Isoelectric Focusing of Glutathione S-Transferases from Rat Liver and Kidney

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The glutathione *S*-transferases that were purified to homogeneity from liver cytosol have overlapping but distinct substrate specificities and different isoelectric points. This report explores the possibility of using preparative electrofocusing to compare the composition of the transferases in liver and kidney cytosol. Hepatic cytosol from adult male Sprague-Dawley rats was resolved by isoelectric focusing on Sephadex columns into five peaks of transferase activity, each with characteristic substrate specificity. The first four peaks of transferase activity (in order of decreasing basicity) are identified as transferases AA, B, A and C respectively, on the basis of substrate specificity, but the fifth peak (pI 6.6) does not correspond to a previously described transferase. Isoelectric focusing of renal cytosol resolves only three major peaks of transferase activity, each with narrow substrate specificity. In the kidney, peak 1 (pI 9.0) has most of the activity toward 1-chloro-2,4-dinitrobenzene, peak 2 (pI 8.5) toward *p*-nitrobenzyl chloride, and peak 3 (pI 7.0) toward *trans*-4-phenylbut-3-en-2-one. Renal transferase peak 1 (pI 9.0) appears to correspond to transferase B on the basis of pI, substrate specificity and antigenicity. Kidney transferase peaks 2 (pI 8.5) and 3 (pI 7.0) do not correspond to previously described glutathione *S*-transferases, although kidney transferase peak 3 is similar to the transferase peak 5 from focused hepatic cytosol. Transferases A and C were not found in kidney cytosol, and transferase AA was detected in only one out of six replicates. Thus it is important to recognize the contribution of individual transferases to total transferase activity in that each transferase may be regulated independently.

The activity of the glutathione *S*-transferases (EC 2.5.1.18) from rat liver cytosol reflects the cumulative activity of each of a group of proteins, five of which have been purified to homogeneity (transferases AA, A, B, C and E; Fjellstedt *et al.*, 1973; Habig *et al.*, 1974; Pabst *et al.*, 1974; Habig *et al.*, 1976; Askelöf *et al.*, 1975). Although the purified transferases are all dimeric proteins of mol.wt. approx. 45000, they have been separated from each other by ion-exchange chromatography (Habig *et al.*, 1974; Jakoby *et al.*, 1976a,b). Except for transferases A and C, each is distinguishable both by immunological methods and by amino acid composition. Each transferase has a distinct isoelectric point (pI 7-10). Their specificities toward electrophilic substrates are broad and overlapping, yet distinctive for each form of the enzyme. By catalysing the conjugation of a variety of electrophilic compounds with glutathione, the transferases are thought to play an important role in xenobiotic detoxification (Chasseaud, 1973; Jakoby *et al.*, 1976a), and even toxification (Ahmed & Anders, 1976).

Other biological functions have also been attributed to the glutathione *S*-transferases in rat liver.

Transferase B, the predominant transferase in rat liver, is identical with ligandin, previously known as Y-protein, azocarcinogen-binding protein and cortisol-metabolite binder I (Litwack *et al.*, 1971); a role for this protein in the uptake, retention and/or secretion of organic anions by liver and kidney has been proposed (Levi *et al.*, 1969). In addition, transferase B can act as a glutathione peroxidase (Burk *et al.*, 1977; Prohaska & Ganther, 1977) and a 3-oxo Δ^5 -steroid isomerase (Benson *et al.*, 1977). Transferases AA, A and C also bind a broad spectrum of non-substrate ligands such as bilirubin and bromosulphophthalein (Ketley *et al.*, 1975).

The actual biological role(s) of these proteins has received much attention, but remains enigmatic. The usual approach to the question has been to correlate changes in transferase activity under various conditions, e.g. age, sex, tissue and exposure to xenobiotics or hormones, with alterations in function, e.g. detoxification or transport. An intrinsic assumption in most of these studies is that a change in any single measurement of the transferases, e.g. catalytic activity toward a given substrate, accurately gauges changes in the amount and activity of all of the

glutathione *S*-transferases. Data previously obtained in our laboratory indicate that this is not the case in rat liver. The proportion of transferase B relative to other transferases varies with sex and hypophysectomy (Hales & Neims, 1976a), age (Hales & Neims, 1976b) and pretreatment with 3-methylcholanthrene (Hales & Neims, 1977). Further, differences in substrate specificity suggest that the composition (identity and/or proportion) of the transferases depends also on tissue. Renal and hepatic cytosol exhibit major differences in transferase substrate specificity (Clifton *et al.*, 1975; Kaplowitz & Clifton, 1976; Kaplowitz *et al.*, 1976). To attempt to explain these findings, we have used preparative electrofocusing as a means of assessing and comparing the various forms of glutathione *S*-transferase that contribute to activity in male rat kidney and liver cytosols.

Experimental

Animals

Male Sprague-Dawley rats (200–250 g) obtained from the Canadian Breeding Laboratories (St. Constant, Quebec, Canada) were housed in the McIntyre Animal Centre (McGill University, Montreal, Quebec, Canada) and given water and Purina rat chow *ad libitum*. The animals were killed by decapitation. Excised livers and kidneys were homogenized (1:5, w/v) in ice-cold 20% (v/v) glycerol with a motor-driven Teflon pestle in a glass homogenizer. Each homogenate was centrifuged at 105000g for 60 min to prepare cytosol for use in enzyme assays and isoelectric focusing.

Enzyme assays

Assay conditions for measurement of the conjugation of 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Eastman Kodak Co., Rochester, NY, U.S.A.), *p*-nitrobenzyl chloride (K & K Laboratories, Plainview, NY, U.S.A.), *p*-nitrophenethyl bromide and *trans*-4-phenylbut-3-en-2-one (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were identical with those of Habig *et al.* (1974). Assays were conducted at room temperature (21–23°C) and were followed with a Beckman Acta III double-beam recording spectrophotometer or a Beckman model 35 recording spectrophotometer. Reaction rates were linear with protein concentration and time for more than 2 min.

Isoelectric focusing in Sephadex columns

The procedure of O'Brien *et al.* (1976) for isoelectric focusing in a Sephadex column was modified for use with the glutathione *S*-transferases. Sephadex G-75 (superfine grade) (10–40 µm pore size; Pharm-

acia, Uppsala, Sweden) was swollen in 20% (v/v) glycerol. The Sephadex was packed by gravity into a 1.6 cm inner-diameter water-jacketed glass column to a height of 36 cm by the continuous flow of at least 2 column volumes of 20% (v/v) glycerol containing 2% (v/v) Ampholines. The Ampholine mixture before dilution consisted of: 20% (v/v) Bio-Lyte 3/10 (40%, w/v, solution; Bio-Rad Laboratories, Mississauga, Ont., Canada); 40% (v/v) Bio-Lyte 8/10 (20%, w/v, solution; Bio-Rad Laboratories); and 40% (v/v) pHisolyte 9–11 (20%, w/v, solution; Brinkmann Instruments, Westbury, NY, U.S.A.) or 40% (v/v) Ampholine 9–11 (20%, w/v, solution; LKB, Fisher Scientific, Montreal, Canada). The occasional interchange of the Brinkmann and LKB 9–11 Ampholine preparations was necessitated by supply problems, and did not alter results. Immediately after preparation, samples (6–10 ml) of cytosol were adjusted to 2% (v/v) with the Ampholine mixture and applied to columns. Each sample was washed into the Sephadex column with approx. 20 ml of the Ampholine/glycerol solution described above, and a Millipore filter (0.45 µm) was placed on top of the column. The top of the column was then layered with 5 ml of acrylamide solution containing 14% (w/v) acrylamide, 0.3% (w/v) *NN'*-methylenebisacrylamide, 0.07% (w/v) ammonium persulphate and 0.5% (v/v) *NNN'*-tetramethylethylenediamine. After polymerization, the column was inverted and the procedure was repeated to plug the other end of the column. The lower end of the column was immersed into the anode buffer, 1% (w/v) phosphoric acid. A reservoir of 0.4% (v/v) diethanolamine attached to the upper end of the column served as the cathode buffer. Cathode and anode platinum electrodes were immersed in the upper and lower reservoirs respectively, and connected to an LKB model 2103 power supply. The column was electrofocused at 800 V for 40 h with continuous circulation of water at 4°C through the jacket. After removal of electrodes and plugs, the Sephadex bed was extruded from the column by use of the column's flow adapter and cut into 0.5 cm sections. The pH in each gel section was measured before mixture of the gel with 3 ml of 20% (v/v) glycerol. The Sephadex was removed from the suspension by centrifugation at 2000g for 10 min, and the extract of each section was assayed for glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing in polyacrylamide disc gels was performed as described by Wrigley (1972) with use of the same Ampholine mixture described above.

After focusing, gels were cut into 0.3 cm sections and each was extracted into 500 μ l of 0.1M-potassium phosphate, pH 6.5. Extracts were assayed for transferase activity as described above.

Immunodiffusion

The preparation of our antiserum to purified hepatic glutathione S-transferase B has been described previously (Hales & Neims, 1976a). Ouchterlony plates (Ouchterlony, 1958) contained 1% (w/v) agar, 2% (w/v) poly(ethylene glycol) 6000 (Matheson Coleman and Bell, Norwood, OH, U.S.A.), 0.1% (w/v) NaN_3 and 0.9% (w/v) NaCl. Antiserum (40 μ l) was placed in the centre well, and outside wells were filled as indicated.

Results

Comparative substrate specificities of overall glutathione S-transferase activity in hepatic and renal cytosol

The glutathione S-transferase activities toward 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one in male rat liver and kidney cytosols are presented in Table 1; both cytosols had very low activity toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrophenethyl bromide. Hepatic transferase activities toward the four substrates are 6–45-fold higher than the respective renal activities (1-chloro-2,4-dinitrobenzene 6-fold, *p*-nitrobenzyl chloride 10-fold, 1,2-dichloro-4-nitrobenzene and *trans*-4-phenylbut-3-en-2-one 45-fold).

Isoelectric focusing pattern of hepatic glutathione S-transferase activities

The cytosols prepared from six male rat livers were electrofocused individually. A representative

profile of the focused glutathione S-transferase activities toward four substrates is shown in Fig. 1. Five major peaks of transferase activity (pI 9.6, 9.1, 8.4, 7.7 and 6.6) were resolved, and each peak exhibited distinctive substrate specificity. On the basis of substrate specificity and relative isoelectric point, the first four peaks of transferase activity (in order of decreasing basicity) correspond to transferases AA, B, A and C (Habig *et al.*, 1974, 1976; Pabst *et al.*, 1974). On Ouchterlony immunodiffusion a line of identity between the second peak and homogeneous transferase B was formed against anti-transferase B. The fifth peak (pI 6.6) does not seem to correspond to a previously described transferase. Transferase E is reported to have activity toward both 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrophenethyl bromide, but not toward *trans*-4-phenylbut-3-en-2-one (Fjellstedt *et al.*, 1973). Peak 5 (pI 6.6) has activity toward *trans*-4-phenylbut-3-en-2-one, but not toward either 1,2-epoxy-3-(*p*-nitrophenoxy)propane or *p*-nitrophenethyl bromide.

To assess the extent of overlap between the various transferase peaks and the possibility that some forms of transferase activity might interconvert, each of the five individual peaks of activity was refocused on polyacrylamide gels after dialysis against 20% (v/v) glycerol. Four of the five peaks of transferase activity refocused with the same isoelectric point and substrate specificity as the source peak (Fig. 2). The fractions corresponding to the fourth peak (mostly transferase C) refocused into three peaks of activity, the sum of which indicated significant contamination of the original peak with adjacent peak 3 (transferase A) and peak 5 (pI 6.6).

Isoelectric-focusing pattern of renal glutathione S-transferase activity

Renal cytosols prepared from six male rats were electrofocused individually, and a representative profile of the transferase activity is shown in Fig. 3. Only three major peaks of transferase activity (pI 9.0, 8.5 and 7.0) were observed. In contrast with liver, where transferase activity toward each substrate was distributed among two or more focused peaks, glutathione S-transferase activity toward each substrate in kidney tended to be highly localized into one or another of the peaks. Transferase activity toward 1-chloro-2,4-dinitrobenzene focused at pI 9.0, activity toward *p*-nitrobenzyl chloride predominantly at pI 8.5, and activity toward *trans*-4-phenylbut-3-en-2-one at pI 7.0. Activity of the focused fractions toward 1,2-dichloro-4-nitrobenzene was at the limit of detection.

When the three peaks of glutathione S-transferase activity from renal cytosol were refocused on polyacrylamide gels (Fig. 4), the peaks designated pI 9.0 and 7.0 refocused with their original isoelectric

Table 1. *Comparative substrate specificities of glutathione S-transferase activity in hepatic and renal cytosols*
Enzyme activities towards 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one, assayed as described in the text, are expressed in μ mol/min per ml of cytosol (1 g/wet wt. of tissue per 5 ml of 20% glycerol). Values are means $\pm$ S.E.M. for cytosol preparations from three to seven animals.

Substrate	Glutathione S-transferase activity	
	Liver	Kidney
1-Chloro-2,4-dinitrobenzene	20.05 $\pm$ 1.54	3.45 $\pm$ 0.10
1,2-Dichloro-4-nitrobenzene	0.91 $\pm$ 0.07	0.02 $\pm$ 0.01
<i>p</i> -Nitrobenzyl chloride	6.26 $\pm$ 0.88	0.63 $\pm$ 0.16
<i>trans</i> -4-Phenylbut-3-en-2-one	0.35 $\pm$ 0.05	0.008 $\pm$ 0.002

Fig. 1. Isoelectric-focusing pattern of glutathione *S*-transferase activities in liver cytosol

Male rat liver cytosol [1 g wet wt. of liver per 5 ml of 20% (v/v) glycerol; 6 ml] prepared as described in the text was applied to a column of Sephadex G-75 (superfine grade) pre-equilibrated with 20% (v/v) glycerol containing 2% (v/v) Ampholine mixture [20% (v/v) Bio-Lyte 3/10, 40% (v/v) pHisolyte 8-10 and 40% (v/v) pHisolyte 9-11]. Electrofocusing was carried out at 4°C and 800 V for 40 h. The pH (----) was measured in sections (0.5 cm) of the extruded Sephadex bed before elution into 3 ml of 20% (v/v) glycerol. Each eluted fraction was assayed for catalytic activity toward: ▲, 1-chloro-2,4-dinitrobenzene; ●, 1,2-dichloro-4-nitrobenzene; ○, *p*-nitrobenzyl chloride; Δ, *trans*-4-phenylbut-3-en-2-one. These activities (expressed in $\mu\text{mol}/\text{min}$ per ml) were assayed as described in the text.

Fig. 2. Refocusing on polyacrylamide disc gels of the five peaks of glutathione *S*-transferase activity resolved from liver cytosol by column electrofocusing

The fractions comprising the five peaks of glutathione *S*-transferase activity obtained after isoelectric focusing of male rat liver cytosol (Fig. 1) were each pooled. Each peak was dialysed for 24 h against 3×2 litres of 20% (v/v) glycerol. Ampholine mixture was added to a final concentration of 2% (v/v) and the sample was refocused overnight on polyacrylamide gels at an initial current of 1 mA/gel. The pH (----) was measured. Enzyme activity ($\mu\text{mol}/\text{min}$ per ml) toward 1-chloro-2,4-dinitrobenzene (▲), *p*-nitrobenzyl chloride (○) and *trans*-4-phenylbut-3-en-2-one ($\times 10^{-1}$) (Δ) was eluted and assayed as described in the text.

Fig. 3. Isoelectric-focusing pattern of glutathione *S*-transferase activity in kidney cytosol

Male rat kidney cytosol (7 ml or 1.4 g kidney wet wt.), prepared as described in the text, was applied to columns of Sephadex G-75 preequilibrated with 20% (v/v) glycerol containing 2% (v/v) Ampholine mixture and then electrofocused at 4°C and 800 V for 40 h. The pH (----) was measured in sections (0.5 cm) of the extruded Sephadex beds before elution into 3 ml of 20% (v/v) glycerol. Each eluted fraction was assayed for catalytic activity (expressed in $\mu\text{mol/min per ml}$) toward 1-chloro-2,4-dinitrobenzene (▲), 1,2-dichloro-4-nitrobenzene (●) *p*-nitrobenzyl chloride (○) and *trans*-4-phenylbut-3-en-2-one (Δ) as described in the text.

point and substrate specificity. The peak originally at pI 8.5 with activity toward *p*-nitrobenzyl chloride did not refocus as a single peak; total recovery of catalytic activity toward *p*-nitrobenzyl chloride was poor (approx. 30%) among the several peaks. These derived peaks of activity toward *p*-nitrobenzyl chloride do not correspond to described transferases, and we do not know the explanation for the behaviour of this peak on refocusing. The renal peaks of transferase activity designated pI 9.0 and 7.0 are similar to hepatic transferase B and transferase pI 6.6 with respect to isoelectric point and substrate specificity (Table 2). On Ouchterlony immunodiffusion, renal transferase pI 9.0 also forms a line of identity with hepatic transferase B against anti-transferase B.

Discussion

In this report we have used isoelectric focusing to ascertain the forms of glutathione *S*-transferase present in hepatic and renal cytosol from adult male rats. This approach has been used in the comparison of insect and mammalian liver transferases (Clark *et al.*, 1973). For our purposes, we selected four substrates having differing activities toward the known hepatic transferases. Recovery of glutathione *S*-transferase activity after electrofocusing whole cytosol from liver or kidney varied from 70 to 100% as a function of substrate. To explore the possibility that the transferase forms separated by preparative electrofocusing could be interconvertible, we have refocused all peaks of transferase activity on polyacrylamide gels. Four of the five peaks present in liver

cytosol refocused with the same isoelectric point and exhibited the same substrate specificity as did the original peak. The remaining transferase peak was contaminated with activity from adjacent peaks. This overlap is probably due to increased steepness of the pH gradient in the relevant region of the column.

The four most basic peaks in liver cytosol can be identified as glutathione *S*-transferases AA, B, A and C on the basis of isoelectric points and substrate specificities. The fifth peak (pI 6.6) may represent a new form of glutathione *S*-transferase. It is also possible that this form is identical with either transferase D or M (Gillham, 1973), neither of which has been described sufficiently to allow definitive comparison.

When comparing the glutathione *S*-transferases found in kidney with those in liver, four major differences are apparent. First, the total transferase activity (per g wet weight of tissue) is substantially lower in kidney than in liver. Secondly, there are no detectable transferases in kidney equivalent to transferases A and C in liver; renal transferase activity equivalent to hepatic transferase AA is detected in only one of six replicate experiments. Thirdly, two of the renal transferases are apparently equivalent to two of the hepatic transferases. Renal transferase pI 9.0 corresponds to hepatic transferase B; this is consistent with earlier reports of the presence of ligandin in both liver and kidney (Levi *et al.*, 1969; Kirsch *et al.*, 1975). Renal transferase pI 7.0 seems similar to hepatic transferase pI 6.6. Finally, in kidney there is a transferase of pI 8.5 with activity toward *p*-nitrobenzyl chloride that apparently has no equivalent in liver. These four observations emphasize the extent of tissue differences with respect

Fig. 4. Isoelectric focusing on polyacrylamide disc gels of the three peaks of glutathione *S*-transferase activity obtained by electrofocusing kidney cytosol

The fractions comprising the three peaks of glutathione *S*-transferase activity from an isoelectric-focusing column of kidney cytosol (Fig. 3) were pooled. Each peak was dialysed for 24 h against 3×2 litres of 20% (v/v) glycerol, before refocusing on polyacrylamide gels as described in the legend to Fig. 2. The pH (----) was measured. Enzyme activities (expressed in $\mu\text{mol}/\text{min}$ per ml) toward 1-chloro-2,4-dinitrobenzene ($\blacktriangle$), *p*-nitrobenzyl chloride ($\circ$) and *trans*-4-phenylbut-3-en-2-one (Δ) were assayed as described in the text.

Table 2. Comparison of the two electrofocused forms of transferase that are found in both liver and kidney cytosol preparations. Enzyme activities toward 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one were assayed as described in the text and are expressed for comparative purposes as ratios of activity towards *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one to activity towards 1-chloro-2,4-dinitrobenzene. Values are means $\pm$ S.E.M. for three to six experiments.

Transferase	pI	Activity ratios	
		1-Chloro-2,4-dinitrobenzene <i>p</i> -nitrobenzyl chloride	1-Chloro-2,4-dinitrobenzene <i>trans</i> -4-phenylbut-3-en-2-one
Liver B	9.1 ± 0.1	30 ± 11	>1000
Kidney pI9.0	9.0 ± 0.1	52 ± 41	>1000
Liver pI6.6	6.6 ± 0.2	2.2 ± 0.4	12 ± 0.1
Kidney pI7.0	7.0 ± 0.2	1.6 ± 0.3	22 ± 0.3

to composition of the various glutathione S-transferases.

In ascertaining the role(s) of these proteins in different tissues, differences in the composition of the glutathione S-transferases must be considered. In kidney, as in liver, the transferases may function in detoxification and/or transport. Renal organic anion uptake can be correlated with ligandin concentration (Kirsch *et al.*, 1975), but not with transferase activity toward 1,2-dichloro-4-nitrobenzene (Pegg & Hook, 1977). Measurement of the individual transferases in kidney may assist in the interpretation of these apparently conflicting results.

Earlier studies on glutathione S-transferase B (Hales & Neims, 1976a,b, 1977) indicate that the relative composition of the transferase family varies quantitatively in liver as a function of sex, endocrine state, age and pretreatment with xenobiotic inducers. It is likely that further studies on the renal transferases may reveal similar changes as a function of some of these parameters (Clifton *et al.*, 1975; Kaplowitz & Clifton, 1976; Kirsch *et al.*, 1975). Differences in the amino acid composition between most of the purified rat transferases suggest that at least some represent separate gene products (Habig *et al.*, 1974). The observation that in kidney cytosol the catalytic activities toward three substrates, 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride and *trans*-4-phenylbut-3-en-2-one, can be resolved into separate fractions may be of experimental value in future studies of the regulation and role(s) of these proteins.

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