Enzyme-Catalysed Conjugations of Glutathione with Unsaturated Compounds

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(*Received* 7 *November* 1966)

1. Rat-liver supernatant catalyses the reaction of diethyl maleate with glutathione. 2. Evidence is presented that the enzyme involved is different from the known glutathione-conjugating enzymes, glutathione *S*-alkyltransferase, *S*-aryltransferase and *S*-epoxidetransferase. 3. Rat-liver supernatant catalyses the reaction of a number of other $\alpha\beta$-unsaturated compounds, including aldehydes, ketones, lactones, nitriles and nitro compounds, with glutathione: separate enzymes may be responsible for these reactions.

Rat-liver supernatant preparations contain at least three enzymes that catalyse the conjugation of GSH with certain compounds: most of the conjugates are excreted from the body either as mercapturic acids or as cysteine derivatives. The enzyme glutathione *S*-aryltransferase is involved in the conjugation of aryl halide and nitro compounds (Booth, Boyland & Sims, 1961; Al-Kassab, Boyland & Williams, 1963; Grover & Sims, 1964) and this enzyme is also concerned in the metabolism of sulphobromophthalein (Combes & Stakelum, 1961). Glutathione *S*-epoxidetransferase is responsible for the formation of glutathione conjugates with several epoxides (Boyland & Williams, 1965). Johnson (1966) has described and partially purified an enzyme, glutathione *S*-alkyltransferase, that catalyses the *S*-alkylation of GSH by methyl iodide and other alkyl halides.

The present paper describes a further glutathione *S*-transferase, which catalyses the conjugation of GSH with $\alpha\beta$-unsaturated esters:

$$GSH + R^1R^2C{:}CR^3 \cdot CO \cdot OR^4 \rightarrow CR^1R^2(SG) \cdot CHR^3 \cdot CO \cdot OR^4 \quad (1)$$

The conjugation of GSH with several other $\alpha\beta$-unsaturated compounds, including aldehydes, ketones and lactones, is also catalysed by liver extracts, but it has not been definitely established whether these reactions are catalysed by the same or separate enzymes.

EXPERIMENTAL

Materials. GSH was obtained from Schwarz BioResearch Inc. (Mount Vernon, N.Y., U.S.A.), L(−)-cysteine from Roche Products Ltd. (Welwyn Garden City, Herts.) and GSSG from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Ethyl sorbate, α-methylcinnamaldehyde and ethyl *p*-nitrocinnamate were from the Eastman Kodak Co. (Rochester, N.Y., U.S.A.). K & K Laboratories Inc. (Plainview, N.Y., U.S.A.) supplied 3-methylcyclohex-2-en-1-one, 1-*p*-methoxyphenylpent-1-en-3-one, isopropyl *p*-methoxystyryl ketone, cinnamaldehyde dimethylacetal and methyl vinyl sulphone. Urocanic acid, acetylene-1,2-dicarboxylic acid, β-angelica lactone, sarkomycin (sodium salt), vinylene carbonate, hex-2-en-1-al and benzylideneacetone were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Ethacrynic acid and its dimethyl analogue [2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid] were from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.). Parasorbic acid was a gift from Dr P. Sims. The other substrates employed were commercially available from British Drug Houses Ltd. (Poole, Dorset), Hopkin and Williams Ltd. (Chadwell Heath, Essex) or Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.). Some of the above compounds were redistilled before use.

Pyrophosphate buffers were prepared from $K_4P_2O_7$ and $Na_2H_2P_2O_7$, and orthophosphate buffers from KH_2PO_4 and Na_2HPO_4. Acetate buffer, prepared from NaOH and acetic acid, was used for incubations involving ω-nitrostyrene and its dimethoxy derivative.

Thiol estimation. In most experiments GSH was estimated by the iodate titration method of Woodward & Fry (1932). The estimation of cysteine and of thiol concentrations below 1mM was carried out with the more sensitive method of Ellman (1959), which uses the 5,5′-dithiobis-(2-nitrobenzoic acid) reagent.

Tissue preparations. The method of Booth *et al.* (1961) was employed in the preparation of rat-liver supernatant fractions except that livers were homogenized in 5vol. of buffer and were not dialysed unless otherwise stated. Dialysis of rat-liver supernatant was carried out in Visking tubing against glass-distilled water neutralized with NaOH solution. The activity of the various glutathione *S*-transferases was lowered by bringing the pH of rat-liver supernatant to 5·0 with acetic acid, leaving at room temperature for 15min. and removing the precipitate by centrifugation; a second precipitate, formed when the pH was raised to

6·5 with NaOH, was removed by centrifugation. Adsorption on Tiselius (1954) 20% (w/v) $Ca_3(PO_4)_2$ gel was carried out by shaking (v/v) dialysed rat-liver supernatant (7 parts) and the gel (11 parts) for 15min. at below 5° and at pH 7·3. The gel was then removed by centrifugation, yielding an almost colourless supernatant that was nearly free of glutathione *S*-alkyltransferase and *S*-epoxidetransferase. The partially purified glutathione *S*-aryltransferase preparation was obtained by the method of Booth *et al.* (1961). Preparations described above were still active after storage for several weeks at approx. −20°.

Liver supernatant preparations from animals of other species were also prepared as above and were stable after storage at low temperature.

Homogenates from various rat tissues were prepared as described by Booth *et al.* (1961) and used immediately.

Enzyme activity. Estimation of the conjugation of GSH and αβ-unsaturated compounds was carried out by incubating equal concentrations of GSH and the second substrate in 0·2M-orthophosphate buffer, pH 6·5 (total vol. 4ml.), in the presence and absence of liver preparations. After the appropriate time-interval had elapsed, an equal volume of aq. 4% (w/v) sulphosalicylic acid was added to stop the reaction and to precipitate protein by lowering the pH to approx. 2. Protein was separated by centrifugation and GSH estimated by the iodate or 5,5′-dithiobis-(2-nitrobenzoic acid) method. The use of Thunberg tubes, providing anaerobic conditions, for longer incubations (1hr.) made no significant difference to the results obtained.

Reactions with methyl iodide and 2,3-epoxypropyl phenyl ether were estimated as described for αβ-unsaturated substrates, except that substrate concentrations of 3mM were employed.

1,2-Dichloro-4-nitrobenzene (0·75mM) was allowed to react with GSH (1mM) under the conditions described above. In the comparison of rat-liver and ferret-liver preparations, the activity towards 1,2-dichloro-4-nitrobenzene was estimated by the method of Grover & Sims (1963) except that a Unicam SP.800 ultraviolet recording spectrophotometer was used.

Most reaction mixtures were examined by chromatography by downward development (16hr.) on Whatman no. 1 paper with butan-1-ol–acetic acid–water (2:1:1, by vol.). The dried chromatograms were treated either with ninhydrin (in acetone) or, for the detection of bivalent sulphur compounds, with the platinic iodide reagent (Toennies & Kolb, 1951).

RESULTS

Catalysis of the reaction between glutathione and αβ-unsaturated compounds. Diethyl maleate was chosen as a substrate for the estimation of the enzyme since it was fairly soluble in water and reacted only slowly with GSH at pH 6·5 in the absence of enzyme, and because the reaction rate was greatly increased by rat-liver supernatant over a convenient time-interval. The catalytic activity of rat-liver supernatant preparations in the reaction of diethyl maleate with GSH was stable to dialysis and was destroyed by heating at 90° for 10min. or at 50° for 1hr.

The pH–activity curve for the enzymic conjugation of diethyl maleate with GSH showed an optimum at pH 7·6 (Fig. 1), but, to lower the rate of the non-enzymic reaction and the aerobic oxidation of GSH, studies with diethyl maleate and related compounds were carried out at pH 6·5. At more alkaline pH values, the rate of the spontaneous reaction between GSH and diethyl maleate increases rapidly and an incubation time of 1min. was employed in pH–activity measurements to ensure that substrate concentrations did not fall by more than 20% (see Dixon & Webb, 1964). At pH 6·5 the optimum substrate concentration for both GSH and diethyl maleate was 5mM (Fig. 2). Lineweaver–Burk plots, obtained under the experi-

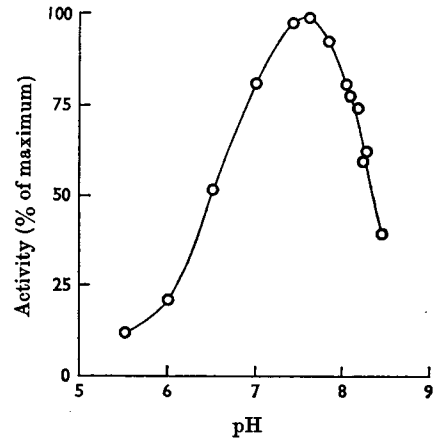

Fig. 1. pH–activity curve. Reaction mixtures contained GSH (5mM), diethyl maleate (5mM) and dialysed rat-liver supernatant in 0·2M-pyrophosphate buffer. Incubation was for 1min. at 25°.

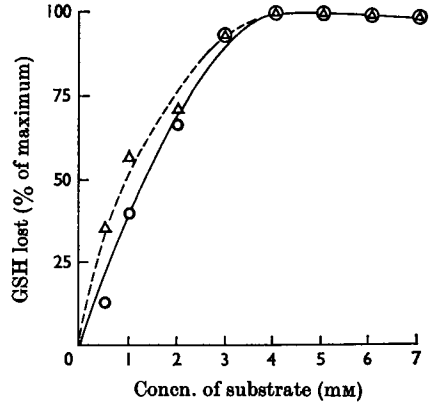

Fig. 2. Velocity–substrate concentration curves for diethyl maleate (○) in the presence of 5mM-GSH and for GSH (△) in the presence of 5mM-diethyl maleate. Incubation was for 5min. at 25° and pH 6·5.

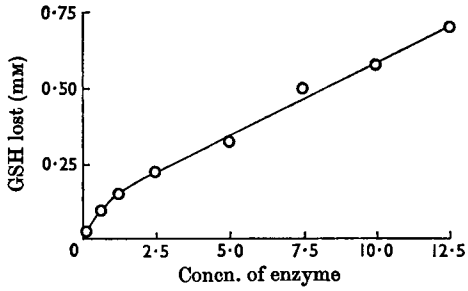

Fig. 3. Velocity–enzyme concentration curve with GSH (5mM) and diethyl maleate (5mM) incubated in 0·2M-orthophosphate buffer, pH6·5, for 5min. at 25°. Enzyme concentrations are expressed as % (v/v) of dialysed rat-liver supernatant in the reaction mixture.

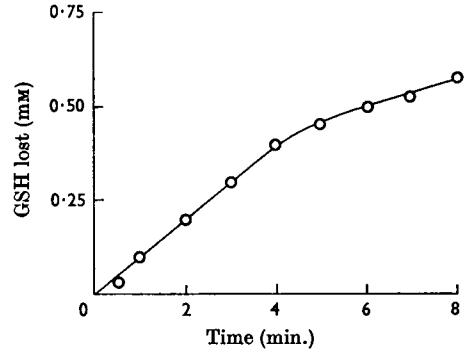

Fig. 4. Time–activity curve obtained with GSH (5mM) and diethyl maleate (5mM) at 25° and pH6·5.

mental conditions employed for the optimum-substrate-concentration curves, gave K_m values 1·8mM for diethyl maleate and 1·2mM for GSH. The difference in these K_m values suggests that the results expressed by Fig. 2 are best represented by two curves. The velocity–enzyme concentration curve (Fig. 3) with dialysed rat-liver supernatant was non-linear at low enzyme concentrations. The time–activity curve (Fig. 4) was non-linear over the time-range studied.

The enzyme was unaffected by addition of up to 5% (v/v) ethanol, but with 15% (v/v) ethanol, 7% (v/v) acetone, 8% (v/v) dimethylformamide or 17% (v/v) methylCellosolve the activity was halved.

At pH6·5, the velocity coefficient for the non-enzymic reaction of GSH with diethyl maleate was $0·79 M^{-1} min.^{-1}$ at 25°. The reaction obeyed the bimolecular rate equation.

Dialysed rat-liver supernatant did not catalyse the reaction of L(−)-cysteine with diethyl maleate.

Separation of the enzyme catalysing the conjugation of glutathione with diethyl maleate from glutathione S-alkyltransferase, S-aryltransferase and S-epoxidetransferase. The partially purified glutathione *S*-aryltransferase preparation of Booth *et al.* (1961), from which the activities of glutathione *S*-alkyltransferase and *S*-epoxidetransferase are substantially removed, was relatively as active in catalysing the reaction of GSH with diethyl maleate as a crude rat-liver supernatant. Dialysis of soluble rat-liver supernatant removed about 50% of the activities of glutathione *S*-alkyltransferase and *S*-epoxide-

Table 1. *Separation of the enzyme catalysing the conjugation of glutathione with diethyl maleate from glutathione S-alkyltransferase, S-aryltransferase and S-epoxidetransferase*

The measurement of activity with these substrates and the preparation of rat-liver extracts were as described in the text. The results are expressed as the average of at least five separate experiments, except for the results with partially purified glutathione *S*-aryltransferase, which are averaged from three separate experiments.

Treatment of rat-liver supernatant	% of activity remaining towards: Methyl iodide	2,3-Epoxypropyl phenyl ether	1,2-Dichloro-4-nitrobenzene	Diethyl maleate
Undialysed rat-liver supernatant	100	100	100	100
Dialysis against distilled water	47	50	68	83
Supernatant obtained after lowering to pH5·0 and then raising to pH6·5	36	22	70	83
Supernatant obtained after dialysis followed by absorption with 20% (w/v) $Ca_3(PO_4)_2$ gel	2	6*	48	28
Relative activity of partially purified glutathione *S*-aryltransferase and rat-liver supernatant: the activity ratio towards 1,2-dichloro-4-nitrobenzene was taken as 100% in each case	11	13	100	40

* This value was not consistent, ranging from 0 to 15.

transferase, but over 80% of the activity towards diethyl maleate was retained. Similar results were obtained whether dialysis was carried out over a 16hr. period with no change of distilled water, or for 3–4 days with three changes of distilled water. Table 1 shows that the enzyme catalysing the reaction of diethyl maleate with GSH is distinct from the three known glutathione *S*-transferases and outlines the procedures employed in their separation.

Distribution of the enzyme in rat tissues. The activities shown by homogenates obtained from various rat tissues are given in Table 2. Liver was four times as active as any other tissue. Activity in tissues other than liver fluctuated somewhat with different rats, but the results shown in Table 2 are typical. The results in Table 2 may be compared with those obtained by Johnson (1966), who showed that glutathione *S*-alkyltransferase in kidney was almost as active as in liver preparations, whereas that of glutathione *S*-aryltransferase was some 20-fold less in kidney preparations when compared with liver extracts.

Distribution of the diethyl maleate-conjugating enzyme in vertebrate species. Table 3 shows the distribution of the enzyme in liver fractions obtained from various species. The highest activity was shown by mouse-liver preparations. Guinea-pig preparations were also more active than rat preparations, but those of the ferret and rat were comparable. The ferret-liver preparations examined showed the same degree of activity towards 1,2-dichloro-4-nitrobenzene as did rat-liver preparations. This result differs from that obtained with ferret-liver preparations by Boyland & Williams (1965), who found no glutathione-*S*-aryltransferase activity.

Formation of glutathione conjugates with αβ-unsaturated compounds. Several αβ-unsaturated compounds were assayed as possible substrates and the results obtained have all been corrected for the non-enzymic reaction. Aldehydes, esters, lactones and ketones are grouped in Table 4, since

Table 2. *Distribution of the enzyme in the homogenates obtained from various rat tissues*

Incubation mixtures contained GSH (5mM), diethyl maleate (5mM) and tissue homogenates in 0·2M-orthophosphate buffer, pH6·5 (total vol. 4ml.). The results given are the average of three experiments.

Tissue	Activity (μmoles of SH lost/min./g. of tissue)
Liver	9·3
Lung	2·7
Kidney	2·1
Heart	1·2
Spleen	1·0
Blood	1·0

Table 3. *Catalysis of the conjugation of glutathione with diethyl maleate by liver preparations obtained from different species*

Supernatant preparations from various species were incubated with GSH (5mM) and diethyl maleate (5mM) at pH6·5 and 25°.

Species	No. tested	Activity (μmoles of SH lost/min./g. of tissue)
Mouse (CBA/H strain) (♀)	4*	26·0
Mouse (CBA/H strain) (♂)	1	18·4
Guinea pig (♂)	2	14·8, 16·4
Ferret (♀)	2	9·3, 12·0
Ferret (♂)	2	6·7, 8·0
Rat (Chester Beatty strain) (♀)	23†	9·0–11·0
Rat (Chester Beatty strain) (♂)	2*	8·7
Hamster (♂)	1	9·6
Hamster (♀)	1	8·3
Pigeon (♂)	1	5·4
Pigeon (♀)	1	5·0
Dog (♂)	1	3·3
Rabbit (♂)	1	5·3
Rabbit (♀)	3	1·2, 2·0, 2·6
Man		
Foetus (14 weeks) (♀)	2*	0·6
Foetus (16 weeks) (♂)	2	0·8, 1·7
Foetus (23 weeks) (♀)	2	1·1, 1·1

* Pooled tissues.

† Some rat livers were pooled.

Table 4. *Catalysis of the conjugation of αβ-unsaturated compounds with glutathione in the presence of dialysed rat-liver supernatant*

Where possible, uniformity of conditions was employed for related substances. In certain cases, however, owing to the insufficient reactivity of some of these compounds, both incubation times and enzyme concentrations were increased. A negative result indicates no detectable activity with dialysed rat-liver supernatant for at least 1 hr. Incubation was at pH 6·5 and 25°. The results shown are the average of two experiments.

Compound	Concn. (mM)	Incubation time (min.)	Supernatant concn. (%, v/v)	Activity (μmoles of SH lost/min./g. of tissue)
Diethyl maleate	5	5	6·25	10·4
Diethyl fumarate	5	5	6·25	4·8
Ethyl acrylate	5	5	6·25	2·5
Dimethyl itaconate	5	5	6·25	0·5
Methyl methacrylate	5	60	25·0	0
Ethyl crotonate	1	60	25·0	0·02
Ethyl *p*-nitrocinnamate	1*	60	25·0	0·04
Ethyl cinnamate	1	60	25·0	0·01
Ethyl sorbate	1	60	25·0	0·008
Ethyl vinyl ketone	1	1	0·5	~145
2,3-Dichloro-4-(2-methylenebutyryl)-phenoxyacetic acid (ethacrynic acid)	1	1·5	5·0	5·6
2,3-Dimethyl-4-(2-methylenebutyryl)-phenoxyacetic acid	1	5	12·5	1·1
Benzylideneacetone	1	5	12·5	1·7
Benzylideneacetophenone	1*	5	12·5	0·4
1-*p*-Methoxyphenylpent-1-en-3-one	1	5	12·5	0·7
Isopropyl *p*-methoxystyryl ketone	1	5	12·5	0·3
Mesityl oxide	3	60	25·0	0·06
Cyclopent-2-en-1-one	3	5	0·5	32
Cyclohex-2-en-1-one	3	5	0·5	44
3-Methylcyclohex-2-en-1-one	3	60	25·0	0·02
3,5,5-Trimethylcyclohex-2-en-1-one (isophorone)	3	60	25·0	0·02
Sarkomycin (sodium salt)	3	60	25·0	0
Santonin	0·5	60	25·0	0
Coumarin	0·5	60	25·0	0
β-Angelica lactone	3	5	12·5	0·8
Parasorbic acid	3	5	0·5	27
Dehydroacetic acid	3	60	25·0	0
Crotonaldehyde	3	2	1·0	50
Hex-2-en-1-al	3	2	1·0	134
Cinnamaldehyde	3	2	1·0	45
α-Methylcinnamaldehyde	3	60	25·0	0·14
Cinnamaldehyde dimethylacetal	3	2	1·0	62
Acrolein diethylacetal	3	0·25	1·0	~350

* GSH concn. 1 mM and second substrate concn. 0·25 mM.

all contain the carbonyl activating group. Carboxylic acids are grouped with nitriles, nitro compounds and miscellaneous other substrates in Table 5. Owing to the differing degrees with which these substances reacted with GSH, reaction conditions were different for groups of compounds and, in some cases, for the individual compounds in a particular group. The fact that reaction times and enzyme concentrations were dissimilar for particular substrates reflected on the reactivity of these substrates and in some cases strict comparisons cannot be made. The results do, however, permit inferences to be drawn as to the effect of substituents or activating groups on reactivity. Where the use of ethanol was necessary to ensure complete solubility of substrate, this did not exceed 5% (v/v) in the reaction mixture. For this reason lower substrate concentrations were necessary in many cases.

To confirm that the loss of GSH as measured by the methods outlined above was due to conjugation with the αβ-unsaturated compounds and not to

Table 5. *Catalysis of the conjugation of αβ-unsaturated compounds with glutathione in the presence of dialysed rat-liver preparations*

The conditions of reaction are as outlined for the substrates in Table 4. With ω-nitrostyrene and dimethoxy-ω-nitrostyrene, pH 5·0 was used owing to the very rapid spontaneous reaction that occurred at pH 6·5.

Compound	Concn. (mM)	Incubation time (min.)	Supernatant concn. (%, v/v)	Activity (μmoles of SH lost/min./g. of tissue)
Maleic acid	3	60	25·0	0·11
Fumaric acid	3	60	25·0	0
Citraconic acid	3	60	25·0	0
ββ-Dimethylacrylic acid	1	60	25·0	0
Sorbic acid	1	60	25·0	0
Urocanic acid	3	60	25·0	0
Cinnamic acid	3	60	25·0	0
p-Nitrocinnamic acid	0·5	60	25·0	0
Coumalic acid	3	60	25·0	0
Acetylene-1,2-dicarboxylic acid	3	60	25·0	0
Acrylonitrile	1	60	25·0	0·04
o-Chlorobenzylidenemalononitrile	1	30	25·0	0·09
o-Hydroxybenzylidenemalononitrile	1	60	25·0	0·04
ω-Nitrostyrene	1*	1	0·5	95
3,4-Dimethoxy-ω-nitrostyrene	1*	1	0·5	68
3-Nitrostyrene	1	60	25·0	0
Methyl vinyl sulphone	3	5	25·0	1·4
Vinyl acetate	3	60	25·0	0·1
Vinylene carbonate	3	60	25·0	0·02
Dihydropyran	3	60	25·0	0
Maleic hydrazide	3	60	25·0	0

* GSH concn. 1 mM and second substrate concn. 0·5 mM.

oxidation of the thiol, the reaction mixtures were examined on chromatograms: each substrate gave only one additional ninhydrin- and sulphur-positive spot, other than those given by the controls, with the exception of the group of aldehydes assayed. After cinnamaldehyde, for example, was incubated for 1 hr. the reaction mixture showed one ninhydrin- and sulphur-positive conjugate spot. With further incubation an additional ninhydrin- and sulphur-positive spot appeared and on longer incubation (16 hr.) the latter spot was dominant. This result suggests that some breakdown of conjugate was occurring. A similar pattern was observed for crotonaldehyde and cinnamaldehyde dimethylacetal, but hex-2-en-1-al gave only one ninhydrin- and sulphur-positive spot.

DISCUSSION

The formation of glutathione conjugates has been shown to be the initial step in the excretion of foreign compounds as mercapturic acids (Bray, Franklin & James, 1959; Booth *et al.* 1961). The intermediate steps involved breakdown of the glutathione conjugate to the cysteine derivative and acetylation of this to yield the mercapturic acid. Barnes, James & Wood (1959) found that GSH concentrations in the liver fell when compounds excreted as mercapturic acids were administered to rats. Johnson (1965) obtained similar results with certain alkyl halides.

Among the compounds assayed as possible substrates for conjugation with GSH by Booth *et al.* (1961) and Boyland & Williams (1965) were several known to be excreted as mercapturic acids. None of the substrates examined in the present work have been shown to be excreted as mercapturic acids: studies of the metabolism of these compounds have, however, been few. Three, at least, of the substrates examined in this paper occur naturally: cinnamaldehyde occurs in cinnamon oils, parasorbic acid has been isolated from the ripe berries of the mountain ash and hex-2-en-1-al has been found in several sources (Forss, Dunstone, Ranshaw & Stark, 1962; Watanabe, 1958). The substrates catalysed by glutathione *S*-alkyltransferase, *S*-aryltransferase and *S*-epoxidetransferase do not include any that are naturally occurring.

Unsaturated esters such as ethyl acrylate exhibit lachrymatory and irritant properties. Treon, Sigmon, Wright & Kitzmiller (1949) noted that ethyl acrylate is ten times as toxic as the saturated

ethyl propionate, but found no signs of poisoning in rabbits, guinea pigs and rats after inhalation of the ester at amounts up to and including 75 p.p.m. There was also an absence of a cumulative effect on repeated oral ingestion of amounts approaching a tenth of the oral LD_{50}. This lack of toxic effect may be due to a glutathione-conjugating system, which metabolizes the lower doses administered. The position of the double bond relative to the activating carbonyl moiety and the position of a substituent on the α- or β-carbon atom of the carbon–carbon double bond have been cited as important factors in the biological action of these esters (Fassett, 1963). The same criteria apply to chemical reactivity and enzyme activity, since ethyl crotonate, with a methyl group β to the carbonyl moiety, was less active than ethyl acrylate. Methyl methacrylate, with a methyl group α to the carbonyl group, was inactive (Table 4). Vinyl acetate, in which the carbonyl group is separated from the carbon–carbon double bond by an oxygen atom, is a less reactive substrate than ethyl acrylate. Additional activating groups at either the α- or β-carbon atoms of the carbon–carbon double bond should increase the activity because of increased polarization of the double bond: a comparison of the activities of diethyl maleate with that of ethyl acrylate illustrates this.

The reactivities of $\alpha\beta$-unsaturated compounds with nucleophiles may, to some degree, be influenced by steric effects (Patai & Rappoport, 1964), but the present study does not permit any assessment of steric contributions to enzyme action to be made. However, the behaviour of the geometric isomers, diethyl maleate and diethyl fumarate, may, in part, be governed by steric factors, since the enzymic reaction is slower with the chemically more reactive *trans*-isomer, diethyl fumarate, than with the chemically less reactive *cis*-isomer, diethyl maleate. In the non-enzymic reaction with GSH, maleic acid was more reactive than fumaric acid (no reaction detected for fumaric acid after 1hr. incubation with GSH). With the corresponding esters, for non-enzymic reaction with GSH diethyl fumarate is more reactive than diethyl maleate, but the converse occurred in the presence of enzyme.

Haynes (1948) outlined several physiological properties shown by parasorbic acid and other unsaturated lactones that contained the $\alpha\beta$-unsaturated structure. Of the carcinogenic lactones containing the $\alpha\beta$-unsaturated system (Dickens, 1964), two reacted rapidly with GSH in the presence of rat-liver preparations (Table 4). The lower reactivity of β-angelica lactone may be due to the compound's dimerizing in solution. The effect of substitution by electron donor groups on the α- and β-carbon atoms cannot be the only important factor defining reactivity. It is probable that steric and electronic effects due to adjacent rings and substituents within the molecule also play a vital part. Coumarin, for example, did not react with GSH in the presence of dialysed rat-liver supernatant.

Ethacrynic acid and related compounds (derivatives of aryloxyacetic acids) are useful diuretics (Baer & Bayer, 1966). Ethacrynic acid, which is excreted as the cysteine conjugate (Baer & Beyer, 1966), reacted very rapidly with GSH in the presence of rat-liver preparations (Table 4); the formation of a glutathione conjugate is the first step in the production of the cysteine adduct *in vivo*.

The ketones 1-*p*-methoxyphenylpent-1-en-3-one and isopropyl *p*-methoxystyryl ketone, which are permitted food additives, were both substrates for the enzyme (Table 4).

Crotonaldehyde reacted spontaneously with GSH more rapidly than did hex-2-en-1-al, but the latter was a better substrate for conjugation in the presence of enzyme. This result shows that there is no strict relationship between reactivity with and without enzyme. Cinnamaldehyde, a compound used in foods and perfumery, reacted at about the same rate as the corresponding dimethylacetal (Table 4), indicating that the addition of GSH occurs on the $\alpha\beta$-double bond rather than on the aldehyde grouping, to give a thiazolidine, a possibility suggested by Libermann (1946).

Maleic acid was the only unsaturated acid studied that reacted spontaneously with GSH and even more rapidly in the presence of rat-liver preparations. Angielski & Rogulski (1961) examined the reaction of maleic acid with GSH in the presence of kidney homogenate and obtained an increase in reaction rate; they concluded that GSH was broken down by the kidney preparation to cysteine, which then reacted with maleic acid more rapidly than the tripeptide. Calam & Waley (1963) have found cysteine and GSH conjugates of maleic acid in extracts from calf lens, and Kuwaki & Mizuhara (1966) have demonstrated the presence of the cysteine derivative in human urine and animal kidney.

With the nitro olefins the reaction was exceedingly rapid and catalysis by rat-liver preparations could be seen at pH 5·0. Certain other nitro olefins occur as air pollutants, probably being formed from the reaction of unsaturated hydrocarbons in petrol with the nitrogen oxides formed during combustion (Deichmann *et al.* 1965).

The reaction of GSH with $\alpha\beta$-unsaturated compounds involves the addition of the nucleophile GS^- to the β-carbon atom of the double bond polarized by conjugation with strongly electron-withdrawing groups. The reaction is probably governed by electronic and steric factors. The nitro group was

the most potent of the electron-withdrawing groups studied, as illustrated by ω-nitrostyrene (Table 5). In most cases it was ascertained by paper chromatography that the loss of thiol was not due to the production of GSSG.

Preliminary experiments, including results obtained from heat-inactivation experiments, activity ratios with separate enzyme preparations, tissue specificity and the activity in different species, have indicated that the reactions of $\alpha\beta$-unsaturated compounds with GSH are catalysed by different enzymes (E. Boyland & L. F. Chasseaud, unpublished work). The enzyme catalysing the reaction of GSH with unsaturated esters may be distinct from that involved with unsaturated aldehydes or ketones.

L.F.C. is grateful to the Nuffield Foundation for a Food Safety Scholarship. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign for Research and by Public Health Service Grant no. CA-03188-09 from the National Cancer Institute, U.S. Public Health Service.

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