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Purification and Characterization of Two Glutathione *S*-Aryltransferase Activities from Rat Liver

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Two forms of glutathione *S*-aryltransferase were purified from rat liver. The only differences noted between the two forms were in the chromatographic and electrophoretic properties, which permitted the separation of the two species. The molecular weights of the enzyme and its subunits were estimated as about 50000 and 23000 respectively. The steady-state kinetics did not follow Michaelis–Menten kinetics when one substrate concentration was kept constant while the second substrate concentration was varied. Several *S*-substituted GSH derivatives were tested as inhibitors of the enzymic reaction. The enzyme was inactivated by thiol-group reagents.

Glutathione *S*-transferases play an important role in the biotransformation of xenobiotics, i.e. compounds which are foreign to living cells. Many enzymic reactions involving conjugation of GSH with different organic compounds have been described (Boyland & Chasseaud, 1969; Wood, 1970; Boyland, 1971; Chasseaud, 1974), but the number of truly different transferases and their substrate specificities are unknown. Booth *et al.* (1961) and Combes & Stakelum (1961) independently discovered enzymic reactions involving conjugation of GSH with aromatic compounds containing suitable substituents. It seems likely that the reaction involving sulphobromophthalein and GSH described by Combes & Stakelum (1961) is catalysed by the enzyme glutathione *S*-aryltransferase (EC 2.5.1.13), defined by Booth *et al.* (1961) on the basis of other aromatic substrates, but no clear evidence for this assumption has been provided (Chasseaud, 1974).

Glutathione *S*-aryltransferase in rat liver, as defined by its activity with 3,4-dichloro-1-nitrobenzene and GSH, is separable into at least two forms by ion-exchange chromatography or isoelectric focusing (Pabst *et al.*, 1973; Ketterer *et al.*, 1974; Eriksson *et al.*, 1974*a*). The relation of these two activities to each other and to the enzymic conjugation of GSH with sulphobromophthalein is unknown. The present investigation was undertaken to purify further and characterize the two forms of glutathione *S*-aryltransferase. A preliminary report of this work has been presented (Jakobson *et al.*, 1974).

Materials and Methods

Chemicals

3,4-Dichloro-1-nitrobenzene was obtained from Schuchardt, Munich, Germany, and GSH and GSSG were from Boehringer, Mannheim, Germany. Sodium sulphobromophthalein was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K., and 1,2-epoxy-3-(*p*-nitrophenoxy)propane was from Eastman Kodak Co., Rochester, N.Y., U.S.A. 1-Menaphthyl sulphate (sodium naphth-1-ylmethyl sulphate) was a gift from Dr. B. Gillham, Department of Biochemistry, St. Thomas's Hospital Medical School, London, U.K. 1,2-Dibromoethane and 5,5′-dithiobis-(2-nitrobenzoic acid) were from BDH, Poole, Dorset, U.K., benzyl chloride was from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A., and diethyl maleate was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *N*-Ethylmaleimide, iodoacetamide, iodoacetate, *p*-hydroxymercuribenzoate and *S*-methylglutathione were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and 2,2′-dithiopyridine and 4,4′-dithiopyridine from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. *N*-Phenylmaleimide and *o*-iodosobenzoate were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., mercuric acetate was from Baker Chemical Co., Phillipsburg, N.J., U.S.A., potassium arsenite was from Hopkin and Williams, and cadmium acetate was from E. Merck A.G., Darmstadt, Germany. *N*-3,5-Dinitrophenylmaleimide was a gift from Dr. U. Östner, Wenner–Gren Institute, Stockholm, Sweden. *S*-Alkyl- and *S*-aralkylglutathione derivatives were synthesized by method A of Vince *et al.* (1971).

Materials for separation

Sephadex G-25 (coarse grade) and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and CM-cellulose and hydroxy-

apatite were from Whatman Biochemicals, Maidstone, Kent, U.K., and Bio-Rad Laboratories, Richmond, Calif., U.S.A. respectively. Membrane filters for ultrafiltration were from Amicon NV, The Hague, Holland.

Animals

Male Sprague–Dawley rats, free from specific pathogens and weighing 200–250 g, were used as a liver source.

Enzyme assays

Enzyme assays during purification, as well as those with thiol-group reagents, were made with a DB-G Beckman Grating Spectrophotometer or a Unicam SP. 1800 Ultraviolet Spectrophotometer. Glutathione *S*-aryltransferase was assayed spectrophotometrically at 344nm ($\varepsilon = 10 mM^{-1} \cdot cm^{-1}$) by the method of Booth *et al.* (1961) at a reaction temperature of 30°C. The final concentrations of GSH and 3,4-dichloro-1-nitrobenzene were 5.0 and 1.0mM respectively. A unit of enzyme is defined as the amount which will form 1 μmol of *S*-(2-chloro-4-nitrophenyl)-glutathione/min and the specific activity is expressed in units/mg of protein. Protein concentration was calculated on the basis of the absorbance at 260 and 280nm (Kalckar, 1947). The conjugation of different substrates with GSH was measured essentially by published methods: sulphobromophthalein as described by Goldstein & Combes (1966), and 1,2-epoxy-3-(*p*-nitrophenoxy)propane as described by Fjellstedt *et al.* (1973) at a final GSH concentration of 18mM. The enzyme activity with 1-menaphthyl sulphate was assayed by the method of Gillham (1971), modified by using 0.2M-Tris–HCl buffer (pH8.3) instead of triethanolammonium chloride. The reaction with 1,2-dibromoethane, benzyl chloride and diethyl maleate was measured by determination of disappearance of GSH essentially as described by Nachtomi (1970).

Enzyme purification

During the purification the enzyme was kept at about 4°C.

(1) *Centrifugation.* Livers from 40 rats were homogenized in 0.25M-sucrose with an Ato-Mix blender and diluted to make a 10% (w/v) suspension. The homogenate was centrifuged for 45min at 14000rev./min with a JA-14 rotor in a Beckman J-21 centrifuge (corresponding to 30000*g* at the bottom of the tubes). The pellets were resuspended in 0.25M-sucrose and re-centrifuged as described above. The supernatants so obtained were adjusted to pH5.5 with 5% (w/v) acetic acid and centrifuged once more for 45min. The pellets were discarded.

(2) *Sephadex G-25 gel filtration.* The supernatant from step (1) (4.5 litres) was chromatographed on a Sephadex G-25 (coarse grade) column (29cm×40cm, 25 litres), which had been equilibrated with 10mM-sodium phosphate buffer, pH6.1. The proteins were eluted with the same buffer.

(3) *CM-cellulose chromatography.* The pooled fractions from the previous step (9.7 litres) were applied to a CM-cellulose column (9cm×10cm), equilibrated with buffer as described above. After washing with about 2 bed vol. of buffer, a linear concentration gradient made up from 2 litres of 10mM-sodium phosphate buffer–1mM-EDTA and 2 litres of 50mM buffer containing 0.2M-NaCl and 1mM-EDTA, pH6.1, was applied. Fractions (15ml) were collected.

(4) *Sephadex G-75 gel filtration.* The sample was concentrated to 25ml in an Amicon 402 ultrafiltration cell with a PM-10 membrane and then applied to a Sephadex G-75 column (4cm×110cm). The column was equilibrated with 10mM-sodium phosphate buffer, pH6.7. After introduction of the sample, the enzyme was eluted with the same buffer.

(5) *Hydroxyapatite chromatography.* The pooled fractions from the gel-filtration step (78ml) were concentrated as described above to 25ml. Afterwards the sample was applied to a hydroxyapatite column (3cm×20cm) previously equilibrated with 10mM-sodium phosphate buffer, pH6.7, containing 1mM-EDTA. The column was washed with the starting buffer and a linear concentration gradient, 10–350mM-sodium phosphate (pH6.7)–1mM-EDTA, total volume 900ml, eluted the adsorbed enzyme activities. The eluate was collected in 5ml fractions.

Electrophoretic systems

(*a*) Disc electrophoresis was performed essentially as described by Ornstein (1964) and Davis (1964). The electrolyte buffer was 5mM-Tris–30mM-glycine, pH8.3. A current of 3mA/gel for 4.5h was required to move the tracking dye, Bromothymol Blue, to the end of the 6cm gel. The gels were fixed in 12.5% (w/v) trichloroacetic acid for 5min and stained in 0.1% Coomassie Brilliant Blue–12.5% trichloroacetic acid. After 15min the gels were destained in 5% (v/v) acetic acid.

(*b*) Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969). Solutions containing about 0.04mg of protein were introduced under the upper gel buffer and a current of 4mA/gel was applied for 14h (or alternatively, 8mA for 4h).

(*c*) Isoelectric focusing was carried out in a 110ml column according to the instructions of the manufacturer (LKB Produkter AB, Stockholm, Sweden). The ampholyte (1%, w/v) covered a pH range of 3.5–10 in a 0–50% (w/v) sucrose density gradient. The isoelectric focusing was run with a power consumption

of less than 2.5W and at 4°C for 2 days. The contents of the column were collected in 2ml fractions.

Determinations of molecular weights

The determinations of molecular weights were carried out on a Sephadex G-150 column (2.5cm× 75cm) equilibrated with 10mM-Tris–HCl buffer (pH7.5)–0.1M-KCl (Andrews, 1970). References and sample were passed through simultaneously. The reference proteins were assayed by standard techniques and were assumed to have the molecular weights indicated: yeast alcohol dehydrogenase (150000), L-lactate dehydrogenase (140000), L-malate dehydrogenase (70000), bovine erythrocyte carbonic anhydrase (29000), and cytochrome *c* (12400). The molecular weights of the subunits of the purified GSH *S*-aryltransferase were estimated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by using bovine serum albumin (mol.wt. 68000), ovalbumin (43000), carbonic anhydrase and cytochrome *c* as standards.

Steady-state kinetics

The initial reaction rates, with one substrate concentration kept constant (either GSH or 3,4-dichloro-1-nitrobenzene) and the second substrate concentration varying over a 1000-fold range, were determined on an Aminco DW-2 UV/VIS spectrophotometer. The enzyme was preincubated for 5min with the desired concentration of GSH in 0.1M-potassium phosphate buffer, pH8.0, and the reaction initiated by addition of 3,4-dichloro-1-nitrobenzene. At a GSH concentration of 3.5mM, the 3,4-dichloro-1-nitrobenzene concentration was varied from 0.5μM to 1.2mM, and at a 3,4-dichloro-1-nitrobenzene concentration of 0.8mM, the GSH concentration was varied from 2μM to 12mM. The concentration of ethanol, used as a solvent, was kept constant, at 10% (v/v). To ascertain that the pH of the incubation mixture would not decrease on addition of GSH, the stock solution was adjusted with NaOH to pH5 when necessary. To prevent oxidation of GSH, the stock solution was kept under N_2 in an ice bath. Thus the decrease in the GSH concentration was limited to 2.5% over a period of 4h.

Inhibition studies

Inhibition studies with GSH derivatives as inhibitors were made with a Unicam SP.1800 ultraviolet spectrophotometer. In experiments with GSSG, 200μl of GSSG solution of variable concentrations, adjusted to pH8.0, was added to preincubated solutions (30°C) of enzyme and GSH in 0.1M-phosphate buffer–1mM-EDTA, pH8.0. The final volume was 1.0ml. The reactions were started by the addition of 3,4-dichloro-1-nitrobenzene. The analyses were performed in duplicate or triplicate. Inhibition of the enzyme by *S*-alkyl- and *S*-aralkyl-glutathione derivatives was measured in the standard assay system at an inhibitor concentration of 0.2mM. The reversibility of the inhibition was checked by preincubation of enzyme and 0.2mM inhibitor at 30°C for 1h. The enzyme activity was then determined after removal of the inhibitor by dilution and was found not to differ significantly from a control incubated in the absence of inhibitor.

Inactivation studies with thiol-group reagents

The inactivation reactions were run in a separate incubation system. The system contained 75μl of 100mM-sodium phosphate buffer, pH8.0, 20μl of enzyme solution (4.2 units/ml), and 10μl of thiol-group reagent of different concentrations (or water in the control). After 30min at 22°C, a 25μl sample was taken for measuring the residual activity in the standard assay system.

Results

Purification

Table 1 summarizes the results of the purification of the enzyme catalysing the conjugation of 3,4-dichloro-1-nitrobenzene with GSH. The CM-cellulose column (Fig. 1) resolved two GSH *S*-aryltransferase activities. As the separation was not complete, the fractions containing the two activities were pooled for further purification by gel filtration on Sephadex G-75. All GSH *S*-transferase activities measured appeared in a single peak. The most active fractions from the Sephadex G-75 column were, after concentration, applied to a hydroxyapatite column. The elution pattern of the hydroxyapatite column, developed with a linear phosphate gradient, displayed two clearly separated GSH *S*-aryltransferase activities (Fig. 2). The peaks were analysed by disc electrophoresis and each was found to contain a major protein component and a trace amount of protein in the form of a hazy zone surrounding the main band. The two components of the enzyme were designated as forms I and II respectively, the numerals indicating the order of appearance from the hydroxyapatite column. Numerous attempts were made to purify the two enzyme components further by chromatography on QAE- [diethyl-(2-hydroxypropyl)aminoethyl]-Sephadex A-50, SP- (sulphopropyl)-Sephadex A-50 (Pharmacia) and Bio-Gel P-150, brushite and Cellex-P (Bio-Rad Laboratories) columns as well as by isoelectric focusing. However, although some minor protein components disappeared, as revealed by disc electrophoresis, no increase in the specific activity of the enzyme was noted. The same result was obtained

Table 1. *Purification of GSH S-aryltransferase*

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Liver supernatant	4500	1080	0.020	100
Sephadex G-25 pool	9700	1120	0.033	103
CM-cellulose pool	760	570	0.31	53
Sephadex G-75 pool	78	436	0.92	40
Hydroxyapatite				
Peak I	56	127	1.42	24
Peak II	52	124	2.08	

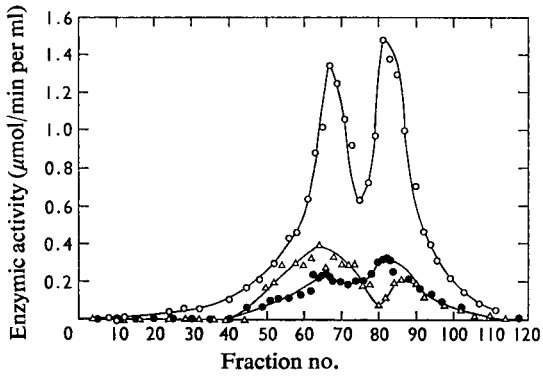

Fig. 1. *Elution pattern of the CM-cellulose chromatography*

The experimental details are described in the Materials and Methods section. Enzymic activities with GSH and one of the following substrates are shown: ○, 3,4-dichloro-1-nitrobenzene; ●, sulphobromophthalein; △, 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

after affinity chromatography on agarose containing GSSG as a ligand, a gel bed which binds GSH *S*-aryltransferase better than many other GSH-linked enzymes tested (experiments carried out in collaboration with Miss Inger Carlberg). The two peaks of activity obtained after hydroxyapatite chromatography were pooled separately and rechromatographed on hydroxyapatite under the conditions previously used. In these experiments the activities appeared as single components in the chromatograms, a finding indicating that the two forms are not in equilibrium with each other. A mixture of both components was resolved into two peaks when rechromatographed on hydroxyapatite or subjected to isoelectric focusing. The crude post-microsomal rat liver supernatant was also analysed by isoelectric focusing to verify that the two components of GSH *S*-aryltransferase are present originally and not produced by modification of a single enzyme species. Fig. 3 demonstrates that at least two components exist from the beginning of the purification. The total activities of the two peaks have been found to be approximately equal throughout the purification in all preparations made. However, the specific activity of form I was always lower than that of form II (see Table 1).

The purification was also followed by measuring other enzymic GSH conjugations. The reactions involving sulphobromophthalein or 1,2-epoxy-3-(*p*-nitrophenoxy)propane were studied most extensively. The conjugation of GSH with sulphobromophthalein closely followed the activity profiles of the reaction of GSH and 3,4-dichloro-1-nitrobenzene throughout the purification procedure (see Figs. 1–3). The activity obtained with GSH and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, on the other hand, had a different elution pattern. Most of the activity was not retained by the CM-cellulose column, but the adsorbed GSH *S*-epoxide transferase activity was recovered in the fractions containing GSH *S*-aryltransferase. However, the ratio of the activity obtained with 1,2-epoxy-3-(*p*-nitrophenoxy)propane to that with 3,4-dichloro-1-nitrobenzene decreased during purification of the GSH *S*-aryltransferase in contrast with the ratio of the activities determined with sulphobromophthalein and 3,4-dichloro-1-nitrobenzene, which was constant.

Other substrates were also used to measure GSH *S*-transferase activities during the purification. These included 1,2-dibromoethane, benzyl chloride and diethyl maleate, giving activities which, like the activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, were due to proteins mainly unadsorbed on CM-cellulose. The adsorbed proteins, which gave enzymic activity with these substrates, were not completely separable from GSH *S*-aryltransferase. 1-Menaphthyl sulphate gave a low activity in the early stages of the purification, which, however, was lost during the purification.

Molecular weight and subunit structure

The molecular weights of the two forms of purified GSH *S*-aryltransferase were estimated by gel filtration as described in the Materials and Methods section. Both components had a value of about 50000. Electrophoresis on sodium dodecyl sulphate–polyacrylamide gels resulted in one major and two minor

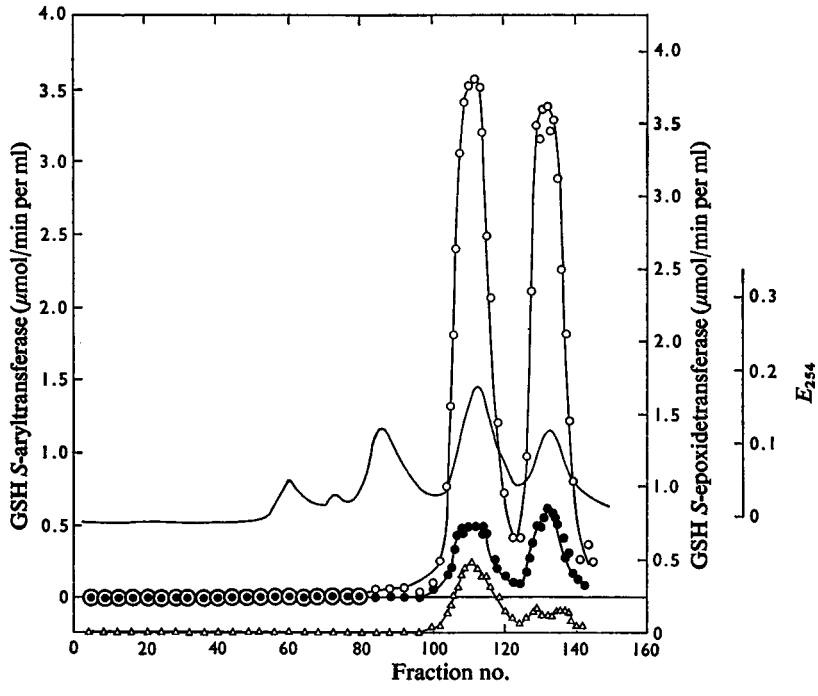

Fig. 2. *Elution pattern of the hydroxyapatite chromatography*

The experimental details are described in the Materials and Methods section. ——, E_{254}; for other symbols, see legend to Fig. 1. Peaks I and II in Table 1 correspond to the pools of fractions no. 106–116 and 128–138 respectively.

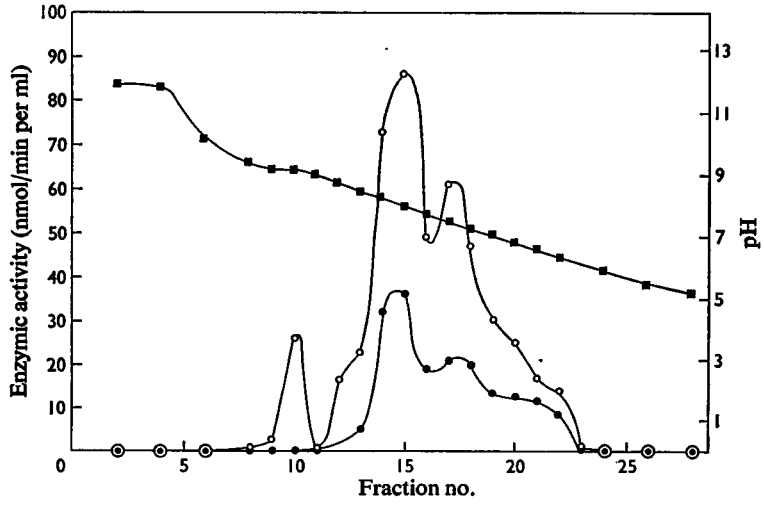

Fig. 3. *Isoelectric focusing of a gel-filtered rat liver cytosol*

The experimental details are described in the Materials and Methods section. ■, pH; for other symbols, see legend to Fig. 1.

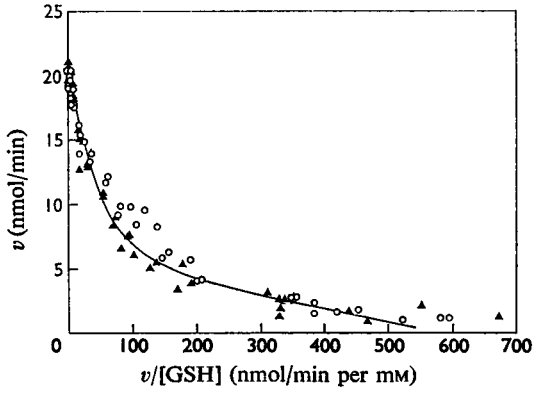

Fig. 4. *Eadie–Augustinsson–Hofstee plot of the GSH S-aryltransferase-catalysed conjugation of GSH with 3,4-dichloro-1-nitrobenzene*

The points denote experimental data from experiments with form I (▲) and form II (○) of the enzyme. The GSH concentration varied from 0.5 μM to 1.2 mM at a constant 3,4-dichloro-1-nitrobenzene concentration (0.8 mM). The curve was fitted by non-linear regression to the data obtained with form I under the assumption that the second-power rate law (see the text) applies.

Table 2. *Kinetic constants estimated by fitting (by non-linear regression) a second-power rate law to experimental data*

The data are presented in Fig. 4 (number of experimental points = 40). Units of the constants are expressed in their appropriate dimensions by using μmol/min and mM as basic units of velocity and concentration.

		Parameter values (±S.E.)	
Constant	Enzyme	... Form I	Form II
V_1		0.409±0.048	0.846±0.084
V_2		4.92 ±0.46	2.70 ±0.64
K_1		0.008±0.002	0.026±0.005
K_2		2.84 ±0.28	1.60 ±0.39

protein bands for each enzyme form. The minor components were presumably due to contaminating protein. The molecular weights of the major components were estimated as about 23000.

Steady-state kinetics

The steady-state kinetics were investigated with both forms of the purified GSH *S*-aryltransferase, with GSH and 3,4-dichloro-1-nitrobenzene as substrates. The initial velocity pattern was determined by varying one substrate concentration at different constant concentrations of the other one. The data were displayed in Eadie–Augustinsson–Hofstee plots (see Mannervik, 1974). To a first approximation the data seemed to follow the pattern of a sequential mechanism, but the lines corresponding to fixed concentrations of one substrate were not linear. A detailed analysis of the kinetics has not been made in the present investigation, which only documents the non-linearities obtained (Fig. 4). It was demonstrated by non-linear regression analysis and statistical criteria (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973) that the second-power rate law

$$v = \frac{V_1[A] + V_2[A]^2}{K_1 + [A] + K_2[A]^2}$$

where V_1, V_2, K_1, and K_2 are composite kinetic constants and [A] the substrate concentration varied, fitted the experimental data significantly better than the Michaelis–Menten equation. Table 2 gives the values of the kinetic constants obtained when the best rate equation was fitted to the data displayed in Fig. 4. The parameter values given in Table 2 are similar for the two enzyme forms, but it should be noted that they are only descriptive and depend on the constant substrate concentration as well as on the concentration of the enzyme (for the numerator constants, V_1 and V_2). The enzyme concentrations of the stock solutions of the two forms were adjusted to give equal velocities at 3.50 mM-GSH and 0.40 mM-3,4-dichloro-1-nitrobenzene.

Inhibition with GSSG

GSSG can be formed from one of the substrates, GSH, and it was therefore important to evaluate its inhibitory properties. The non-linearities demonstrated in plots of v versus v/[GSH] (see Fig. 4) or of v versus v/[3,4-dichloro-1-nitrobenzene] precluded a rigorous evaluation of the inhibition pattern of experiments involving GSSG and variable concentrations of the substrates. The apparent inhibition pattern was for both enzyme forms non-competitive with both substrates. When the GSSG concentration was varied in the range 0.1–20.0 mM at three different combinations of constant substrate concentrations, however, it was clear that the inhibitory effect was linear with the GSSG concentration. The inhibition of enzyme form II is shown at two different combinations of substrate concentrations in Fig. 5. No significant difference between the kinetic patterns of the two enzyme forms could be detected. The effect of GSSG inhibition owing to GSH oxidation in stock solutions was found to be negligible.

Inhibition with S-substituted GSH derivatives

A series of *S*-*n*-alkylglutathione derivatives were screened as inhibitors of both forms of GSH *S*-aryltransferase. The inhibitory effect increased with the length of the alkyl group, maximal inhibition being obtained with *S*-*n*-octylglutathione (Table 3). Three

S-aralkyl derivatives tested were less inhibitory than the most active alkyl derivatives. In no case could any clear-cut difference between the inhibitory effects on the two enzyme forms be demonstrated. GSSG (0.2mM) tested under identical conditions gave no significant inhibition with any of the two enzyme forms. The compounds were also tested as inhibitors of GSH *S*-aryltransferase in the crude rat liver supernatant fraction. The inhibitory effects were not significantly different from the results obtained with the purified enzyme fractions, indicating that the catalytic properties of the enzyme are unchanged after purification.

Effect of thiol-group reagents

The effect of different classes of thiol-group reagents was tested with both forms of the purified GSH *S*-aryltransferase (Table 4). Iodoacetamide and iodoacetate were much less active as inactivators than the second group of alkylating agents, i.e. maleimides.

Table 3. *Inhibition with S-substituted GSH derivatives*

The inhibition was determined with 0.2mM inhibitor as described in the Materials and Methods section. The stock solutions of the two enzyme forms had approximately equal activities. The results given are means±S.E. based on three separate experiments, each involving duplicate or triplicate measurements.

S-Substituent	Enzyme ...	Inhibition (%) Form I	Form II
Methyl		3±4	1±13
Ethyl		19±9	11±17
n-Propyl		34±3	25± 7
n-Butyl		51±6	49± 4
n-Pentyl		56±5	52±10
n-Hexyl		64±4	61± 8
n-Heptyl		69±6	69± 5
n-Octyl		81±5	78± 5
Phenylpropyl		43±6	48±12
Benzyl		50±6	52± 5
p-Bromobenzyl		53±7	67±12

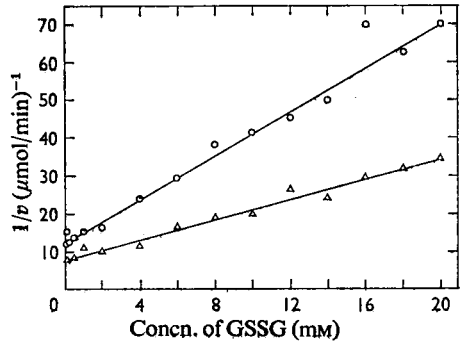

Fig. 5. *Inhibition of GSH S-aryltransferase (form II) with GSSG (Dixon plot)*

The experimental details are described in the Materials and Methods section. The two substrate combinations shown were: ○, 0.5mM-GSH and 0.2mM-3,4-dichloro-1-nitrobenzene; △, 0.5mM-GSH and 0.5mM-3,4-dichloro-1-nitrobenzene.

Table 4. *Effect of thiol-group reagents*

The inhibition of the enzyme was determined after incubation for 30min at 22°C in the presence of thiol-group reagents. For further details see the Materials and Methods section.

Reagent	Concentration (mM)	Enzyme ...	Inhibition (%) Form I	Form II
Alkylating				
Iodoacetate	10		13	16
Iodoacetamide	10		30	30
N-Ethylmaleimide	0.1		58	60
N-Phenylmaleimide	0.1		84	82
N-3,5-Dinitrophenylmaleimide	0.1		>90	>90
Oxidizing				
2,2′-Dithiopyridine	1		68	72
4,4′-Dithiopyridine	1		60	60
o-Iodosobenzoate	10		0	0
Mercaptide-forming				
Mercuric acetate	1		49	48
p-Hydroxymercuribenzoate	1		34	37
Potassium arsenite	10		5	4
Cadmium acetate	10		0	0

The aromatic maleimides were more potent than *N*-ethylmaleimide. The oxidizing dithiopyridines were, next to the maleimides, the most active inactivators tested, whereas *o*-iodosobenzoate had no effect. Of the mercaptide-forming reagents, the mercurials were the most active compounds. Arsenite and Cd^{2+} ions, which have a high affinity for dithiol groups but not for monothiol groups, had no clearly demonstrable effect. The inhibitory effect of the mercurials is stronger than indicated by the numbers given in Table 4, because at the start of the measurement of residual enzyme activity the velocity was zero. The activity then increased owing to re-activation of the enzyme by GSH in the assay system, and the values given in the table are quasi-steady-state velocities obtained when maximal velocity had been attained. No significant differences between the two enzyme forms were noted in any of the experiments.

Discussion

The present work extends previous observations (Eriksson *et al.*, 1974*a*) on the properties and possible relation to thiol–disulphide interchange of two GSH *S*-aryltransferase activities in rat liver cytosol to a large-scale purification and characterization of the two enzyme forms. This enzyme has previously been prepared on a smaller scale by several investigators (Booth *et al.*, 1961; Ishida, 1968; Boyland & Speyer, 1970; Troxler *et al.*, 1973; Ketterer *et al.*, 1974). Pabst *et al.* (1973) have reported the preparation of a homogeneous GSH *S*-aryltransferase, but the experimental details have not been published so far. However, they give a specific activity of 4.3 units/mg for the purified enzyme, which is somewhat higher than the highest values reached in the present work. Gel electrophoresis of our purest fractions indicates that the two forms of the enzyme are not homogeneous, in spite of the fact that we have been unable to raise the specific activity to values significantly higher than those reported in the present paper. Nevertheless, the purified enzyme forms seem to be more than 50% pure, a value which in combination with the purification factor (about 100) indicates that about 1% of the total protein of the rat liver cytosol consists of GSH *S*-aryltransferase.

It has been explicitly or tacitly assumed that the activity obtained with sulphobromophthalein and GSH was due to the same enzyme as the activity obtained with 3,4-dichloro-1-nitrobenzene and GSH, although no clear evidence for this assumption has been provided (see Chasseaud, 1974). The results presented in Figs. 1–3 and the finding that the ratio of the two activities was constant during the purification give strong evidence for the view that the two reactions are catalysed by the same enzyme.

Other GSH *S*-transferases which have been purified extensively utilize 1-menaphthyl sulphate (Gillham, 1973) or epoxides (Fjellstedt *et al.*, 1973; Hayakawa *et al.*, 1974) as donor substrates. In the present investigation, most of the activities obtained with these substrates were lost during purification, in agreement with the view that enzymes distinct from GSH *S*-aryltransferase catalyse the conjugation of these substrates with GSH. The activities obtained with 1,2-dibromoethane, benzyl chloride and diethyl maleate were also separated to a great extent from the GSH *S*-aryltransferase activity in the different steps of purification. On the other hand, the purest enzyme fractions had residual activity with all substrates except 1-menaphthyl sulphate, indicating that the specificity of the enzyme is not limited to a single class of organic compounds. A similar conclusion has previously been reached by Pabst *et al.* (1973). However, a detailed delineation of the relationships between the different GSH *S*-transferases requires further investigation.

An important question about the GSH *S*-aryltransferase studied in the present investigation is the relation of the two enzyme forms to one another. In most respects their properties were indistinguishable or very similar. Thus the molecular weights of the intact enzymes and their subunits were estimated as 50000 and 23000 respectively for both forms, indicating a dimeric structure of the native enzyme. These findings exclude an association–dissociation relationship between the two enzyme forms. The kinetic properties were also very similar, as demonstrated in Fig. 4 and in Tables 2 and 3. Further, the sensitivity to thiol-group reagents appear to be the same for both forms (Table 4). Consequently, it seems justifiable to conclude that the two GSH *S*-aryltransferase species are functionally very similar, at least with respect to the catalytic properties. The only clear-cut differences noted are expressed in the electrophoretic and chromatographic properties demonstrated in Figs. 1, 2 and 3. These differences indicate that the ionic charges of the two enzyme forms are different. We have previously obtained evidence for a GSH-dependent interconversion between a GSH-containing and a GSH-deficient species of a thiol transferase in rat liver (Eriksson *et al.*, 1974*b*). Further, GSH has been reported to change the electrophoretic mobility of aged GSH *S*-epoxide transferase (Fjellstedt *et al.*, 1973). However, no evidence whatsoever indicates a similar relationship between the two forms of GSH *S*-aryltransferase, nor do any data suggest that they are at all interconvertible.

The initial-rate data obtained show clearly that the kinetics do not follow Michaelis–Menten kinetics when either the GSH concentration (Fig. 4) or the 3,4-dichloro-1-nitrobenzene concentration was varied at a fixed concentration of the second substrate. The data in Fig. 4 seem to be compatible with the assumption of a mixture of two enzymes following Michaelis–Menten kinetics, which act on the same substrates,

but an inflexion point at high [GSH] in a plot of v versus v/[GSH] (not visible in Fig. 4 owing to the scale of the abscissa) contradicts this possibility. The kinetics show nonlinearities which can all be explained by the assumption of a random two-substrate mechanism (Ferdinand, 1966), but establishment of this kinetic model must await further confirmation.

The inhibition studies demonstrate that glutathione derivatives inhibit the enzyme and that the alkyl derivatives with the longest carbon chains are the strongest inhibitors (see Table 3). GSSG, which can be regarded as a GSH derivative containing a polar *S*-substituent, had no inhibitory effect at 0.2mM. The aromatic character of the three *S*-aralkyl derivatives does not provide better inhibitory properties than those of the most potent *S*-alkyl derivatives. Further, an *S*-aryl compound, probably *S*-2-chloro-4-nitrophenylglutathione (synthesized from GSH and 3,4-dichloro-1-nitrobenzene under alkaline conditions), gave 44 ± 12 and $47\pm7\%$ inhibition of enzyme forms I and II respectively at 0.2mM concentration. These results indicate that hydrophobicity of the *S*-substituent of the inhibitor is more important than aromatic character.

The inactivation experiments with thiol-group reagents (Table 4) indicate strongly that thiol groups are important for the function of both forms of the enzyme. This conclusion is based on the finding that members of all three classes of thiol reagents are inhibitory and that the effect of the two mercurials can be reversed by GSH. The effect of the different maleimides indicates that a hydrophobic site of the enzyme is the target of the inactivators. This finding is in accordance with the results with the reversible inhibitors, but the question of whether they act at the same site, and if this is the active site of GSH *S*-aryltransferase, has not yet been answered. The original aim of the experiments described in the present paper was to explore the possible differences between the two forms of the enzyme. However, no functional differences have been discovered in this investigation.

Note Added in Proof (Received 18 March 1975)

Recently two papers have appeared describing the purification from rat liver of three GSH *S*-transferases denoted A, B, and C (Habig *et al.*, 1974; Pabst *et al.*, 1974). These enzymes, as well as the previously studied transferase E (active with epoxides), demonstrate wide and partially overlapping substrate specificities. Transferases A and C are distinguished by having considerably higher activities with 3,4-dichloro-1-nitrobenzene and sulphobromophthalein than transferases B and E. The specificity for these substrates and the chromatographic and electrophoretic properties indicate strongly that transferases A and C correspond to forms II and I respectively described in the present paper. If so, it is noteworthy that Habig *et al.* (1974) demonstrate that transferases A and C display great similarities in amino acid composition and share a common immunochemical determinant. These and other data are consonant with the similarities between forms I and II shown in the present paper.

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