Enzymes Catalysing Conjugations of Glutathione with $\alpha\beta$ -Unsaturated Carbonyl Compounds

By E. BOYLAND AND L. F. CHASSEAUD

Chester Beatty Research Institute, Institute of Cancer Research:
Royal Cancer Hospital, London, S.W. 3

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1. Heat-inactivation experiments, ammonium sulphate-fractionation studies, enzyme-inhibition studies with *S*-($\alpha\beta$ -diethoxycarbonyl-ethyl)glutathione, and evidence from the distribution of activities in rat liver, in rat kidney and in the livers of other animals, indicate that reactions of glutathione with (i) *trans*-benzylideneacetone, (ii) cyclohex-2-en-1-one, (iii) *trans*-cinnamaldehyde, (iv) diethyl maleate, (v) diethyl fumarate and (vi) 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid are catalysed by different enzymes. 2. Evidence is presented that the enzymes catalysing the reactions of glutathione with substrates (i)–(iv) are different from glutathione *S*-alkyltransferase, *S*-aryltransferase and *S*-epoxidettransferase. 3. The name 'glutathione *S*-alkenyltransferases' is proposed for enzymes catalysing reactions of glutathione with $\alpha\beta$ -unsaturated compounds. 4. The Arrhenius plot for the enzyme-catalysed reaction of diethyl maleate with glutathione is discontinuous, with lower energy of activation at 38°.

Certain foreign compounds are metabolized to mercapturic acids (derivatives of *N*-acetylcysteine). An initial stage in mercapturic acid formation, conjugation of the foreign compound with GSH, is catalysed by glutathione *S*-transferases. These include: glutathione *S*-alkyltransferase, catalysing the *S*-alkylation of GSH by alkyl halides and related compounds (Johnson, 1966); glutathione *S*-aryltransferase, catalysing the replacement of labile aryl halogen or nitro groups by GSH (Booth, Boyland & Sims, 1961; Al-Kassab, Boyland & Williams, 1963); glutathione *S*-epoxidettransferase catalysing the addition of GSH to various epoxides (Boyland & Williams, 1965); several enzymes catalysing the addition of GSH to $\alpha\beta$ -unsaturated compounds containing activated carbon–carbon double bonds (Boyland & Chasseaud, 1966, 1967; Chasseaud, 1967).

The present paper gives evidence to show that at least six separate enzymes catalyse conjugations of GSH with different $\alpha\beta$ -unsaturated carbonyl compounds according to the reaction:

EXPERIMENTAL

Materials. Parasorbic acid (δ -lactone of 5-hydroxyhex-2-en-1-oic acid) was a gift from Dr P. Sims of this Institute; 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid

was a gift from Dr J. E. Baer (Merck Institute for Therapeutic Research, West Point, Pa., U.S.A.); samples of hex-2-en-1-al were gifts from Compagnie Parento Inc. (Croton-on-Hudson, N.Y., U.S.A.) and Norda Essential Oil and Chemical Co. (New York, N.Y., U.S.A.); GSH was obtained from Schwarz BioResearch Inc. (Mount Vernon, N.Y., U.S.A.); cinnamaldehyde dimethylacetal was from K & K Laboratories Inc. (Plainview, N.Y., U.S.A.); *trans*-benzylideneacetone (4-phenylbut-3-en-2-one) was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.); ethyl acrylate, diethyl fumarate and diethyl maleate were from British Drug Houses Ltd. (Poole, Dorset); cyclohex-2-en-1-one was from Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.); *trans*-cinnamaldehyde (3-phenylprop-2-en-1-al) was from Hopkin and Williams Ltd. (Chadwell Heath, Essex). The last three chemicals were redistilled before use.

S-($\alpha\beta$ -Diethoxycarbonyl-ethyl)glutathione, monosodium salt. A Thunberg tube containing GSH (614 mg.) and diethyl maleate (1 ml.) in aq. 50% (v/v) ethanol (20 ml.) neutralized to pH 8.5 with 1*N*-NaOH was incubated at 37° for 50 hr., until all the GSH had reacted. The reaction mixture was partially evaporated to remove ethanol and extracted with ether to remove excess of diethyl maleate. The aqueous solution was evaporated to dryness *in vacuo*, and the solid was reprecipitated several times from boiling ethanol by cooling. A hygroscopic white amorphous solid was obtained, m.p. 178° (decomp.) (Found: C, 42.5; H, 5.8; N, 8.5; S, 6.4; Na, 4.3; $\text{C}_{18}\text{H}_{28}\text{O}_{10}\text{N}_3\text{SNa}$ requires C, 43.1; H, 5.6; N, 8.4; S, 6.4; Na, 4.6%). On descending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (2:1:1, by vol.), the product gave one ninhydrin- and sulphur-positive spot at R_F 0.67 (R_F of GSH = 0.43). A similar spot was given by reaction mixtures containing GSH and diethyl maleate at pH 6.5 in the presence or

absence of rat liver supernatant. Bivalent sulphur compounds were detected with platonic iodide reagent (Toennies & Kolb, 1951). The methods of Ellman (1959) and Woodward & Fry (1932) showed no free thiol group in the conjugate. Paper chromatograms showed that some samples of the conjugate contained traces of GSSG (R_F 0.18) and an unidentified compound (R_F 0.49), the amounts of which depended on the quality of the GSH used.

Buffers. Pyrophosphate buffers were prepared from $K_4P_2O_7$ and $Na_2H_2P_2O_7$, phosphate buffers from KH_2PO_4 and Na_2HPO_4 , and acetate buffers from sodium acetate and acetic acid.

Thiol estimation. In all experiments, GSH was estimated by titration with iodate in the presence of iodide in sulphosalicylic acid; sodium starch glycolate was used as an indicator (Woodward & Fry, 1932).

Tissue preparations. Rat liver and rat kidney supernatants from female adult rats (Chester Beatty strain) were prepared as described by Booth *et al.* (1961) except that tissues were homogenized in 5 vol. of buffer. Dialysis of the supernatant was carried out in Visking tubing against glass-distilled water at pH 7.0. The small precipitate that separated on dialysis was removed by centrifugation. All operations were carried out below 10°. Liver supernatants from adult animals of different species were prepared as described above and were all stable on storage at -20°. All samples were prepared from freshly killed animals except for human liver samples, which were obtained within 6 hr. after death.

Adjustment of the pH of rat liver supernatants to pH 5.0 and calcium phosphate-gel adsorptions were carried out as described by Boyland & Chasseaud (1967), except that various supernatant/gel ratios were used.

Heat inactivation. Dialysed rat liver supernatant was added to a round-bottomed flask immersed in a water bath at 45°. The supernatant was constantly stirred, and samples were withdrawn at various intervals and immediately cooled in ice. The activities of the glutathione *S*-transferases in each sample were measured by the method outlined for the second substrate examined. Zero time was taken to be when the contents of the flask had attained water-bath temperature (about 5 min.). The pH of dialysed rat liver supernatants was 7.4 ± 0.2 and did not change during the inactivation process.

Acetone drying. Ice-cold acetone (9 vol.) was added quickly to stirred, ice-cold dialysed rat liver supernatant at pH 6.5 (1 vol.). The resulting precipitate was removed by centrifugation at 2000g for 5 min. The precipitate was filtered and thoroughly washed with ice-cold acetone to yield a dry powder containing glutathione *S*-transferases.

For estimations of enzyme activity, the powder was homogenized in 0.2 M-phosphate buffer, pH 6.5. Undissolved protein was removed by centrifugation and the supernatant assayed for activity.

Acid and ammonium sulphate fractionation. This was carried out by modifying (P. L. Grover, personal communication) the method of Booth *et al.* (1961) for the preparation of partially purified glutathione *S*-aryltransferase. Livers from female adult rats (Chester Beatty strain) were homogenized in 5 vol. of 0.1 M-phosphate buffer, pH 7.4, in an Ato-Mix blender (Measuring & Scientific Equipment, London, S.W. 1). The homogenate was centrifuged and the precipitate discarded. The supernatant was adjusted to pH 5.2 with acetic acid and, after 1.5 hr., centrifuged. The

precipitate was discarded, and the supernatant adjusted to pH 8.0 with 5 N-NaOH soln. Then $(NH_4)_2SO_4$ (as required) was added, with stirring, and after 30 min. the precipitate was removed by centrifugation and discarded. The supernatant was adjusted to pH 4.5 with acetic acid and, after 30 min., centrifuged. The precipitate, which contained glutathione *S*-transferases, was dissolved in 0.1 M-pyrophosphate buffer, pH 8.0, and the solution was adjusted to pH 8.0 with 5 N-NaOH soln. and dialysed for 18 hr. against glass-distilled water at pH 8.0. The precipitate that separated was removed by centrifugation and the supernatant assayed for activity. All procedures were carried out between 5° and 10°, and preparations were stable for at least 1 year when stored at -20°. Centrifugations were carried out at approx. 2000g for 30 min.

Fractionation with Sephadex G-200 gel. Rat liver homogenate was fractionated with 37% (w/v) $(NH_4)_2SO_4$ as described above; acetic acid was added to adjust the pH to 5.4, the precipitate was removed by centrifugation, and the supernatant was dialysed against 0.05 M-acetate buffer, pH 5.4. After centrifugation, the supernatant was concentrated by ultrafiltration to contain 100 mg. of protein/ml., and 1 ml. of this preparation was applied to a Sephadex G-200 column (20 cm. $\times$ 1 cm.) and eluted with 0.05 M-acetate buffer, pH 5.4, as described by Cohen, Smith & Turbert (1964). Fractions (1 ml.) were collected and assayed for activity.

Enzyme activity. Conjugations of GSH with $\alpha\beta$ -unsaturated substrates and other substrates were measured as described by Boyland & Chasseaud (1967). The amount of enzyme added was such that not more than 20% of the substrate reacted (see Dixon & Webb, 1964, p. 10) during the incubation. All determinations were corrected for any non-enzymic reaction.

RESULTS

To determine whether distinct enzymes catalyse reactions of GSH with $\alpha\beta$ -unsaturated compounds, four rather different substrates were studied, namely diethyl maleate (IA), cyclohex-2-en-1-one (IIIA), *trans*-benzylidenacetone (V) and *trans*-cinnamaldehyde (VIA) (Table 1 and Fig. 1).

Rat liver preparations catalysing reactions of these substrates with GSH were treated in various ways. Procedures that distinguished between the enzymes catalysing reactions of GSH with the above substrates were also used to study reactions of GSH with six other $\alpha\beta$ -unsaturated compounds (Table 1 and Fig. 1).

From observations reported below, it seems likely that six different enzymes catalysing conjugations of GSH with $\alpha\beta$ -unsaturated compounds have been detected (Table 1).

Heat inactivation. Heating of dialysed rat liver supernatant at 45° separated the catalytic activities towards reactions of GSH with compounds IA, IIIA, V and VIA (Fig. 2), indicating that different enzymes catalyse these reactions. At 40° the activities were poorly separated and at 50° all the enzymes were rapidly inactivated.

Fig. 1. $\alpha\beta$ -Unsaturated carbonyl compounds that are substrates for glutathione *S*-alkenyltransferases.

Table 1. *Glutathione S-alkenyltransferases*

The glutathione *S*-alkenyltransferases listed have not been individually named since the second substrate specificities have not been established. The enzymes listed were differentiated as described in the text.

Enzyme no.	Type of unsaturated substrate	Example	Compound no.
1	<i>cis</i> -Ester	Diethyl maleate	IA
		Ethyl acrylate	IB
2	<i>trans</i> -Ester	Diethyl fumarate	II
3	Cyclic ketone and possibly some lactones	Cyclohex-2-en-1-one	IIIA
		Parasorbic acid*	IIIB
4	Vinyl ketone	2,3-Dimethyl-4-(2-methylene-butyl)phenoxyacetic acid	IV
5	Open-chain <i>trans</i> -ketone (substituted vinyl ketone)	<i>trans</i> -Benzylideneacetone	V
6	<i>trans</i> -Aldehyde and possibly acetals	<i>trans</i> -Cinnamaldehyde	VIA
		<i>trans</i> -Hex-2-en-1-al	VIB
		<i>trans</i> -Cinnamaldehyde dimethyl-acetal	VIC

* δ -Lactone of 5-hydroxyhex-2-en-1-oic acid.

It is possible that only one enzyme catalysed reactions of GSH with $\alpha\beta$ -unsaturated compounds, and that the differences shown in Fig. 2 were due to altered enzyme-substrate affinities caused by heating; however, K_m values were unaltered by heat-treatment that caused loss of half the activity.

Fig. 3 shows the inactivation of glutathione

S-alkyltransferase, *S*-aryltransferase and *S*-epoxide-transferase at 45°. Inactivation of the enzyme catalysing the reaction of the $\alpha\beta$ -unsaturated compound IA with GSH is also shown in order that Figs. 2 and 3 may be compared. Glutathione *S*-aryltransferase and *S*-epoxidettransferase were not differentiated by this method.

Fig. 2. Decrease in the activities of enzymes catalysing reactions of GSH with diethyl maleate (IA) (○), cyclohex-2-en-1-one (IIIA) (●), *trans*-benzylideneacetone (V) (□) and *trans*-cinnamaldehyde (VIA) (△), by heating dialysed rat liver supernatant for 0-50 min. at pH 7.4 and 45°. The activities towards these substrates were measured as described in the text.

Fig. 3. Decrease in the activities of glutathione *S*-alkyltransferase (●), *S*-aryltransferase (□) and *S*-epoxidetransferase (△), and of the enzyme catalysing the reaction of GSH (5 mM) with diethyl maleate (IA) (5 mM) (○), by heating dialysed rat liver supernatant for 0-50 min. at pH 7.4 and 45°. The activities of these enzymes were measured as described in the text.

Fig. 4 shows results comparing esters IA, IB and II. The results indicate that the reactions of GSH with ester IA and ester II are catalysed by different enzymes. Boyland & Chasseaud (1967) showed that, although the non-enzymic reaction rate with GSH was greater for the *trans*-isomer II (velocity coefficient $5.3 \text{ M}^{-1} \text{ min}^{-1}$ at pH 6.5 and 25°) than for the *cis*-isomer IA (velocity coefficient $0.79 \text{ M}^{-1} \text{ min}^{-1}$ at pH 6.5 and 25°), the latter was a better substrate for enzymic conjugation. A possible explanation for this result is the existence of a different enzyme for each isomer. Heat-inactivation curves for esters IA and IB coincide, indicating

Fig. 4. Decrease in the activities of enzymes catalysing reactions of GSH with diethyl maleate (IA) (○), ethyl acrylate (IB) (△) and diethyl fumarate (II) (□), by heating dialysed rat liver supernatant for 0-50 min. at pH 7.4 and 45°. The activities towards these substrates were measured as described in the text.

Fig. 5. Decrease in the activity of the enzyme catalysing reactions of GSH with *trans*-cinnamaldehyde (VIA) (●), *trans*-hex-2-en-1-al (VIB) (□) and cinnamaldehyde dimethylacetal (VIC) (△), by heating dialysed rat liver supernatant for 0-50 min. at pH 7.4 and 45°. The activities towards these substrates were measured as described in the text.

that these substrates may be catalysed by the same enzyme (Fig. 4).

Reactions of GSH with aldehydes VIA and VIB and the acetal VIC (Fig. 5) may be catalysed by the same enzyme. The results in Fig. 6 indicate that different enzymes catalyse the reactions of GSH with the ketones IIIA, IV and V (which can occur in the less stable *cis*-form; Schinz, 1955). The results indicate that the same enzyme may catalyse the reactions of GSH with the lactone IIIB and the structurally similar compound IIIA (Fig. 6).

Comparison of Figs. 4, 5 and 6 showed that some

heat-inactivation curves overlapped, and separate experiments established that the curves for compounds II and V were almost identical. The heat-inactivation curves for compounds IA and IV were similar over 50 min. but were distinguished when heat-inactivation was continued for 200 min. at 45° (Table 2). Some activity towards compound IA remained, but the enzyme catalysing the reaction of GSH with compound IV was inactivated between 50 min. and 100 min. Table 2 shows that negligible activity remained towards compounds IIIA, IIIB, VIA, VIB and VIC after a dialysed rat liver supernatant had been heated for 100 min. at 45°.

The slopes of the heat-inactivation curves

Fig. 6. Decrease in the activities of enzymes catalysing reactions of GSH with cyclohex-2-en-1-one (IIIA) (○), parascorbic acid (IIIB) (△), 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid (IV) (▲) and *trans*-benzylideneacetone (V) (●), by heating dialysed rat liver supernatant for 0–50 min. at pH 7.4 and 45°. The activities towards these substrates were measured as described in the text.

(Figs. 2, 3, 4, 5 and 6) suggest that inactivation may be a two-stage process: an initial rapid inactivation followed by a slower one. Some of the curves may be composite, representing the inactivation of one or more enzymes catalysing the same reaction. In all the experiments, where results obtained for various substrates were compared, the substrates were always assayed with the same specimen of treated supernatant at each time-interval.

Ammonium sulphate fractionation. Booth *et al.* (1961) obtained a partially purified glutathione *S*-aryltransferase preparation by ammonium sulphate fractionation of rat liver homogenates. This preparation also contains other glutathione *S*-transferases. In a modification of the procedure of Booth *et al.* (1961), the quantities of ammonium sulphate used were varied in an attempt to separate activities towards the $\alpha\beta$ -unsaturated substrates.

Ammonium sulphate (47%, w/v) separated activities in rat liver preparations into two major groups in agreement with results obtained from heat-inactivation experiments. On the one hand the activity ratio (untreated/fractionated, A/B, Table 3) for substrates such as compounds IA and V was about 1, whereas for substrates such as compounds IIIA and VIA it was considerably greater than 1 (Table 3); this suggests that different enzymes are concerned with these two groups. The substrates for which an activity ratio of about 1 was obtained can be further subdivided into those with activity ratio slightly greater than 1, such as compounds IA and IB, and slightly less than 1, (as with glutathione *S*-aryltransferase) such as compounds II, IV and V: this result indicates that different enzymes catalyse the reactions of GSH with the substrates in these two sub-groups. Low activities were present in 47% (w/v) ammonium

Table 2. Heat-inactivation of dialysed rat liver supernatant for 200 min. at 45°

Equimolar amounts of GSH and second substrate were incubated for 4 min. at pH 6.5 and 25° with dialysed rat liver supernatant heated at 45° for various intervals of time.

Second substrate	Compound no.	Time (min.)	...	...	Activity remaining (%)				
					0	50	100	150	200
Diethyl maleate (5 mm)	IA	100			100	36	23	14	9
Ethyl acrylate (5 mm)	IB	100			100	35	18	18	—
Diethyl fumarate (5 mm)	II	100			100	56	39	28	22
Cyclohex-2-en-1-one (3 mm)	IIIA	100			100	4	0	0	—
Parascorbic acid (3 mm)	IIIB	100			100	0	0	0	—
2,3-Dimethyl-4-(2-methylenebutyryl)-phenoxyacetic acid (2 mm)	IV	100			100	37	0	0	—
<i>trans</i> -Benzylideneacetone (2 mm)	V	100			100	72	50	31	25
<i>trans</i> -Cinnamaldehyde (3 mm)	VIA	100			100	15	4	4	—
<i>trans</i> -Hex-2-en-1-al (3 mm)	VIB	100			100	17	0	0	—
Cinnamaldehyde dimethylacetal (3 mm)	VIC	100			100	16	3	3	—

Table 3. *Ammonium sulphate fractionation of rat liver preparations*

Preparation of $(\text{NH}_4)_2\text{SO}_4$ -fractionated rat liver supernatant was as described in the text. Equimolar amounts of GSH and second substrate were incubated for 4 min. at pH 6.5 and 25° in the presence of dialysed rat liver supernatant and $(\text{NH}_4)_2\text{SO}_4$ -fractionated rat liver supernatant. The activity ratio of glutathione *S*-aryltransferase was 0.4.

Second substrate	Compound no.	Activity ($\mu\text{moles of thiol lost/min./ml. of supernatant}$)		
		With untreated supernatant (A)	With ammonium sulphate-fractionated supernatant (B)	Activity ratio (A/B)
Diethyl maleate (5 mm)	IA	0.95	0.80	1.2
Ethyl acrylate (5 mm)	IB	0.40	0.35	1.1
Diethyl fumarate (5 mm)	II	0.50	0.75	0.7
Cyclohex-2-en-1-one (3 mm)	IIIA	8.5	0.75	11.3
Parasorbic acid (3 mm)	IIIB	3.45	0.48	7.2
2,3-Dimethyl-4-(2-methylenebutyl)-phenoxyacetic acid (2 mm)	IV	0.17	0.21	0.8
<i>trans</i> -Benzylideneacetone (2 mm)	V	0.32	0.48	0.7
<i>trans</i> -Cinnamaldehyde (3 mm)	VI A	6.75	1.35	5.0
<i>trans</i> -Hex-2-en-1-al (3 mm)	VI B	20.9	3.0	7.0
Cinnamaldehyde dimethylacetal (3 mm)	VIC	6.75	1.40	4.8

Table 4. *Distribution of enzymes catalysing reactions of glutathione with $\alpha\beta$ -unsaturated carbonyl compounds in dialysed rat liver and rat kidney supernatants*

The supernatants were prepared as described in the text. Equimolar amounts of GSH and second substrate were incubated for 4 min. at pH 6.5 and 25° in the presence of dialysed rat liver or rat kidney supernatants. Results were averaged from five separate experiments carried out with the same preparations for all substrates. The activity ratio for glutathione *S*-aryltransferase was about 30.

Second substrate	Compound no.	Activity ($\mu\text{moles of thiol lost/min./g. of tissue}$)		Activity ratio (L/K)	Non-enzymic loss of GSH (approx. % in 4 min.)
		Liver (L)	Kidney (K)		
Diethyl maleate (5 mm)	IA	6.9	3.1	2.2	2
Ethyl acrylate (5 mm)	IB	2.7	1.0	2.7	4
Diethyl fumarate (5 mm)	II	3.6	1.2	3.0	10
Cyclohex-2-en-1-one (3 mm)	IIIA	61.8	37.1	1.7	9
Parasorbic acid (3 mm)	IIIB	24.0	14.5	1.7	3
2,3-Dimethyl-4-(2-methylenebutyl)-phenoxyacetic acid (2 mm)	IV	1.3	0.6	2.2	6
<i>trans</i> -Benzylideneacetone (2 mm)	V	2.3	0.4	5.8	2
<i>trans</i> -Cinnamaldehyde (3 mm)	VI A	40.1	22.7	1.8	6
<i>trans</i> -Hex-2-en-1-al (3 mm)	VI B	136.2	81.7	1.7	10
Cinnamaldehyde dimethylacetal (3 mm)	VIC	47.2	25.2	1.9	6

sulphate-fractionated preparations towards substrates with activity ratios considerably greater than 1, such as compounds IIIA and VI A, so that although an activity ratio of 11.3 (compound IIIA) may be considered significantly different from 5.0 (compound VI A), an activity ratio of 7.0 (com-

pound VI B) is not significantly different from either 5.0 on the one hand or 11.3 on the other (Table 3).

Concentrations of ammonium sulphate in the range 37–47% (w/v) did not improve the separation of activities towards substrates IA, IIIA, V and

VI4; concentrations below 37% (w/v) were ineffective, and inconsistent results were obtained with concentrations above 47% (w/v).

Activity ratios obtained by comparing dialysed rat liver and rat kidney supernatants. Considerable activity towards $\alpha\beta$ -unsaturated substrates was present in kidney preparations (Table 4), and this was lowest towards compound V, when compared with liver preparations. Activity ratios (liver/kidney) obtained distinguished the enzyme catalysing the reaction of GSH with compound V from enzymes concerned with the remaining compounds (Table 4). Of the remaining compounds, no significant differences in the activity ratio were obtained except for compound II. The results indicate that compound II may be catalysed by an enzyme separate from the enzymes concerned with substrates, such as compounds IIIA and VIA, for which an activity ratio of less than 2.0 was obtained (Table 4).

The liver/kidney activity ratio obtained for the enzyme catalysing the reaction of compound IA with GSH was 4.4 when the activities of homogenates were measured (Boyland & Chasseaud, 1967), possibly because rat kidney homogenates contain enzymes that convert GSH into cysteine (Binkley, Davenport & Eastall, 1959), resulting in a decreased concentration of GSH for enzymic conjugation with compound IA in the incubation mixture. Kidney homogenates therefore show lower activity than the corresponding supernatant. About one-fifth as much activity towards substrates IA, IIIA, V and VI4 was present in lung, heart, blood and spleen (Chasseaud, 1967) as in liver supernatants.

Distribution in vertebrate species. The enzymes catalysing the reactions of compounds IIIA and VIA with GSH differed in their distribution in the livers of various animal species (Table 5). The values of the ratio of activities towards compounds IIIA and VIA varied more than fivefold. Similar

Table 5. *Distribution of enzymes catalysing reactions of glutathione with cyclohex-2-en-1-one (IIIA) and trans-cinnamaldehyde (VIA) in the livers of several animal species*

Liver supernatants were prepared as described in the text for rat liver. GSH (3mm) and second substrate (3mm) were incubated for 5 min. at pH 6.5 and 25° with liver supernatants from various animals. Both second substrates were always assayed with the same preparations. Mice were CBA/H strain and rats Chester Beatty strain.

Species	Sex	No. tested	Activity (μ moles of thiol lost/min./g. of tissue)		Activity ratio (IIIA/VIA)
			Cyclohex-2-en-1-one (IIIA)	trans-Cinnamaldehyde (VIA)	
Mouse	M	4*	71.3	22.5	3.2
Mouse	F	4*	64.0	14.4	4.4
Rat	F	24†	30.0-76.8	37.0-60.0	—
Rat	M	2*	54.0	36.0	1.5
Rat	F	—	61.8‡	40.1‡	1.5
Pigeon	M	1	32.5	6.3	5.2
Pigeon	F	1	15.6	4.5	3.5
Ferret	F	2	15.8, 22.8	19.2, 26.4	0.8, 0.9
Ferret	M	2	9.6, 10.4	10.8, 10.8	0.9, 1.0
Guinea pig	M	2	16.8, 21.5	24.0, 26.4	0.7, 0.8
Rabbit	M	1	18.6	12.0	1.5
Rabbit	F	2	0.4, 1.8	0.8, 2.6	0.5, 0.7
Dog	M	1	9.0	9.0	1.0
Hamster	F	1	6.7	7.8	0.9
Hamster	M	1	5.5	2.1	2.6
Man					
Foetus (14-week)	F	2*	1.7	0.7	2.4
Foetus (16-week)	M	2	1.4, 1.6	0.4, 0.7	3.5, 2.3
Foetus (23-week)	F	2	1.7, 1.7	0.8, 0.9	2.1, 1.9
Post-mortem specimen	F	2	0.8, 1.0	0.8, 1.0	1.0, 1.0

*Pooled tissues.

†Some rat livers were pooled.

‡Results from Table 4.

variations were observed when activities for compounds IIIA and VIA were compared with those obtained for compound IA by Boyland & Chasseaud (1967).

Inhibition by the glutathione conjugate of diethyl maleate (IA) of enzyme-catalysed conjugations of glutathione with diethyl maleate (IA), diethyl fumarate (II) and trans-benzylideneacetone (V). Some GSH conjugates are probably inhibitors of glutathione *S*-transferases (Chasseaud, 1967). *S*-($\alpha\beta$ -Diethoxycarbonyl)ethylglutathione, prepared by the reaction of GSH with diethyl maleate (IA), inhibited the enzyme-catalysed conjugations of GSH with compounds IA, II and V. A linear competitive Dixon (1953) plot was obtained for the inhibition of the enzymic reaction of GSH with compound IA; K_i was 0.5 mM (Fig. 7). For enzymic reactions of GSH with compounds II and V, Dixon (1953) plots were non-linear for inhibitor concentrations above 0.5 mM, and the apparent inhibitor constants (K'_i) were 0.05 mM and 0.44 mM respectively (Figs. 8 and 9). Greatly increased inhibition was observed with increasing inhibitor concentrations, showing either that the enzyme-inhibitor reaction was not truly reversible under these conditions, or that enzyme-inhibitor affinities increased with increasing inhibitor concentrations. It is also possible that trace amounts of a second inhibitor were present, affecting the reaction only at higher inhibitor concentrations.

The different nature of the Dixon (1953) plots obtained with a competitive inhibitor suggests

that the enzyme catalysing the reaction of GSH with compound II is different from the enzymes concerned with compounds IA and V.

Other fractionation procedures. Acetone-dried dialysed rat liver supernatant showed activity with all the substrates examined. The powders also contained glutathione *S*-aryltransferase and *S*-epoxidettransferase, although these were inactivated in similar experiments by Boyland & Williams (1965).

Adsorption of dialysed rat liver supernatant on calcium phosphate gel, with various supernatant/gel ratios, did not separate the activities towards the $\alpha\beta$ -unsaturated substrates IA, IIIA, V and VIA. However, the results showed that these compounds

Fig. 7. Competitive-type Dixon (1953) plots obtained with diethyl maleate (IA) (○, 5 mM; Δ, 2.5 mM) as substrate and *S*-($\alpha\beta$ -diethoxycarbonyl)ethylglutathione as inhibitor. Incubation was for 5 min. at pH 6.5 and 25° with GSH (5 mM) and (dialysed rat liver supernatant). *i*, Concn. of inhibitor (mM); *v*, initial velocity (GSH lost, mM).

Fig. 8. Competitive-type Dixon (1953) plots obtained with diethyl fumarate (II) (○, 5 mM; Δ, 2.5 mM; □, 1.75 mM) as substrate and *S*-($\alpha\beta$ -diethoxycarbonyl)ethylglutathione as inhibitor. Incubation was for 3 min. at pH 6.5 and 25° with GSH (5 mM) and dialysed rat liver supernatant. *i*, Concn. of inhibitor (mM); *v*, initial velocity (GSH lost, mM).

Fig. 9. Competitive-type Dixon (1953) plots obtained with trans-benzylideneacetone (V) (○, 2 mM; Δ, 1 mM; □, 0.7 mM) as substrate and *S*-($\alpha\beta$ -diethoxycarbonyl)ethylglutathione as inhibitor. Incubation was for 6 min. at pH 6.5 and 25° with GSH (2 mM) and dialysed rat liver supernatant. *i*, Concn. of inhibitor (mM); *v*, initial velocity (GSH lost, mM).

are not substrates for glutathione *S*-alkyltransferase and *S*-epoxidettransferase, which were distinguished from the enzyme catalysing the reaction of compound IA with GSH by the use of calcium phosphate gel (Boyland & Chasseaud, 1967).

Activities towards the $\alpha\beta$ -unsaturated substrates IA, IIIA, V and VIA were not separated by fractionation of the partially purified rat liver preparation described by Booth *et al.* (1961) on Sephadex G-200 gel.

Dialysis of rat liver supernatant or the lowering of the pH to 5.0 did not separate the activities towards the substrates IA, IIIA, V and VIA, and over 80% of the activity was retained in each case. The same procedures decreased the activities of glutathione *S*-alkyltransferase and *S*-epoxidettransferase by at least 50% (Boyland & Williams, 1965; Boyland & Chasseaud, 1967), suggesting that these enzymes are distinct from those concerned with the $\alpha\beta$ -unsaturated substrates IA, IIIA, V and VIA.

Effect of temperature on the rate of the enzyme-catalysed reaction of GSH with diethyl maleate (IA). Since optimum substrate concentrations have been determined for the enzyme-catalysed reaction of diethyl maleate (IA) with GSH (Boyland & Chasseaud, 1967), the effect of temperature on the rate of this reaction was also examined. Fig. 10 shows a break in the plot of the logarithm of velocity against the reciprocal of absolute temperature, corresponding to a change of activation energy at the transition temperature (32–35°). The energy of activation over the lower temperature range was 10.3 kcal./mole, and that over the higher range was 3.7 kcal./mole. The cause of this discontinuity is unknown, but experiments comparing the enzymic and the non-enzymic rates of reaction of GSH with diethyl maleate (compound IA) and diethyl fumarate (compound II) showed that the change was not due to a two-enzyme

process whereby isomerization of maleate to fumarate preceded conjugation with GSH. The latter would have occurred if the heat-inactivation curve (Fig. 4) for diethyl maleate (IA) was a composite of that for diethyl fumarate (II) and a *cis-trans*-isomerizing enzyme.

DISCUSSION

Previous work (Boyland & Chasseaud, 1967; Chasseaud, 1967) had shown that compounds of the general formula $RR'C:CR''A$ are conjugated with GSH by glutathione *S*-transferases present in the livers of various animal species and in different rat tissues. Reactions with GSH depend on the strength of the electron-attracting group A and on electron repulsion or attraction and probably steric effects exerted by the groups R, R' and R''. Until the specificities of the various glutathione *S*-transferases are established, it is proposed that a general name, glutathione *S*-alkenetransferases, be used to describe these enzymes.

The enzymes catalysing reactions of GSH with diethyl maleate (IA), cyclohex-2-en-1-one (IIIA), *trans*-benzylideneacetone (V) and *trans*-cinnamaldehyde (VIA) have been distinguished by their different sensitivities to heat, by ammonium sulphate fractionation and by consideration of the distribution of activities in rat liver and rat kidney and in the livers of several animal species.

The behaviour towards dialysis, lowering of pH, and calcium phosphate-gel adsorption showed that the glutathione *S*-alkenetransferases concerned with substrates IA, IIIA, V and VIA are different from the glutathione *S*-alkyltransferase and the *S*-epoxidettransferase previously described by Johnson (1966) and by Boyland & Williams (1965) respectively. Glutathione *S*-aryltransferase, first described by Booth *et al.* (1961), is different from these glutathione *S*-alkenetransferases, since activities in dialysed rat liver supernatants compared with 47% (w/v) ammonium sulphate-fractionated rat liver preparations were lower for the former enzyme and greater for the latter enzymes, with the exception of the enzyme catalysing the reaction of GSH with *trans*-benzylideneacetone (V) (Table 3); liver/kidney activity ratios also differed (see Table 4 and below). Heat-inactivation studies (cf. Figs. 2 and 3) showed that these glutathione *S*-alkenetransferases were different from the three previously described glutathione *S*-transferases, except that the enzyme catalysing the reaction of diethyl maleate (IA) with GSH was not distinguished from glutathione *S*-alkyltransferase, and the enzyme catalysing the reaction of *trans*-benzylideneacetone (V) with GSH was not distinguished from glutathione *S*-aryltransferase and *S*-epoxidettransferase.

Heat-inactivation studies suggested that at least

Fig. 10. Arrhenius plot for the effect of temperature (25–42°) on the rate of the enzyme-catalysed reaction of GSH (5mM) with diethyl maleate (IA) (5mM) in the presence of dialysed rat liver supernatant incubated for 3min. at pH 6.5. The enzyme was stable for at least 40 min. at 40°. T , absolute temperature (degrees); v , initial velocity (GSH lost, mM/5).

five glutathione *S*-alkenyltransferases, probably specific for second substrates containing $\alpha\beta$ -carbon-carbon double bonds activated by the carbonyl group, are present in rat liver. Inhibition by *S*-($\alpha\beta$ -diethoxycarbonyl)glutathione of enzyme-catalysed conjugations of GSH with diethyl fumarate (II) and *trans*-benzylideneacetone (V), and different activity ratios obtained for rat liver and rat kidney preparations, indicated that a sixth glutathione *S*-alkenyltransferase probably occurs (Table 1). Preliminary results suggest that reactions of GSH with $\alpha\beta$ -unsaturated compounds containing nitrile, nitro and sulphone activating groups may be catalysed by different enzymes (E. Boyland & L. F. Chasseaud, unpublished work).

Compounds containing aldehyde or acetal groups probably react with the same enzyme (Fig. 5). In aqueous solutions aldehydes exist in equilibrium with their hydrated forms:

(Dixon & Webb, 1964, p. 261), which may be the form binding to the enzymic active site. As different enzymes are required by the *cis* and *trans* isomers diethyl maleate (IA) and diethyl fumarate (II) (Fig. 4), glutathione *S*-alkenyltransferases may be specific for one conformation.

Liver is the best source of glutathione *S*-transferases in the rat but, with the exception of glutathione *S*-aryltransferase, the kidney also contains appreciable amounts. Johnson (1966) reported a liver/kidney activity ratio of 1.4 for glutathione *S*-alkyltransferase, Boyland & Williams (1965) found an activity ratio of 2.2 for glutathione *S*-epoxidettransferase, and an activity ratio of about 20 has been given for glutathione *S*-aryltransferase (Booth *et al.* 1961; Johnson, 1966; Suga, Ohata, Kumaoka & Akagi, 1967).

Al-Kassab *et al.* (1963) suggested that the enzyme catalysing conjugations of GSH with certain aryl halides and nitro compounds activated GSH, which could then react with electrophilic (acceptor) second substrates. The discovery of several glutathione *S*-transferases now shows that both GSH and the second substrate are specifically bound: further evidence for this has been provided by a study of the enzyme-catalysed reaction of GSH with a homologous series of alk-2-en-1-als (Chasseaud, 1967), which showed that non-enzymic reaction rates decreased with increasing alkyl chain length, as expected, but enzyme activity increased to a maximum with the C₉ member. The work of Johnson (1966) on glutathione *S*-alkyltransferase and of Clark, Darby & Smith (1967) on glutathione *S*-aryltransferase also showed that both GSH and second substrate are bound to the enzyme.

Several enzymes probably catalyse the conjugations of GSH with $\alpha\beta$ -unsaturated compounds. The identities of glutathione *S*-alkyltransferase, *S*-aryltransferase and *S*-epoxidettransferase have not been intensively studied, and it is possible that the wide second-substrate specificity attributed to these enzymes is narrower than was at first thought. The work of Booth *et al.* (1961), for example, suggested that reactions of GSH with 1,2-dichloro-4-nitrobenzene and benzyl chloride may be catalysed by different enzymes, since the latter non-competitively inhibits the reaction of the former with GSH. Suga *et al.* (1967) obtained a liver/kidney activity ratio of 1.4 for the enzyme catalysing the reaction of GSH with benzyl chloride, compared with 12.5 for that catalysing reaction with 1,2-dichloro-4-nitrobenzene.

The Arrhenius plot (Fig. 10) for the enzyme-catalysed reaction of diethyl maleate (IA) with GSH was discontinuous. A lower energy of activation was obtained at 38° (physiological temperatures). The causes of such phenomena are seldom clear although several explanations have been suggested (see Dixon & Webb, 1964, p. 158).

Whereas other detoxication processes, such as glucuronide or sulphate conjugation, require the initial formation of a 'high-energy' or 'active' intermediate involving ATP, the reaction of foreign compounds with GSH does not appear to do so.

Some substrates for enzyme-catalysed conjugations with GSH react rapidly, non-enzymically, with thiol groups, and a probable function of glutathione *S*-transferases in the cell is to protect important thiol-containing enzymes and other cellular proteins.

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