Distribution of Enzymes that Catalyse Reactions of Glutathione with $\alpha\beta$ -Unsaturated Compounds

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1. A study of the distribution of glutathione *S*-alkenyltransferases in the livers of vertebrate species suggests that different enzymes may catalyse reactions of GSH with (i) *trans*-benzylidenacetone, (ii) 2,3-dimethyl-4(2-methylenebutyl)phenoxyacetic acid, (iii) cinnamionitrile, (iv) *o*-chlorobenzylidenemalononitrile, (v) methyl vinyl sulphone, and (vi) 3-(β -nitrovinyl)indole. 2. Glutathione *S*-alkenyltransferase activity was generally greatest in rat liver, but the enzyme in hamster liver was more active towards *o*-chlorobenzylidenemalononitrile, and the enzyme in rabbit, hamster, guinea-pig and mouse livers was more active towards methyl vinyl sulphone. 3. Results from studies of the distribution of activities in rat liver and rat kidney, heat inactivation of rat liver supernatants, and $(\text{NH}_4)_2\text{SO}_4$ fractionation and acid-precipitation experiments, differentiated further between some of the enzymes concerned with substrates (i)-(vi). 4. The infrequent detection of mercapturic acids *in vivo* is discussed.

Reaction of foreign compounds with GSH is an initial stage in mercapturic acid biosynthesis *in vivo* (Barnes *et al.*, 1959; Boyland & Chasseaud, 1969a).

Boyland & Chasseaud (1968) showed that at least six separate enzymes (glutathione *S*-alkenyltransferases) appeared to catalyse reactions of GSH with $\alpha\beta$ -unsaturated carbonyl compounds. Reactions of GSH with some $\alpha\beta$ -unsaturated nitriles, sulphones, and nitro compounds are also catalysed by enzymes (Boyland & Chasseaud, 1967). The present paper describes the distribution of some of the enzymes involved.

Experimental

Materials

The sources of the materials used in these studies have been described previously (Boyland & Chasseaud, 1967, 1968). 3-(β -Nitrovinyl)indole was obtained from K & K Laboratories Inc. (Plainview, N.Y., U.S.A.), and *o*-chlorobenzylidenemalononitrile from R. N. Emanuel Ltd. (Wembley, Middx., U.K.).

Buffers

Phosphate buffers were prepared from KH_2PO_4 and Na_2HPO_4 .

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Thiol determination

In most experiments, GSH was determined by titration with iodate in the presence of iodide in sulphosalicylic acid (Woodward & Fry, 1932); sodium starch glycollate was used as an indicator. In incubation mixtures containing *o*-chlorobenzylidenemalononitrile as the second substrate, GSH was measured as described above or, more often, by the method of Ellman (1959), which uses the 5,5'-dithiobis-(2-nitrobenzoic acid) reagent. Quantitative recoveries of GSH were obtained by either method. Incubation mixtures containing 3-(β -nitrovinyl)indole were extracted with chloroform (2×3 ml) immediately before titration so as to remove the interfering yellow colour. On being left to stand, the yellow colour of the extracted incubation mixture returned, indicating decomposition of the reaction product of GSH and 3-(β -nitrovinyl)indole.

Tissue supernatants

The source and preparation of the dialysed tissue supernatants employed in these studies have been described by Boyland & Chasseaud (1969b).

Rat liver supernatant fractionation

Heat inactivation and $(\text{NH}_4)_2\text{SO}_4$ fractionation followed by acetic acid precipitation were carried out as described by Boyland & Chasseaud (1968). The

results obtained are based on at least three separate experiments.

Enzyme activity

This was measured as described by Boyland & Chasseaud (1967) except that equal concentrations of GSH and second substrate were always made to react. The following second substrate concentrations were used: *trans*-benzylideneacetone, 2mM; 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid, 2mM; cinnamionitrile, 2mM; *o*-chlorobenzylidenemalononitrile, 0.5mM; methyl vinyl sulphone, 2mM; 3-(β -nitrovinyl)indole, 0.5mM. The amount of enzyme added was such that less than 20% of the substrate reacted during the incubation, except when the tissue supernatant was very active and up to 30% of the substrate reacted. All determinations were corrected for the non-enzymic reaction occurring during the incubation. This was approx. 2% of *trans*-benzylideneacetone in 5 min, 6% of 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid in 5 min, 1% of cinnamionitrile in 60 min, 6% of *o*-chlorobenzylidenemalononitrile in 15 min, 5% of methyl vinyl sulphone in 6 min, and 3% of 3-(β -nitrovinyl)indole in 1 min at pH6.5 and 25°C. In any related set of experiments,

substrates were always assayed with the same specimen of treated supernatant.

Chromatography

T.l.c. was carried out on pre-layered Kieselgel F₂₅₄ plates of layer thickness 0.25mm obtained from E. Merck A.-G., Darmstadt, Germany. Paper chromatography was carried out by downward development on Whatman no. 1 paper. Butan-1-ol-acetic acid-water (2:1:1, by vol.) was used as the solvent system. Dried chromatograms were treated either with ninhydrin (in butan-1-ol or acetone) or with the platonic iodide reagent for the detection of bivalent sulphur compounds (Toennies & Kolb, 1951).

Results

The second substrates were chosen (Table 1) because their non-enzymic reaction with GSH was relatively slow at pH6.5, and because the rate of the enzymic reaction could be accurately measured except for the dinitrile (IV) which was included because of its toxicological interest [Report of the

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

Compound no.	Name	Formula
(I)	<i>trans</i> -Benzylideneacetone	$\text{PhCH}=\text{CHCOMe}$
(II)	2,3-Dimethyl-4-(2-methylenebutyryl)-phenoxyacetic acid
(III)	Cinnamionitrile	$\text{PhCH}=\text{CHCN}$
(IV)	<i>o</i> -Chlorobenzylidenemalononitrile
(V)	Methyl vinyl sulphone	$\text{CH}_2=\text{CHSO}_2\text{Me}$
(VI)	3-(β -Nitrovinyl)indole

Enquiry into the Medical and Toxicological Aspects of CS, Part II (1971) H.M.S.O., London].

Distribution of glutathione S-alkenotransferases in vertebrate species

The distribution of the enzymes catalysing reactions of GSH with compounds (I)–(VI) differed (Table 2) indicating that the enzymes concerned may be different. The enzymes least distinguished were those concerned with the ketone (I) and the nitrile (III) (Table 2) except that the mouse liver enzyme was relatively twice as active towards the nitrile (III). Also the human foetal liver enzyme was more active towards the nitrile (III) than the ferret liver and rabbit liver enzymes, whereas the converse obtained towards the ketone (I) (Table 2).

The activities of the enzyme catalysing the reaction of GSH with the ketone (II) were similar in rat, dog and mouse liver supernatants (Table 2), whereas the enzyme concerned with the nitro compound (VI) was at least twice as active in rat liver supernatants as in the other liver supernatants examined (Table 2).

The activities of the enzyme catalysing the reaction of GSH with the sulphone (V) were lower in rat liver supernatants than in rabbit, hamster, mouse and guinea-pig liver supernatants (Table 2). By contrast, activities of the other enzymes were relatively low in rabbit liver supernatant (Table 2).

The enzyme catalysing the reaction of GSH with the dinitrile (IV) was at least twice as active in hamster liver supernatant than in rat liver supernatant and fivefold more active when compared with other liver supernatants. Rabbit liver supernatant appeared to be inactive (Table 2).

Activities of glutathione S-alkenotransferases in dialysed rat liver and rat kidney preparations

The ratio of activities in liver and kidney of enzymes catalysing the reactions of GSH with compounds (I), (II), (III), (IV), (V) and (VI) were 5.8, 2.2, 7.0, 2.2, 4.9 and 1.6 respectively. Thus, enzymes concerned with substrates (II), (IV) and (VI) may be different from the remainder. The results were based on a minimum of four separate experiments. Comparable activity ratios for other glutathione S-alkenotransferases, catalysing reactions of GSH with $\alpha\beta$ -unsaturated carbonyl compounds, were in the range 1.7–5.8 (Boyland & Chasseaud, 1968), whereas that for glutathione S-alkyltransferase was 5.1, S-epoxidotransferase, 4.1, S-alkyltransferase, 8.3 and S-aryltransferase 47.6 (Boyland & Chasseaud, 1969b).

Other fractionation procedures

Boyland & Chasseaud (1968) distinguished between enzymes catalysing reactions of GSH with $\alpha\beta$ -unsaturated carbonyl compounds in rat liver supernatants by heat inactivation and by $(\text{NH}_4)_2\text{SO}_4$ fractionation followed by acid precipitation.

Heating of dialysed rat liver supernatants at 45°C separated the activity towards reaction of GSH with the nitro compound (VI) from that with the nitrile (III) and the sulphone (V) which were not distinguished (Fig. 1). The heat-inactivation curves of the enzymes conjugating the nitrile (III), the sulphone (V) and the nitro compound (VI) were dissimilar to those reported for the enzymes conjugating the ketones (I) and (II), which were also dissimilar (Boyland & Chasseaud, 1968). Results from heat-inactivation

Table 2. *Distribution of glutathione S-alkenotransferases in the livers of various vertebrate species*

Tissue preparations and measurements of enzyme activity are described in the text. The following were the mean activities of dialysed rat liver supernatant $\pm$ S.E.M. (μmol of thiol lost/min per g of tissue) (>20 rats): *trans*-benzylideneacetone (I), 2.25 ± 0.10 ; 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid (II), 1.28 ± 0.06 ; cinnamonnitrile (III), 0.12 ± 0.01 ; α -chlorobenzylidenemalononitrile (IV), 0.08 ± 0.02 ; methyl vinyl sulphone (V), 0.75 ± 0.04 ; and 3-(β -nitrovinyl)indole (VI), 24.5 ± 1.6 . The activities of the tissue supernatants are expressed relative to the activity of dialysed rat liver supernatant which was taken as 1.

Species	Compound no. ...	Activity					
		(I)	(II)	(III)	(IV)	(V)	(VI)
Rat ($\varphi + \delta$)		1.00	1.00	1.00	1.00	1.00	1.00
Mouse (δ)		0.45	0.93	0.74	0.51	2.98	0.25
Ferret (δ)		0.21	0.48	0.13	0.35	0.73	0.45
Rabbit (δ)		0.18	0.16	0.04	0	6.46	0.23
Pigeon (δ)		0.35	0.54	0.31	0.26	0.99	0.50
Human adult (φ)		0.10	0.26	0.07	0.26	0.33	0.02
Human foetus (δ)		0.15	0.41	0.21	0.26	0.29	0.07
Dog ($\varphi + \delta$)		0.35	1.03	0.27	0.33	0.65	0.27
Guinea pig (δ)		0.66	0.53	0.36	0.52	1.58	0.34
Hamster (δ)		0.69	0.51	0.61	2.72	4.68	0.20

studies of the enzyme catalysing the conjugation of GSH with the dinitrile (IV) were inconclusive because differences between the rates of the enzymic and the non-enzymic reaction were not sufficiently large.

By comparison of the activities to substrates (I)–(VI) in dialysed rat liver supernatants with those in partially purified $[(\text{NH}_4)_2\text{SO}_4\text{-fractionated-acid-precipitated}]$ rat liver supernatants, the activity ratios obtained were 0.7, 0.8, 1.2, 2.2, 0.9 and 5.2 respectively. Thus, enzymes concerned with substrates (IV) and (VI) were probably different from those concerned with the other substrates.

Nature of the non-enzymic and enzymic reaction of glutathione with $\alpha\beta$ -unsaturated compounds

The initial reaction with GSH probably occurs by addition of the nucleophile GS^- to the electrophilic β -carbon atom of the $\alpha\beta$ -double bond; thiols are known to react with $\alpha\beta$ -unsaturated compounds in this way (Mayo & Walling, 1940; Hurd & Gershbein, 1947; Knuth *et al.*, 1954; Patai & Rappoport, 1964; Friedman *et al.*, 1965; Black, 1966; Lough *et al.*, 1968).

At pH 6.5 and 25°C, non-enzymic reaction of GSH with compounds (I), (II) and (V) followed second-order kinetics with rate constants of about 1.8, 7.2 and $3.3 \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively. Reaction of GSH with the nitro compound (VI) followed second-order kinetics only for about the initial 10 min (rate constant of

about $48 \text{ M}^{-1} \cdot \text{min}^{-1}$) and appeared to reach equilibrium when about one-third of the GSH had reacted.

At pH 7.4 and 25°C, reaction of GSH with the dinitrile (IV) only followed second-order kinetics (rate constant of about $12.9 \text{ M}^{-1} \cdot \text{min}^{-1}$) when the dinitrile (IV) was preincubated with the buffer for at least 30 min. It is possible that in aqueous solution compound (IV) is in equilibrium with the hydrated form and the conversion of the hydrate into compound (IV) is the rate-limiting stage in the overall reaction of GSH with the dinitrile (IV).

Paper chromatography and/or t.l.c. of incubation mixtures containing GSH and any of the second substrates (I)–(VI) in the presence or in the absence of dialysed rat liver supernatant showed that only one ninhydrin- and sulphur-positive reaction product was formed. In reactions involving substrate concentrations of 2 mM, more of this product was formed in the presence of dialysed rat liver supernatant than in its absence.

Discussion

Application of procedures that distinguish between different enzymes (Dixon & Webb, 1964, p. 201) to enzyme-catalysed reactions of GSH with $\alpha\beta$ -unsaturated compounds containing carbonyl-, sulphone-, nitrile- and nitro-activating groups (Table 1) showed that several different enzymes (or enzyme combinations) were probably involved. This was initially deduced from differences in the distribution of activities in the livers of different vertebrate species (Table 2). By itself, this criterion is insufficient evidence of a multiplicity of enzymes since, for example, different forms of the same enzyme, with differing affinities for the substrate, may exist in different species, although this apparently seldom occurs (Dixon & Webb, 1964, p. 651). However, these results (Table 2) were further substantiated by those obtained from measurement of liver/kidney activity ratios, heat-inactivation studies (Fig. 1; see also Boyland & Chasseaud, 1968) and $(\text{NH}_4)_2\text{SO}_4$ -fractionation-acid-precipitation experiments.

The detailed metabolism of the second substrates used in these studies has not been reported, and none are known to be metabolized to mercapturic acids. However, the diuretic ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid], which is the dichloro analogue of the ketone (II) (Table 1), has been shown to be excreted as a cysteine conjugate by several species (C. C. Porter, unpublished work cited by Baer & Beyer, 1966), which presumably arises from initial conjugation of the ketone with GSH followed by catabolism of the GSH conjugate to the cysteine derivative. Conjugates with glutathione have the required properties (Brauer, 1959; Smith, 1966; Millburn, 1970) to be secreted in the bile. These conjugates and their related catabolites

Fig. 1. Decrease in the activities of the enzymes catalysing reactions of GSH with cinnamionitrile (III) (Δ), methyl vinyl sulphone (V) ($\circ$) and 3-(β -nitrovinyl)-indole (VI) ($\square$), by heating dialysed rat liver supernatant at pH 7.4 and 45°C

The activities towards these substrates were measured as described in the text.

(cysteine derivatives and mercapturic acids), if secreted in the bile, could be eliminated from the body via the faeces after probable deconjugation in the gut (by the flora). Such a mechanism would account for the infrequent detection of glutathione conjugates, cysteine conjugates or mercapturic acids after administration of some likely precursors.

The expected metabolic pathway for the riot-control agent, *o*-chlorobenzylidenemalononitrile (IV) would be hydrolysis at the $\alpha\beta$ -double bond to the corresponding aldehyde and malononitrile (Cucinell *et al.*, 1971), particularly since benzylidenemalononitriles have been reported to be spontaneously hydrolysed in aqueous solutions (Patai & Rappoport, 1962). However, there was no evidence of hydrolysis in the present study, but evidence of hydration of the double bond. Production of malononitrile is thought to account for one of the two distinct loci of toxicity observed after treatment of mice with the dinitrile (Jones & Israel, 1970). The other locus, which was of long-term duration, may be the result of the reaction with important thiol groups *in vivo*. The dinitrile (IV) causes extreme lacrimation in exposed persons (Rengstorff, 1969), a property of other reagents for thiol groups (Dixon, 1948).

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