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Isoenzymes of glutathione transferase in rat small intestine

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The major glutathione transferases in the rat small-intestine cytosol were isolated and characterized. The enzymes active with 1-chloro-2,4-dinitrobenzene as second substrate were almost quantitatively recovered after affinity chromatography on immobilized *S*-hexylglutathione. The different basic forms of glutathione transferase, which account for 90% of the activity, were resolved by chromatofocusing. Fractions containing enzymes with lower isoelectric points were not further resolved. The isolated fractions were characterized by their elution position in chromatofocusing, apparent subunit M_r, reactions with specific antibodies, substrate specificities and inhibition characteristics. The major basic forms identified were glutathione transferases 1–1, 4–4 and 7–7. In addition, evidence for the presence of a variant form of subunit 1, as well as trace amounts of subunits 2 and 3, was obtained. A significant amount of transferase 8–8 in the fraction of acidic enzyme forms was demonstrated by immunoblot and Ouchterlony double-diffusion analysis. In the comparison of the occurrence of the different forms of glutathione transferase in liver, lung, kidney and small intestine, it was found that the small intestine is the richest source of glutathione transferase 7–7.

INTRODUCTION

The glutathione transferases (GSTs) constitute a family of detoxification enzymes that catalyse the reaction of GSH with numerous electrophiles. Thus they play an essential role in biotransformation of xenobiotics and are believed to be involved in carcinogen metabolism (Chasseaud, 1979; Smith & Litwack, 1980; Mannervik, 1985). The enzymes are present in a variety of tissues and, in most instances, they occur in multiple forms. The most extensively studied tissue is rat liver, in which the predominant forms have basic isoelectric points (Ålin *et al.*, 1985). The proteins are dimeric, and the major isoenzymes in liver cytosol have been described as binary combinations of four different subunits (Mannervik & Jensson, 1982). A systematic nomenclature for these enzymes has been proposed that reflects the subunit composition of the isoenzymes (Jakoby *et al.*, 1984). On the basis of our studies of the enzymes in man (Warholm *et al.*, 1983), mouse (Warholm *et al.*, 1986) and rat (Guthenberg *et al.*, 1983, 1985*a*; Ålin *et al.*, 1985; Jensson *et al.*, 1986) the cytosolic GSTs have been grouped into three species-independent classes, which are recognized by similarities in substrate specificities, sensitivities to inhibitors, cross-reactivity with antisera and homologies in amino sequences (Mannervik *et al.*, 1985, 1987).

The distribution of GSTs in different tissues is not uniform. Certain forms that are absent in one organ may be present as major components in another tissue. For example, rat GST 6–6, which is not present in significant amounts in most tissues, constitutes 50% of the transferase activity in the cytosol from rat testis (Guthenberg *et al.*, 1983, 1985*b*). GST 7–7, which is hardly detectable in normal rat liver, has been shown to be present in kidney (Guthenberg *et al.*, 1985*a*), lung (Robertson *et al.*, 1985) and placenta (Sato *et al.*, 1984). The trace amounts of this form present in liver cytosol can be largely attributed to its presence in the bile duct (Hayes *et al.*, 1987; Power *et al.*, 1987; Sato *et al.*, 1987). GST 7–7 occurs in high concentration in chemically induced preneoplastic rat hepatocyte nodules (Satoh *et al.*, 1985*a*; Jensson *et al.*, 1985) and in rat primary hepatomas (Meyer *et al.*, 1985). The same protein is expressed at high concentrations in many cancer cells and has been proposed to be used as a marker for preneoplastic tissues (Satoh *et al.*, 1985*a*; Sato *et al.*, 1987).

The small intestine is a primary site of metabolism and entry into the organism of ingested xenobiotics. GSTs have been shown to be present in the gastrointestinal tract (Pinkus *et al.*, 1977; Clifton & Kaplowitz, 1978; Hayes & Mantle, 1986) and to have an approx. 30-fold higher concentration in the upper small intestine than in the terminal ileum (Ogasawara *et al.*, 1985). However, no definitive study of the enzyme forms present has been carried out. The present work describes the composition of GSTs in rat small intestine. A significant finding was that GST 7–7 is one of the major enzyme forms in small intestine, accounting for approx. 40% of the total cytosolic enzyme activity. Thus this tissue appears to be the richest source of GST 7–7 among the normal rat tissues studied so far.

MATERIALS AND METHODS

S-Hexylglutathione was synthesized and linked to epoxy-activated Sepharose 6B as described by Mannervik & Guthenberg (1981). Chromatography materials were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were standard commercial products.

Abbreviations used: GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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Table 1. Purification of GSTs from rat small intestine

Activity was determined with CDNB as substrate. The amount of protein was determined by the method of Kalckar (1947).

Fraction	Volume (ml)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min per mg)	Yield (%)
Cytosol	930	1860	16 440	0.11	(100)
Sephadex G-25	932	1864	5517	0.34	100
S-Hexylglutathione–Sepharose 6B + Sephadex G-25	405	1520	67.4	22.5	81.5

Male Sprague–Dawley rats weighing 200–250 g were killed by decapitation and the small intestine was removed from each. The intestine was rinsed with ice-cold iso-osmotic saline, cut into small pieces and homogenized at 0 °C in 0.25 M-sucrose containing 1 mM-EDTA and 0.2 mM-dithioerythritol in an Ultra-Turrax homogenizer for 2 min. The homogenate (25%, w/v) was centrifuged at 105000 *g* for 1 h in order to obtain a microsome-free supernatant fraction. Floating lipids were removed by filtration through cotton. Further purification and characterization were performed as described earlier (Ålin *et al.*, 1985).

The cytosol fraction was passed through Sephadex G-25 equilibrated with Tris/HCl buffer pH 7.8 containing 1 mM-EDTA and 0.2 mM-dithioerythritol (buffer A). Active fractions were applied on *S*-hexylglutathione-coupled affinity matrix equilibrated with buffer A. After the column had been washed with 2 bed volumes of buffer A containing 0.2 M-NaCl, the activity was eluted with buffer A containing 5 mM-*S*-hexylglutathione in addition to 0.2 M-NaCl. The eluent was passed through Sephadex G-25 equilibrated with buffer A without EDTA to remove NaCl and *S*-hexylglutathione. The pool of active fractions was concentrated and applied on Mono P for chromatofocusing in the pH range 10.5–7.5 by f.p.l.c. Activity remaining bound to Mono P was eluted with 2 M-NaCl.

The different purification steps were followed by assaying enzyme activity with 1-chloro-2,4-dinitrobenzene (CDNB) as the second substrate. Measurement of activity with other substrates and inhibition studies were made as previously described (Ålin *et al.*, 1985). The protein concentration in fractions obtained during purification was determined by measurement of A_{280} and A_{260} (Kalckar, 1947), whereas the concentrations of purified isoenzymes were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. SDS/polyacrylamide-gel electrophoresis was performed according to the method of Laemmli (1970), and transfer to nitrocellulose paper and immunoblot analysis were performed as described by Berzins *et al.* (1983).

RESULTS

The GST activity of the small-intestine cytosol fraction with CDNB as electrophilic substrate was about 0.1 μmol/min per mg of protein. This cytosolic specific activity is similar to that of rat lung (Robertson *et al.*, 1985) and is comparatively much lower than that of liver (approx. 1 μmol/min per mg). Affinity chromatography resulted in a 200-fold concentration of the enzyme (Table 1). Chromatofocusing on a Mono P column in the pH interval 10.5–9 gave rise to a single major peak at pH 9.5 (peak I) with a trailing shoulder (peak Ia) (Fig. 1). There was no separation of components corresponding to GST 1–2 or GST 2–2, which are eluted in this pH range in the purification of the liver enzymes. Chromatofocusing with the second gradient from pH 9 to pH 7.5 resolved two major peaks of activity at pH 8.7 (peak II) and 8.2 (peak III) (Fig. 1). Peak II was preceded by a minor shoulder (peak IIa). On the basis of their pH of elution, peaks I, II and III can be expected to contain GST 1–1, GST 3–4 or 7–7 and GST 4–4 respectively. The peaks were further analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2). Peak I contained a subunit with an apparent M_r of 25000. The fractions corresponding to the trailing shoulder on the first peak were separately analysed. These fractions (peak Ia) showed a strong band with an apparent M_r value of 25000 and traces of a band corresponding to M_r 28000 (not visible in Fig. 2). The component in peak II demonstrated the highest electrophoretic mobility among the rat transferases. The

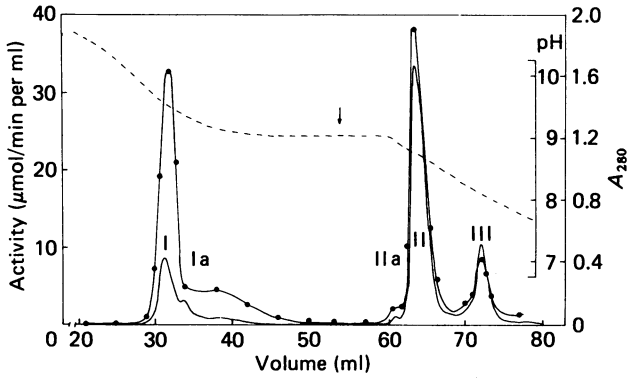

Fig. 1. Separation of basic GSTs from rat small intestine by chromatofocusing

S-Hexylglutathione-affinity-purified material was run by f.p.l.c. on a Mono P chromatofocusing column. The elution was made by application of two pH gradients (----) (for details see the text). The arrow indicates the point of change of the eluent. ——, A_{280}; ●—●, GST activity.

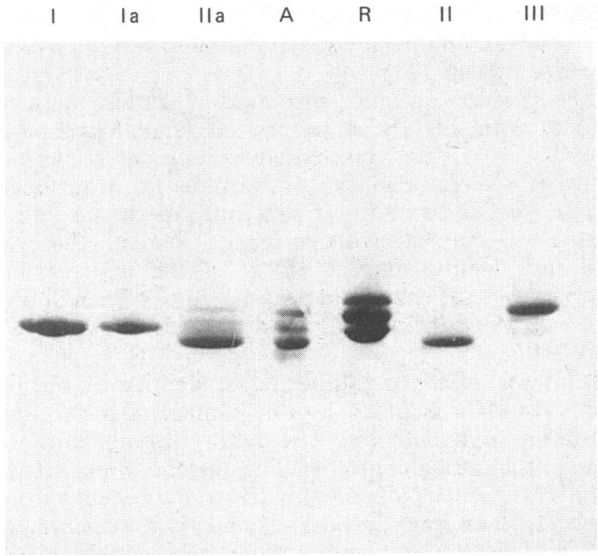

Fig. 2. SDS/polyacrylamide-gel electrophoresis of different GSTs containing fractions obtained by chromatofocusing

The purified transferases after chromatofocusing (cf. Fig. 1) were loaded on an SDS/polyacrylamide slab gel and run by the method described by Laemmli (1970). The protein bands were stained by Coomassie Brilliant Blue G. The order of samples from left to right is as follows: peak I (I); eluted material (concentrated) in different fractions in the shoulder on peak I (Ia), showing traces of subunit 2; eluted material (concentrated) in fractions in the shoulder preceding peak II (IIa), showing traces of subunit 3/4; *S*-hexylglutathione-affinity-purified enzyme from small intestine (A); *S*-hexylglutathione-affinity-purified material from rat liver cytosol (R); peak II (II); peak III (III).

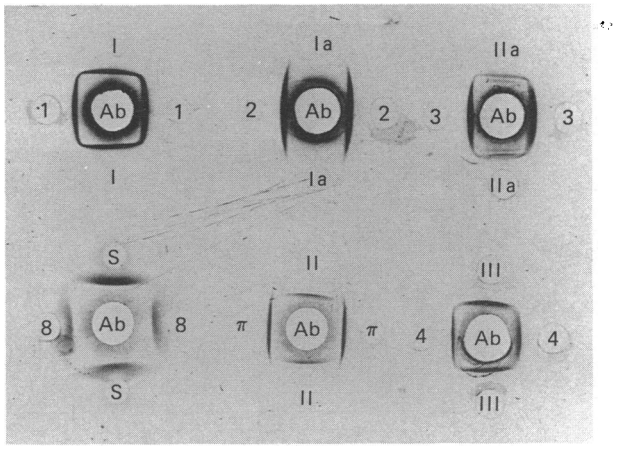

Fig. 3. Ouchterlony double-diffusion analysis of different forms of GST purified from rat small intestine

Antisera (Ab) against rat GST 1–1, GST 2–2, GST 3–3, GST 4–4, and GST 8–8 and human GST π (purified from placenta) were placed in central wells, and reference antigens were loaded horizontally. Purified transferases from small intestine were applied in wells above and below the central well. Fractions in peaks Ia and IIa were checked against antibodies against GST 2–2 and GST 3–3 to detect the possible presence of subunits 2 and 3 respectively. Antiserum against human GST π, which cross-reacts with rat GST 7–7, was used to detect GST 7–7. S represents GSTs eluted with 2 M-NaCl (for details see the text).

apparent subunit M_r was 24000. Peak IIa contained the same component as the major peak, but also included trace amounts of a protein with M_r 26500. Peak III appeared to be homogeneous and composed of subunits with apparent M_r 26500.

In Ouchterlony double-diffusion analysis (Fig. 3) peak I gave a precipitin line only with antiserum against GST 1–1 purified from rat liver (Table 2). The small amount of subunit 2 indicated by gel electrophoresis (Fig. 2) in fractions corresponding to peak Ia was insufficient to give a precipitin line with antiserum against rat GST 2–2 (Fig. 3); an identity reaction was obtained with GST 1–1, as found with the main peak (results not shown). Fractions of peak IIa showed a faint precipitin line with anti-(GST 3–3) antibodies. The proteins in peaks II and III gave positive reaction with antisera against rat GST 7–7 and GST 4–4 respectively. Immuno-cross-reactivity has been observed between different members of the same class of GSTs (Mannervik *et al.*, 1985). Peak II

Table 2. Physical and immunochemical properties of purified GSTs from small intestine

Property	Peak after chromatofocusing...	I	(1–1)*	II	(7–7)†	III	(4–4)*
Apparent subunit M_r		25 000	25 000	24 000	24 000	26 500	26 500
pH of elution in chromatofocusing on f.p.l.c.		9.5	> 9.0	8.7	8.7	8.2	8.3
Precipitin reaction with antiserum against:							
Rat GST 1–1		+	+	−	−	−	−
Rat GST 2–2		−	−	−	−	−	−
Rat GST 3–3		−	−	−	−	−	−
Rat GST 4–4		−	−	−	−	+	+
Human GST π		−	−	+	+	−	−

* Data from Ålin *et al.* (1985).
† Data from Guthenberg *et al.* (1985*a*).

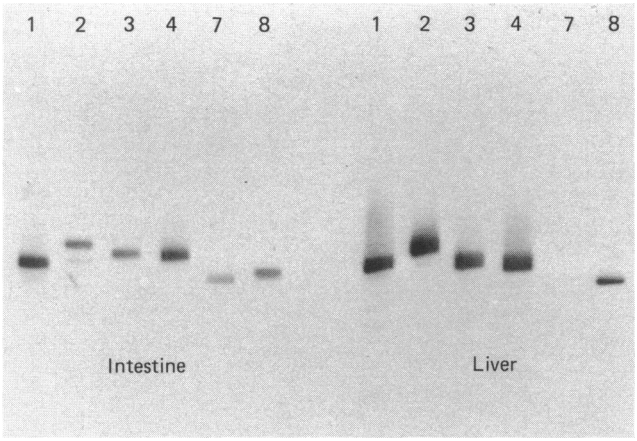

Fig. 4. Immunoblot analyses of enzymes purified from rat small intestine and liver by *S*-hexylglutathione affinity chromatography

Equal amounts of affinity-purified material from small intestine and liver were subjected to SDS/polyacrylamide-gel electrophoresis. The proteins were transferred on to nitrocellulose paper by a method described previously (Berzins *et al.*, 1983). Nitrocellulose paper was cut into strips and treated with different antisera. Immunocomplexes were radiolabelled with ^{125}I-Protein A and detected by autoradiography. The antisera used for detection of different subunits are labelled by the arabic numerals of corresponding enzyme subunits. The relative intensities of the protein bands depend on the antisera, and comparisons can be made only between corresponding subunits in intestine and liver.

gave a strong precipitate with antiserum against GST π purified from human placenta (Guthenberg & Mannervik, 1981).

The enzyme eluted with 2 M-NaCl after chromatofocusing was not further purified because of the limited amounts of catalytically active protein recovered. However, immuno-double-diffusion experiments with anti-(GST 8–8) antibodies demonstrated the presence of subunit 8, or an immunologically related component, in this fraction. A comparison (by immunoblotting) of the relative amounts of different subunits of GSTs in the intestine with those in liver revealed a higher concentration of a component corresponding to subunit 8 in the intestine cytosol (Fig. 4).

The purified enzymes displayed maximum catalytic activity with CDNB as second substrate. Other compounds tested were class-differentiating substrates previously used to identify the various rat transferases (Table 3). The second-best substrate for the enzyme in peak I was cumene hydroperoxide, whereas ethacrynic acid was highly efficient (15% of the activity with CDNB) with the enzyme in peak II. *trans*-4-Phenylbut-3-en-2-one was a distinguishing substrate for the enzyme in peak III.

Inhibitors used to establish the identity of purified forms of GSTs included *S*-(*p*-bromobenzyl)glutathione, haematin, indomethacin, triethyltin bromide and triphenyltin chloride (Table 4). The enzyme in peak I was strongly inhibited by haematin and triphenyltin chloride, whereas the enzymes in peaks II and III were characteristically affected by *S*-(*p*-bromobenzyl)glutathione with I_{50} values of 2 μM and 6 μM respectively. Indomethacin selectively inhibited the enzyme present in peak III.

The substrate specificity and inhibition characteristics of peak Ia were also investigated with the compounds listed in Tables 3 and 4. No significant difference in the catalytic properties between peaks I and Ia were noted. The amount of enzyme in peak IIa was insufficient for similar studies.

DISCUSSION

The cytosolic GST activity per mg of protein with CDNB in intestine is significantly lower than in liver (Ålin *et al.*, 1985) and testis (Guthenberg *et al.*, 1983) and of a similar magnitude to the values for kidney (Guthenberg *et al.*, 1985*a*) and lung (Robertson *et al.*, 1985). Most of the transferases active with CDNB were bound to the *S*-hexylglutathione affinity matrix. Rat GST 5–5 does not bind to this affinity matrix (Meyer *et al.*, 1984) and has very low activity with CDNB. As the whole purification has been monitored with this substrate, it is difficult to say anything about the presence of GST 5–5 in the rat intestine cytosol.

Chromatofocusing in the pH interval 10.5–7.5 resolved three major peaks of activity towards CDNB. All these

Table 3. Relative substrate specificities of purified GSTs from rat small intestine

Absolute specific activities (μmol/min per mg) with CDNB as substrate are given in parentheses. Abbreviation: N.D., not detectable.

Substrate	Peak after chromatofocusing...	Relative activity (% of activity obtained with CDNB)					
		I	1–1*	II	7–7†	III	4–4*
CDNB		100 (66)	100 (50)	100 (20)	100 (24)	100 (14)	100 (17)
Cumene hydroperoxide		5	6	0.4	0.2	3.4	4
Ethacrynic acid		0.3	0.2	14	16	6	4
trans-4-Phenylbut-3-en-2-one		N.D.	0.008	0.4	0.9	7	7

* Data from Ålin *et al.* (1985).
† Data from Guthenberg *et al.* (1985*a*).

Table 4. I_{50} values for GSTs from rat small intestine

Abbreviation: N.A., not available.

Inhibitor	Peak after chromatofocusing... I	(1–1)*	II	(7–7)†	III	(4–4)*
Triethyltin bromide	200	350	95	100	63	70
Triphenyltin chloride	0.8	0.5	10	10	0.9	0.2
Haematin	0.04	0.05	5	4	0.5	1
S-(*p*-Bromobenzyl)glutathione	80	50	2	1	6	6
Indomethacin	40	50	> 100‡	N.A.	0.5	1

* Data from Ålin *et al.* (1985).
† Data from Guthenberg *et al.* (1985*a*).
‡ 18% inhibition.

Table 5. Relative amounts of GST subunits 1, 2, 3, 4 and 7 in different rat tissues

The relative amounts of the various subunits in intestine (present work), kidney (Guthenberg *et al.*, 1985*a*), liver (Ålin *et al.*, 1985) and lung (Robertson *et al.*, 1985) were scored (− to +++) by comparing the protein elution profiles of f.p.l.c. separations of affinity-purified cytosol fractions.

Subunit	Tissue...	Relative amount of subunit: Intestine	Kidney	Liver	Lung
1		++	+++	+++	−
2		+	+++	++	++
3		+	+	+++	++
4		++	++	+++	+++
7		+++	++	−	++

forms appear to be dimers of identical subunits. Peak I was identified as GST 1–1 on the basis of its pH of elution in chromatofocusing, relative mobility on SDS/polyacrylamide-gel electrophoresis, high Se-independent peroxidase activity and characteristic I_{50} values with selective inhibitors. The enzyme present in peak II has no counterpart in normal liver; GST 3–4 is eluted at this position in chromatofocusing (Ålin *et al.*, 1985). However, the apparent M_r of the protein in peak II (M_r 24000) is lower than that for subunits 3 and 4 (M_r 26500). On the basis of its physical, immunochemical and catalytic properties, the enzyme in peak II can be identified as GST 7–7, previously purified from kidney (Guthenberg *et al.*, 1985*a*) and lung (Robertson *et al.*, 1985). This enzyme is highly efficient in the conjugation of GSH with (+)-9α,10α-oxy-7,8,9,10-tetrahydro-7β,8α-dihydroxy-benzo[*a*]pyrene (Robertson *et al.*, 1986). In the rat subunit 7 has been found exclusively in homodimeric form, whereas heterodimers of subunits 1 and 2 or subunits 3 and 4 have been identified. In fact, no heterodimeric structures have been observed among the class Pi GSTs in the different species studied so far.

Peak III has the properties characteristic of GST 4–4 (Ålin *et al.*, 1985). Its relative M_r, reaction with antibodies against GST 4–4, substrate specificity and inhibition characteristics strongly indicate its identity with GST 4–4. This enzyme is present in most tissues investigated, but certain chemically induced preneoplastic hepatocyte nodules do not contain significant amounts of GST 4–4 (Jensson *et al.*, 1985).

In addition to the above-mentioned three major forms, small amounts of subunits 2 and 3 were also detected in the cytosolic fractions from the small intestine. The shoulder on peak I (peak Ia) contains some subunit 2, but the major component has the properties of subunit 1 except for its pH of elution. Previously, kinetic (Sheehan & Mantle, 1984) and chromatographic data (Ostlund Farrants *et al.*, 1987), as well as electrophoretic studies with polyclonal (Satoh *et al.*, 1985*b*) and monoclonal antibodies (Wang *et al.*, 1986), have indicated multiplicity of subunit 1 (Ya) in the rat. Primary structure analyses (Beale *et al.*, 1982; Pickett *et al.*, 1984; Lai *et al.*, 1984) also indicate the existence of two variant forms (for a review see Mannervik, 1985). The data presented in this paper support the interpretation that two discrete forms of subunit 1 exist also in the intestine. The small peak preceding peak II (peak IIa) can be identified as GST 3–3 and/or 3–4 from its position in chromatogram.

The immunocomplexes of proteins in the sample of affinity-purified small intestine cytosol and antibodies directed against GST 2–2 and GST 3–3 indicated relatively high concentrations of the corresponding subunits (Fig. 4). This finding appears to contrast with the relatively low concentrations detected after chromatofocusing (Fig. 1). However, the immunoblot analysis is very sensitive and trace amounts of proteins may give significant bands after autoradiography, a property that makes quantification difficult.

Multiple forms of GST with isoelectric points in the near-neutral and acidic pH range have been purified from rat liver cytosol (H. Jensson & B. Mannervik, unpublished work). The presence of GST 8–8 (Jensson *et al.*, 1986) is worth mentioning. This enzyme is also present in kidney (Guthenberg *et al.*, 1985*a*), lung (Robertson *et al.*, 1985) and testis (C. Guthenberg & B. Mannervik, unpublished work). The present investigation indicates that significant amounts of subunit 8 are present in rat small intestine (Fig. 4). GST 8–8 (a class Alpha enzyme) is highly active with ethacrynic acid; its activity with 4-hydroxynon-2-enal, an endogenous product of lipid peroxidation, is even higher than that with CDNB.

A comparison of the estimated relative contents of GST subunits 1, 2, 3, 4 and 7 in rat kidney, liver and lung demonstrates that the enzyme pattern in small intestine is much different from those of other organs (Table 5). It is particularly noteworthy that the small intestine is the richest source of GST 7–7, considering the relative abundance of GST 7–7 in comparison with other enzyme forms (cf. Fig. 1) as well as the weight of the organ. The high concentration of GST 7–7 in the small intestine may signify a special detoxification function relevant to the gastrointestinal tract. Another interpretation is that a high GST 7–7 concentration is an expression of the rapid growth of the epithelial cells in the gut; cancer cells are other rapidly growing cells that often exhibit a high concentration of the class Pi GST (Mantle *et al.*, 1987). However, further investigations are required to distinguish between these or alternative explanations for the high concentrations of GST 7–7 in the rat small intestine.

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