-[transferase AA (YcYc)] IgG. Lanes 2–10 of the gel and all the immune replicas (a–e) contain identical samples. Lane 1 contains transferase K in panels (a), (b), (d) and (e), but transferase λ was applied in this position in panel (c). Portions of transferases were applied as follows: lane 1, 15 μ g of transferase K (or λ in c); lane 2, 30 μ g of kidney transferases from *S*-hexylglutathione–Sepharose pool; lane 3, 15 μ g of kidney transferases from glutathione–Sepharose pool; lane 4, 30 μ g of lung transferases purified by *S*-hexylglutathione–Sepharose affinity chromatography; lane 5, 15 μ g of lung transferases purified by glutathione–Sepharose affinity chromatography; lane 6, 25 μ g of heart transferases from *S*-hexylglutathione–Sepharose pool; lane 7, 10 μ g of heart transferases from glutathione–Sepharose pool; lane 8, 15 μ g of transferases from small intestine isolated by glutathione–Sepharose affinity chromatography; lane 9, 10 μ g of transferases from colon isolated by glutathione–Sepharose affinity chromatography; lane 10, 10 μ g of transferases B.

Fig. 3. Immuno-blot analysis of the glutathione *S*-transferases from spleen, testis and thymus

The transferases from spleen, testis and thymus were analysed as described in the legend to Fig. 2. Panels (a), (b), (c), (d) and (e) show respectively the polyacrylamide gel and blots developed with anti-(transferase K), anti-(transferase λ), anti-(transferase L) and anti-(transferase AA). Portions of transferases, loaded similarly in each panel, were applied as follows; lane 1, 15 μ g of transferase K (or λ in panel c); lane 2, 45 μ g of spleen transferases from *S*-hexylglutathione-Sepharose pool; lane 3, 15 μ g of spleen transferase from glutathione-Sepharose pool; lane 4, 40 μ g of testis transferases purified by *S*-hexylglutathione-Sepharose affinity chromatography; lane 5, 10 μ g of testis transferases purified by glutathione-Sepharose affinity chromatography; lane 6, 40 μ g of thymus transferases from *S*-hexylglutathione-Sepharose pool; lane 7, 10 μ g of thymus transferases from glutathione-Sepharose pool; lane 8, 15 μ g of transferases from small intestine; lane 9, 10 μ g of transferases from colon; lane 10, 15 μ g of transferase B.

Fig. 4. Analysis of Yb₁ and Yb₂ content of rat tissues

The glutathione *S*-transferases from liver, kidney, lung, heart, spleen, testis and thymus were prepared by *S*-hexylglutathione affinity chromatography. The transferases from small intestine and colon were purified by glutathione affinity chromatography. Panels (a) and (b) show blots developed with anti-[transferase A (Yb₁Yb₁)] and anti-[transferase D (Yb₂Yb₂)] serum respectively. Portions (20 µg) of transferase, loaded similarly in each panel, were applied as follows: lane 1, liver transferases; lane 2, kidney transferases; lane 3, lung transferases; lane 4, heart transferases; lane 5, spleen transferases; lane 6, testis transferases; lane 7, thymus transferases; lane 8, small-intestine transferases; lane 9, colon transferases.

Table 4. Relative organ distribution of glutathione *S*-transferase subunits

The intensity of peroxidase staining was noted and assigned a relative score out of a possible total of 16.

Approx. percentage of cytosolic protein bound to affinity matrix . . . Polypeptide	Intensity of peroxidase staining								
	Liver 4	Kidney 1.2	Lung 0.9	Heart 0.4	Spleen 0.5	Testis 4.2	Thymus 0.7	Small intestine 1.6	Colon 2.0
Yc	+++	+++++	+++++	++	+++++	+++++	+++++	Trace	Trace
Yb ₁	+++++	Trace	++	+++	++	+++	+++	++	+++++
Yb ₂	+++++	+	++	+++	+	+++	++	++	+++++
Yn	Trace	—	—	++	+	+++++	+	—	—
Ya	+++++	+++++	Trace	Trace	Trace	Trace	Trace	+++++	Trace
Yk	+	++	+++	++	++	+	+	++	++
Yf	Trace	+++++	+++++	+++++	+++++	Trace	+++++	+++	+++++

of this polypeptide strongly suggest that it represents a further transferase monomer, presumably structurally related to transferase K. There may be more than one polypeptide.

DISCUSSION

The present study reports the application of 'Western' blotting to help elucidate the immunochemical relationship between individual glutathione *S*-transferases and to establish their distribution in extrahepatic tissues.

We have used antisera to challenge homogeneous enzymes, purified transferase mixtures and whole-tissue cytosols. Three types of experiment were employed. Firstly, 'Western' blotting was used to examine the cross-reactivity of homogeneous enzymes; this showed that transferase K can be immunochemically distinguished

from other enzymes (Table 2), and allowed identification of seven subunit types, Yf, Yk, Ya, Yn, Yb₁, Yb₂ and Yc.

The second way in which we used 'Western' blotting was in order to establish the tissue distribution of the Yf, Yk, Ya, Yn, Yb₁, Yb₂ and Yc subunits in fractions containing mixtures of affinity-purified transferases. These experiments demonstrated a distinct and marked tissue-specific distribution for these subunits (Table 4). Significant differences were also noted in the apparent *M_r* of the species in the *S*-hexylglutathione-affinity-purified pools and in the glutathione-affinity-purified pools that cross-reacted with anti-(transferase YaYa) IgG (Figs 2d and 3d); although the reason for these different electrophoretic mobilities is not known, it is not due to Yk or Yc subunits.

Figs 2 and 3 also show that the small intestine contains high concentrations of the Ya subunit but little of the Yc

Fig. 5. Identification of immunoreactive Yk- and Yc-type polypeptides in the cytosols of extrahepatic tissues

The existence of a new Yc-type polypeptide that cross-reacts with anti-(transferase YkYk) IgG but not with anti-(transferase YcYc) IgG was identified as follows. The 100000 *g* fraction was prepared from various tissues and, after SDS/polyacrylamide-gel electrophoresis, each cytosol was transferred to nitrocellulose paper (Towbin *et al.*, 1979). The nitrocellulose papers were washed with (a) anti-(transferase K) serum and (b) anti-(transferase AA) serum, and the cross-reacting polypeptides were detected by using goat anti-(rabbit IgG) antibodies conjugated with horseradish peroxidase. The lanes on the immune replicas were obtained from polyacrylamide gels loaded as follows: lane 1, 15 μ g of immunogen (i.e. either transferase K or transferase AA); lane 2, 265 μ g of liver cytosol; lane 3, 350 μ g of kidney cytosol; lane 4, 600 μ g of small-intestine cytosol; lane 5, 500 μ g of colon cytosol; lane 6, 700 μ g spleen cytosol; lane 7, 700 μ g of lung cytosol; lane 8, 700 μ g of heart cytosol; lane 9, 600 μ g thymus cytosol; lane 10, 400 μ g of testis cytosol. The top of the paper represents the origin of the SDS/polyacrylamide gel, and peptides were run towards the anode (bottom).

subunit. Our data therefore indicate that the cross-reactivity of intestinal cytosol with antisera raised against transferase B (YaYc protein), reported by Pinkus *et al.* (1977), is due to the presence of either transferase F or L, or both, but not transferase B (YaYc) or AA (YcYc). However, it is still unclear why Levi *et al.* (1969) and Clifton & Kaplowitz (1977) failed to identify 'ligandin' (i.e. Ya-type subunit activity) in intestinal cytosol with a bromosulphthalein-binding assay. The present study has also shown that the Ya subunit is absent from, or present in only trace amounts in, the lung. It is therefore unlikely that the pulmonary transferase with pI 7.2 isolated by Singh *et al.* (1984) comprises YaYa monomers as proposed by these workers. A more likely suggestion is that this enzyme contains Yf-type subunits.

Cytosols from various organs were probed with antiserum against transferase K, in a third set of experiments using 'Western' blotting, to identify polypeptides that are related to Yk and which may represent additional transferase subunits. These experiments showed the existence of a new major polypeptide, present in all tissues examined except small intestine, that cross-reacted strongly with anti-(transferase YkYk) IgG. This species exhibits an electrophoretic mobility similar to that of the Yc-type subunit(s) (M_r 28000–30000). Although it is distinct from the basic Yc monomer, which possesses selenium-independent glutathione peroxidase activity, it may represent a new Yc-type transferase subunit.

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