An Enzyme Catalysing the Conjugation of Epoxides with Glutathione

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1. Liver supernatant preparations from rats and ferrets catalyse the conjugation of some epoxides with glutathione. The enzyme involved might be called 'glutathione *S*-epoxidetransferase', as it is different from glutathione *S*-aryltransferase, the enzyme catalysing the conjugation of 1,2-dichloro-4-nitrobenzene, 4-nitropyridine *N*-oxide and other cyclic compounds with glutathione and from the enzyme catalysing the conjugation of iodomethane and glutathione. 2. The enzyme does not catalyse the reaction with cysteine. It is not inactivated by dialysis but is unstable at pH 5.0. 3. The role of the enzyme in metabolism of foreign compounds is discussed.

Booth, Boyland, Sato & Sims (1960b) and Booth, Boyland & Sims (1961) showed that rat-liver supernatant catalyses the conjugation of certain epoxides, which are possible intermediates of polycyclic hydrocarbon metabolism, with glutathione. An enzyme has been partially purified and characterized as glutathione *S*-aryltransferase (Grover & Sims, 1964), which catalysed the reaction of 1,2-dichloro-4-nitrobenzene and other cyclic compounds with glutathione (reaction 1), and it seemed possible that this enzyme was also responsible for the conjugation of epoxides (reaction 2). There is now evidence that a distinct enzyme is involved in the catalysis of the conjugation of epoxides and glutathione, which may be termed glutathione *S*-epoxidetransferase.

EXPERIMENTAL

Materials. 1,2-Dichloro-4-nitrobenzene was a commercial sample recrystallized from ethanol, 4-nitropyridine *N*-oxide was from L. Light and Co. Ltd., and glutathione (GSH) was from the Sigma Chemical Co.

Iodomethane, iodoethane, 1-iodopropane, 2-iodopropane, 1-iodobutane, 2-iodobutane, 2-iodo-2-methylpropane, diiodomethane, 2-iodo-2-methylbutane, iodoform, chloroform, carbon tetrachloride and trichloroethylene were from British Drug Houses Ltd. and were redistilled if not colourless. Diodone (*N*-carboxymethyl-3,5-di-iodopyrid-4-one)

and HN2 [di(β-chloroethyl)methylamine] were also commercial samples. Chlorambucil [*p*-*NN*-di-(β-chloroethyl)-aminophenylbutyric acid] was a gift from Dr W. J. C. Ross, and melphalan [*L-p*-di-(β-chloroethyl)aminophenylalanine] a gift from Dr J. A. Stock.

DL-1,2:3,4-Diepoxybutane (1,2:3,4-dianhydro-DL-threitol) was prepared according to Feit (1960), and 1,2:5,6-diepoxyhexane by the method of Everett & Kon (1950). 1,2-Epoxy-1,2,3,4-tetrahydronaphthalene was prepared via the bromohydrin from 1,2-dihydronaphthalene (Straus & Rohrbacher, 1921), 2,3-epoxy-1,2,3,4-tetrahydronaphthalene from 1,4-dihydronaphthalene (Cook & Hill, 1940), 1,4-epoxy-1,4-dihydronaphthalene as described by Friedman & Logullo (1963), 1,4:2,3-diepoxy-1,2,3,4-tetrahydronaphthalene by the oxidation of 1,4-epoxy-1,4-dihydronaphthalene with perbenzoic acid (E. Boyland & P. Sims, unpublished work), and 1,2-epoxyindane from indene as described by Brooks & Young (1956). 2,3-Epoxypropyl phenyl ether, 1,2-epoxypropane and 2,3-epoxypropanol were redistilled commercial samples; 2,3-epoxypropyl butyrate, epoxyethane-1,2-dicarboxylic acid, dodecyl 2,3-epoxypropyl ether and 9,10-epoxystearic acid were gifts from Unilever Ltd.; 2,3-epoxypropyl 2-ethylhexyl ether, 3,4-epoxycyclohexanol, 2,3-epoxypropyl methacrylate, resorcinol bis-2,3-epoxypropyl ether and 4,4'-biphenyl bis-2,3-epoxypropyl ether were from Ciba (A.R.L.) Ltd. Triethylene glycol bis-2,3-epoxypropyl ether (Epidyl), 2,3-epoxypropyl 3-methoxyphenyl ether, 2,3-epoxypropyl 3-ethoxyphenyl ether, *N*-(2,3-epoxypropyl)phenothiazine, 2,3-epoxypropyl 1-naphthyl ether and 2-benzylphenyl 2,3-epoxypropyl ether were gifts from Imperial Chemical Industries Ltd. Dieldrin (1,2,3,4,10,10'-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphthalene) was a sample from the Shell Petroleum Co.

Pyrophosphate buffers were prepared from K₄P₂O₇ and Na₂H₂P₂O₇, and phosphate buffer from KH₂PO₄ and Na₂HPO₄. Tris-acetate buffer was used in the experiments in which chloride was determined.

Chloride estimation. This was by the ferric thiocyanate

method described by Al-Kassab, Boyland & Williams (1963). More reliable results were obtained if 0.5% sodium nitrite was added to the mercuric thiocyanate reagent.

Tissue preparations. Liver suspensions, soluble rat-liver supernatant and the partially purified ammonium sulphate-fractionated preparation of glutathione *S*-aryltransferase were prepared as described by Booth *et al.* (1961), except that the supernatant fraction was not dialysed unless otherwise stated. Absorptions on Tiselius (1954) 20% (w/v) $\text{Ca}_2(\text{PO}_4)_3$ gel were carried out by adjusting the pH of the dialysed liver supernatant to 6.5 with HCl or NaOH, adding the gel and shaking for 15 min. at below 5°. The gel was then removed by centrifugation. The liver supernatant was almost freed from activity towards iodomethane and 2,3-epoxypropyl phenyl ether by lowering the pH to 5 with acetic acid, leaving for 15 min. at room temperature and removing the precipitate by centrifugation (10 min. at 600 g). A second precipitate formed on raising the pH to 6.5 with NaOH was removed by centrifugation to give a supernatant containing mainly glutathione *S*-aryltransferase.

Dialysis was carried out in Visking tubing against glass-distilled water that was first neutralized with NaOH solution.

Enzyme activity. Glutathione *S*-aryltransferase was determined by following the conjugation of 1,2-dichloro-4-nitrobenzene and GSH by the increase in the extinction at 344 m μ (Grover & Sims, 1964) and by the release of nitrite from 4-nitropyridine *N*-oxide (Al-Kassab *et al.* 1963). These incubations were at 37° owing to the limited solubility of the substrates.

The enzyme in rat-liver supernatant that catalyses the reaction of alkyl halides with GSH (Johnson, 1963) could be considered as a 'glutathione *S*-alkyltransferase'. It was estimated by measurement of the I^- ions liberated from iodomethane, by a modification of the method for chloride described by Cunningham, Brooks & Kirk (1941). A mixture (2 ml.) containing GSH (10 mM), the enzyme preparation, phosphate buffer, pH 7.4 (0.1 M), and iodomethane (8 mM), added last at zero time, was incubated at 25° for 15 min. The reaction was stopped by the addition of 0.1 ml. of acetic anhydride; 1.9 ml. of 4% (w/v) sulphosalicylic acid was added 5 min. later and the mixture centrifuged. The protein-free supernatant was titrated with 1 mM-AgNO₃ by using silver-silver amalgam electrodes to determine the end point. There was a rapid change in potential when equivalence was reached; if little iodide was present, a small known amount of iodide was added to facilitate the determination of the end point. Three pairs of electrodes were cleaned and freshly amalgamated about 2 hr. before use and tested with potassium iodide solution. The electrodes varied in sensitivity and it was necessary to select a suitable pair by testing. The potential changes were measured using a Pye pH-meter as a value millivoltmeter. Boiled enzyme preparations did not catalyse the reactions. Little inorganic iodide was liberated in the absence of GSH except in alkaline solutions.

In determinations of the catalytic activity of tissue preparations for the conjugation of epoxides, mixtures containing orthophosphate buffer, pH 6.5 (0.1 M), GSH (10 mM) and 2,3-epoxypropyl phenyl ether (8 mM) were incubated anaerobically in Thunberg tubes, the GSH solution being placed in the side arm. After mixing and incubating for 1 hr. at 25° the incubation mixture was added to an equal

volume of 4% (w/v) sulphosalicylic acid. The protein was removed by centrifugation and the supernatant titrated against iodate (Woodward & Fry, 1932) to determine the remaining GSH. Activities toward other epoxides were determined in a similar manner.

The reaction mixtures were examined by paper chromatography by downward development (18 hr.) on Whatman no. 1 paper with butan-1-ol-acetic acid-water (2:1:1, by vol.) or butan-1-ol-propan-1-ol-2N-ammonia (2:1:1, by vol.) as solvent. Chromatograms were examined after treatment with ninhydrin or with the platonic iodide reagent (Toennies & Kolb, 1951) for the detection of sulphur compounds. Protein was estimated by the biuret method of Fincham (1954).

RESULTS

Catalysis of the conjugation of epoxides and glutathione. 2,3-Epoxypropyl phenyl ether was used as substrate for estimation of 'glutathione *S*-epoxide-transferase' because it was soluble in water and reacted only slowly with glutathione at pH 6.5 in the absence of enzyme and because the reaction rate was greatly increased by rat-liver supernatant. The boiling point of ethylene oxide (10.7°) was too low for it to be used in incubations at 25°. The reaction of 1,2-epoxypropane could be followed, with difficulty, at pH 6.5 and 25° in stoppered tubes, but more consistent results could be obtained with 2,3-epoxypropyl phenyl ether since this epoxide was not too volatile to use in evacuated Thunberg tubes. Anaerobic conditions were necessary at alkaline pH values and also gave more consistent results at pH 6.5 since the rate of reaction of epoxides with glutathione was measured by following the loss of reduced thiol groups by iodate titration.

The pH-activity curve for enzymic conjugation of 2,3-epoxypropyl phenyl ether was different from that obtained with 1,2-dichloro-4-nitrobenzene; the peaks were sharper and the optimum was lower at pH 6.5 (Fig. 1). The results obtained with 1,2-dichloro-4-nitrobenzene were identical with those of Booth *et al.* (1961). At pH 6.5 the optimum glutathione concentration was 8 mM and the 2,3-epoxypropyl phenyl ether concentration 6 mM (Fig. 2). The time-activity curve (Fig. 3) was almost linear during the first hour. The velocity-concentration curve (Fig. 4) was not linear at low enzyme concentrations. The velocity-enzyme concentration curve with dialysed rat-liver supernatant was also non-linear.

In these experiments the amount of enzyme employed had to be chosen with care. The reaction was followed by measuring the loss of one of the reactants (glutathione) and it was necessary for sufficient of this to react to permit accurate measurement without the loss of substrate affecting the rate of reaction. Where possible, 10–20% of the glutathione was allowed to react in the determination of the enzyme process.

Fig. 1. pH-velocity curves for undialysed rat-liver supernatant with substrates: (1) 2,3-epoxypropyl phenyl ether (8 mM) and glutathione (10 mM) incubated in 0.1 M-orthophosphate buffer for 1 hr. at 25° (O-O); (2) iodomethane (8 mM) and glutathione (10 mM) incubated in orthophosphate buffer for 15 min. at 25° (●---●); (3) 1,2-dichloro-4-nitrobenzene (1 mM) and glutathione (8 mM) incubated for 5 min. at 37° in pyrophosphate buffer (●—●). The optimum pH with iodomethane was 7.4 on incubation for 30-60 min.

Fig. 2. Velocity-substrate concentration curves for 2,3-epoxypropyl phenyl ether (●) in the presence of 10 mM-glutathione and for glutathione (○) in the presence of 8 mM-2,3-epoxypropyl phenyl ether. Incubation was in 0.1 M-orthophosphate buffer, pH 6.5, for 1 hr. at 25°.

Fig. 3. Time-activity curve in the presence of 2,3-epoxypropyl phenyl ether (8 mM) and glutathione (10 mM). Incubation was in 0.1 M-orthophosphate buffer, pH 6.5, at 25°.

Fig. 4. Enzyme concentration-activity curve obtained with 2,3-epoxypropyl phenyl ether (8 mM) and glutathione (10 mM) incubated in 0.1 M-orthophosphate buffer, pH 6.5, for 30 min. at 25°. Enzyme concentration is expressed as % (v/v) of supernatant in incubation mixture.

The reaction of glutathione with 2,3-epoxypropyl phenyl ether in the absence of enzyme increased rapidly above pH 6.5. No difference was observed in the rate of reaction in the absence or presence of boiled liver supernatant.

Activity in rat liver catalysing the conjugation of iodomethane. Rat-liver supernatant was shown by Johnson (1963) to contain an enzyme ('glutathione *S*-alkyltransferase') distinct from the glutathione *S*-aryltransferase (Grover & Sims, 1964) of Booth *et al.* (1961). Iodomethane probably inactivated the

enzyme responsible for its conjugation since consistent results were obtained only when the alkyl halide was added last. When short incubation times were used (10 min.) the optimum pH appeared to be 8–8.5 (Fig. 1), but with incubation for 30 min. the optimum was about pH 7.4. Erratic results were obtained at higher pH values. For estimation of activity pH 7.4 was therefore employed: at this

Fig. 5. Velocity-substrate concentration curve obtained for iodomethane incubated in 0.1 M-orthophosphate buffer, pH 7.4, with glutathione (10 mM) for 15 min. at 25°.

Fig. 6. Velocity-substrate concentration curve for glutathione in the presence of iodomethane (8 mM). Incubation was in 0.1 M-orthophosphate buffer, pH 7.4, for 15 min. at 25°.

pH the optimum concentration of iodomethane was 6 mM and of glutathione 8 mM (Figs. 5 and 6). Time-activity (up to 1 hr.) and velocity-concentration curves were both linear.

Separation of the enzymes. Prolonged dialysis (3 days at 4°) of a rat-liver supernatant in Visking tubing against three or four changes of distilled water followed by centrifugation of the dialysate gave supernatants that contained only about 5–15% of the original 'glutathione S-alkyltransferase' activity but that retained 70–80% of the 'glutathione-S-epoxidetransferase' (as measured by conjugation of 2,3-epoxypropyl phenyl ether) and 95–100% of the glutathione-S-aryltransferase activities. It was not possible to remove the activity toward iodomethane completely, the last 5–10% remaining after prolonged dialysis.

Separation of glutathione S-aryltransferase. A comparison of the ratios of the activities towards

Table 1. *Separation of the activity in rat-liver supernatant catalysing conjugations of iodomethane, 2,3-epoxypropyl phenyl ether and 1,2-dichloro-4-nitrobenzene with glutathione*

Activity towards these substrates was measured as described in text. The partially purified glutathione S-aryltransferase preparations were as described by Booth *et al.* (1961).

Treatment of rat-liver supernatant	Percentage activity remaining towards		
	Iodo-methane	2,3-Epoxy-propyl phenyl ether	1,2-Di-chloro-4-nitro-benzene
Undialysed rat-liver supernatant	100	100	100
Prolonged dialysis against distilled water	6–13	70–80	90–95
Supernatant obtained after lowering to pH 5 then raising to pH 6.5	7–10	0–3	95–100
Supernatant left after dialysis followed by absorption with an equal volume of 20% (w/v) $\text{Ca}_3(\text{PO}_4)_2$ gel	5–11	100	46–52
Relative activity of partially purified glutathione S-aryltransferase and rat-liver supernatant, activity towards 1,2-dichloro-4-nitrobenzene being taken as 100% in each case	9–17	21–24	100

2,3-epoxypropyl phenyl ether, iodomethane and the glutathione *S*-aryltransferase substrates (1,2-dichloro-4-nitrobenzene and 4-nitropyridine *N*-oxide) of rat-liver supernatant and of the partially purified *S*-aryltransferase preparation of Booth *et al.* (1961) showed that there was a relative decrease in activity towards the two former substrates on treatment by the procedures outlined in Table 1. This loss of activity was due to inactivation during treatment at pH 5.2 and subsequent dialysis. Little *S*-aryltransferase activity was lost by treatment of rat-liver supernatant at pH 5 and 6.5 and dialysis,

but this preparation had no activity toward 2,3-epoxypropyl phenyl ether and only slight activity towards iodomethane.

Partial separation of the epoxide-conjugating activity. Most of the 'glutathione-*S*-alkyltransferase' activity of the liver supernatant was removed by dialysis, but no significant separation of the activity towards epoxides and 1,2-dichloro-4-nitrobenzene was obtained by fractional precipitation with ammonium sulphate, acetone or ethanol; the organic solvents inactivated both enzymes. The best separation was obtained by use of calcium

Table 2. *Catalysis of the reaction of epoxides with glutathione by undialysed rat-liver supernatant*

Incubation was anaerobic in Thunberg tubes (except for epoxypropane) in 0.1 M-phosphate buffer, pH 6.5, and at 25°. The loss of GSH was followed by iodate titration. A negative result refers to no detectable loss of thiol after incubation for 3 hr. with an incubation mixture containing 25% (v/v) of supernatant, and to allow for the non-linear velocity-enzyme concentration curve (Fig. 3) this concentration was used in all curves. The results given are the average of two experiments.

Epoxy compound	Concn. of epoxide (mm)	Concn. of GSH (mm)	Enzymic loss of SH (μ mole/ml. of supernatant/min.)	Percentage loss of SH relative to 2,3-epoxypropyl phenyl ether at the same concn.
2,3-Epoxypropyl phenyl ether	6	6	0.202	100
Styrene oxide	6	6	0.172	85
2,3-Epoxypropyl 3-methoxyphenyl ether	6	6	0.135	67
2,3-Epoxypropyl 3-ethoxyphenyl ether	6	6	0.091	45
1,2-Epoxypropane	6	6	0.073	36
2,3-Epoxypropanol	6	6	0.018	9
1,2:5,6-Diepoxyhexane	6	6	0.083	41
DL-1,2:3,4-Diepoxybutane	6	6	0.075	37
Triethylene glycol bis-2,3-epoxypropyl ether (Epidyl)	6	6	0.022	11
1,2-Epoxyindane	6	6	0.151	75
1,2-Epoxy-1,2,3,4-tetrahydronaphthalene	6	6	0.081	42
2,3-Epoxy-1,2,3,4-tetrahydronaphthalene	6	6	0.037	19
1,4-Epoxy-1,4-dihydronaphthalene	6	6	0	0
1,4-2,3-Diepoxy-1,2,3,4-tetrahydronaphthalene	6	6	0.006	3
Epoxy cyclohexane	6	6	0.030	15
3,4-Epoxyvinyl cyclohexane	6	6	0.018	9
Trimethylene oxide	6	6	0	0
2,3-Epoxypropyl butyrate	6	6	0.022	11
1,2-Epoxyethane-1,2-dicarboxylic acid	6	6	0	0
2,3-Epoxypropyl phenyl ether	1	2	0.048	100
2,3-Epoxypropyl 1-naphthyl ether	1	2	0.050	105
2-Benzylphenyl 2,3-epoxypropyl ether	Sat. soln.	2	0.014	30
<i>N</i> -(2,3-Epoxypropyl)phenothiazine	1	2	0.020	42
Resorcinol bis-2,3-epoxypropyl ether	1	2	0.047	98
4,4'-Biphenyl bis-2,3-epoxypropyl ether	Sat. soln.	2	0.015	31
Dieldrin	1	2	0	0
2,3-Epoxypropyl 2-ethylhexyl ether	1	2	0.072	16
2,3-Epoxypropyl methacrylate	1	2	0.022	45
Methyl methacrylate	1	2	0	0
Dodecyl 2,3-epoxypropyl ether	Sat. soln.	2	0	0
9,10-Epoxy stearic acid	Sat. soln.	2	0	0
16,17-Epoxy pregnenolone acetate	Sat. soln.	2	0	0
1,2-Epoxy-5,6- <i>trans</i> -9,10-cyclododecadiene	1	2	0	0
3,4-Epoxy cyclohexanol (mixed isomers)	1	2	0.0027	5

phosphate gel, but the epoxide-conjugating preparation still contained almost half the original glutathione-*S*-aryltransferase activity.

Specificity of the glutathione-conjugating enzymes. The ability of rat-liver supernatant to catalyse the conjugations of different epoxides with glutathione is shown in Table 2. Dialysis of the supernatant did not decrease the number of epoxides that reacted, and preparations that had been almost freed from activity toward 2,3-epoxypropyl phenyl ether did not catalyse the reaction of the other epoxides. That the loss of reduced glutathione estimated by iodate titration was due to conjugation with the epoxides was shown by chromatography of the incubation mixtures, but to be certain of the

presence of conjugates from the slower-reacting epoxides it was necessary to incubate for 24 hr. in the presence of 25% (v/v) liver supernatant. All eventually gave additional ninhydrin- and sulphur-positive spots; each diepoxide appeared to give only one conjugate even in the presence of an excess of glutathione. Cysteine reacted only slowly with 2,3-epoxypropyl phenyl ether and diepoxyhexane at pH 6.5, whether enzyme was present or not, and no increase in the amount of conjugate formed could be detected by paper chromatography after the addition of enzyme preparations.

Although 0.5 mM-1,2-epoxy-1,2,3,4-tetrahydronaphthalene caused approx. 50% and 0.5 mM-1,2-epoxyindane 85% inhibition of the conjugation of 1 mM-1,2-dichloro-4-nitrobenzene in the presence of 6 mM-glutathione at pH 8 and 25°, 10 mM solutions of 1,2:5,6-diepoxyhexane, DL-1,2:4,6-diepoxybutane and 2,3-epoxypropyl phenyl ether caused 20–25% inhibition and the inhibition by 1 mM solutions of these epoxides was negligible.

The epoxides reacted too slowly with glutathione at pH 6.5 in the absence of enzyme for determination of the rates of the reactions. Although the reactions were more rapid at pH 8, they were neither first-order nor second-order and it is probable that hydrolyses of the epoxides occurred at the same time as their conjugation with glutathione under these conditions.

The ability of rat-liver supernatant to catalyse the conjugation of alkyl halide with glutathione was found, in agreement with Johnson (1963), to be restricted. The rates of catalysis decreased with increase in the chain lengths of the molecules in the series: 1-iodomethane, 1-iodoethane, 1-iodopropane and 1-iodobutane. No enzyme catalysis could be detected with 2-iodopropane or 2-iodo-2-methylpropane; the latter, however, reacted almost instantaneously with glutathione (Table 3). The iodide released from 1-iodomethane and 1-iodoethane by the enzyme was equivalent to the glutathione uptake, and chromatographic analysis showed that ninhydrin- and sulphur-positive compounds had been formed.

The enzyme in pig and rat liver that catalyses the release of nitrite from glycerol trinitrate in the presence of glutathione, described by Heppel & Hilmo (1950), did not produce any new substances giving ninhydrin- and sulphur-positive reactions on chromatograms; it is possible that no conjugation occurs in this reaction.

Other tissue preparations. Rat-kidney supernatant contains much less glutathione *S*-aryltransferase than liver (Booth *et al.* 1961), but has appreciable activity towards iodomethane (Johnson, 1963) and 2,3-epoxypropyl phenyl ether (Table 4). Ferret liver has no detectable conjugating activity towards 1,2-dichloro-4-nitrobenzene (P.

Table 3. *Catalysis of the reaction between alkyl halides and glutathione by undialysed rat-liver supernatant*

Reaction rates were determined by measurement of the liberated halide ion by using 0.1 M-orthophosphate buffer, pH 7.4, or 0.1 M-pyrophosphate buffer, pH 8, for the iodo compounds and 0.1 M-tris-acetate buffer for chloro compounds. Incubation was for 5–15 min. at 25° for the active compounds, depending on the rate of chemical reaction. Compounds where no catalysis could be detected after 3 hr. incubation in the presence of 25% (v/v) supernatant were regarded as inactive. The incubation mixture contained alkyl halide (1 mM) and glutathione (6 mM). The results given are the average of two experiments. The tertiary halides had almost completely reacted in the absence of enzyme in 5 min.

Halide	Activity (μ mole of substrate reacted/min./ml. of supernatant)	
	At pH 7.4	At pH 8
1-Iodomethane	0.51	0.57
1-Iodoethane	0.205	0.266
1-Iodopropane	0.05	0.060
1-Iodobutane	0.043	0.409
2-Iodopropane	0	0
2-Iodobutane	0	0
2-Iodo-2-methylpropane	0	0
2-Iodo-2-methylbutane	0	0
Di-iodomethane	0.219	0.266
Iodoform (sat. soln.)	0	0.003
<i>N</i> -Carboxymethyl-3,5-di-iodopyrid-4-one (diodone)	0	0.001
Tetraiodophenolphthalein	0	0.001
Trichloroethylene	0	0
Chloroform	0	0
Carbon tetrachloride	0	0
Di-(β -chloroethyl)methylamine	0	0
<i>p</i> - <i>NN</i> -Di-(β -chloroethyl)amino-phenylbutyric acid (chlorambucil)	0	0
<i>L</i> - <i>p</i> -Di-(β -chloroethyl)aminophenyl-alanine (melfalan)	0	0

Table 4. *Activity of other tissue preparations towards iodomethane, 2,3-epoxypropyl phenyl ether and 1,2-dichloro-4-nitrobenzene*

The tissue preparations used were undialysed particle-free supernatants prepared according to the method described by Booth *et al.* (1961) for rat liver. Estimations of the activity towards these substrates were as described in the text.

Tissue preparation	Activity (μ moles of substrate reacted/min./ml. of tissue preparation)		
	Iodo-methane	2,3-Epoxy-propyl phenyl ether	1,2-Di-chloro-4-nitrobenzene
Rat-liver supernatant	2.34	0.200	1.63
Ferret-liver supernatant (δ)	0.47	0.097	0
Ferret-liver supernatant (φ)	0.59	0.093	0
Rat-kidney supernatant	0.645	0.089	0.041

Grover, personal communication) or 4-nitropyridine *N*-oxide but contains the other two enzymes (Table 4).

DISCUSSION

Barnes, James & Wood (1959) showed that the administration of compounds that were excreted as mercapturic acid decreased the glutathione content of the livers of rats. Bray, Franklin & James (1959) demonstrated that *S*-(*p*-chlorobenzyl)glutathione was present in the livers of rabbits given *p*-chlorobenzyl chloride. These results indicated that the formation of a glutathione conjugate was involved in the mercapturic acid formation, and Booth *et al.* (1961) described an enzyme in rat-liver supernatant that catalyses the formation of *S*-(2-chloro-4-nitrophenyl)glutathione from 1,2-dichloro-4-nitrobenzene and glutathione. *S*-(2-Chloro-4-nitrophenyl)glutathione was broken down to *S*-(2-chloro-4-nitrophenyl)-L-cysteine by rat-kidney homogenate and this could be acetylated to the mercapturic acid by rat-liver slices.

Studies of the metabolism of naphthalene, 1,2-dihydronaphthalene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene by Booth, Boyland & Sims (1960a) and Booth *et al.* (1960b) had shown that the excretion of naphthalene and 1,2-dihydronaphthalene as mercapturic acids probably involved the initial formation of epoxides that reacted with glutathione to form conjugates. Booth *et al.* (1961) showed that bromobenzene, naphthalene, 1-chloronaphthalene and phenanthrene were only converted into glutathione conjugates if rat-liver microsomes and NADPH were added to the rat-liver super-

natant in the presence of oxygen. Although it was not possible to isolate the intermediates, it is probable that the perhydroxylating system was necessary for the formation of epoxides, since the conjugation of 1,2-epoxy-1,2,3,4-tetrahydronaphthalene was catalysed by rat-liver supernatant alone.

Although rat-liver supernatant catalyses the conjugation of both 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene with glutathione, different enzymes appear to be involved. Booth *et al.* (1961) pointed out this possibility when they emphasized that crude dialysed rat-liver supernatant catalysed the reaction with the epoxide, and that 1,2-epoxy-1,2,3,4-tetrahydronaphthalene behaved as a non-competitive inhibitor of the conjugation of 1,2-dichloro-4-nitrobenzene and not as a competitive inhibitor as would be expected if it were an alternative substrate.

Prolonged dialysis against distilled water removed most of the catalytic activity of rat-liver supernatant towards iodomethane (Table 1), but dialysed preparations still contained most of their original activity towards epoxides and 1,2-dichloro-4-nitrobenzene (glutathione *S*-aryltransferase). This showed that the enzyme described by Johnson (1963) was not responsible for epoxide conjugation.

The epoxide-conjugating enzyme was, however, readily inactivated at pH 5, and glutathione-*S*-aryltransferase preparations almost free of activity towards epoxides, but still containing a little activity towards iodomethane, were obtained by lowering the pH of rat-liver supernatant to 5 for 15 min. followed by centrifugation, after raising the pH to 6.5, and dialysis of the supernatant.

The reactions of 1,2-epoxyindane and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene with glutathione were not catalysed by the above preparation, showing that they were not substrates for glutathione *S*-aryltransferase; but they are much more potent inhibitors of this enzyme than 2,3-epoxypropyl phenyl ether, 1,2:5,6-diepoxyhexane or 1,2:3,4-diepoxybutane. The inhibition by 1,2-epoxy-1,2,3,4-tetrahydronaphthalene was, however, non-competitive (Booth *et al.* 1961), showing that the active site of glutathione *S*-aryltransferase was not involved.

The results shown in Table 2 do not enable a strictly quantitative assessment of the specificity of rat-liver supernatant for epoxides since maximum velocities and Michaelis constants have not been obtained for each substrate, but they give some indication of the nature of the specificity.

All the epoxides containing the epoxypropyl (glycidyl) group and the diepoxides act as substrates, with the exception of the sparingly soluble dodecyl 2,3-epoxypropyl ether. Proximity of an aromatic ring to the epoxy group increases the rate of reaction; this may be due to the electron-

withdrawing effect of the aromatic ring on the epoxy group since 2,3-epoxypropyl methacrylate is more reactive than 2,3-epoxypropyl butyrate. The effect of the aromatic ring is also shown by 1,2-epoxy-1,2,3,4-tetrahydronaphthalene and 2,3-epoxy-1,2,3,4-tetrahydronaphthalene; the latter epoxide has a slow reaction rate comparable with that of epoxycyclohexane.

The presence of the carboxyl groups, however, decreases the activity of epoxides; thus 2,3-epoxypropyl butyrate has a low activity, and no reaction of 1,2-epoxyethane-1,2-dicarboxylic acid could be detected.

The hydroxyl group of 2,3-epoxypropanol also interferes with the action of the epoxide-conjugating enzyme, since the reaction rate of this compound is considerably lower than that of 1,2-epoxypropane.

Only the oxiran ring was acted on by rat-liver supernatant; no reaction could be detected either with trimethylene oxide or with 1,4-epoxy-1,4-dihydronaphthalene. The presence of this 1,4-bridge also inactivates the 2,3-position since the reaction of 2,3-epoxy-1,2,3,4-tetrahydronaphthalene with glutathione was catalysed by rat-liver supernatant but that of 1,4:2,3-diepoxy-1,2,3,4-tetrahydronaphthalene was not.

Dieldrin has a methylene bridge in the same relative position to an epoxy group as the oxygen bridge of the diepoxynaphthalene. This insecticide was not a substrate for rat-liver supernatant and, although it was possible that the methylene bridge had a similar effect to the oxygen bridge, the low solubility of dieldrin could also contribute to its lack of activity.

The epoxide-conjugating enzyme could, however, play an important part in the detoxication of the bifunctional epoxides Epidyl and diepoxybutadiene when these drugs are used in the chemotherapeutic treatment of cancer.

The enzyme coupling glutathione with iodomethane has a very restricted specificity, as suggested by the results of Johnson (1963). The reactions of iodomethane, iodoethane and diiodomethane were catalysed to appreciable extents but there was less effect with 1-iodopropane and 1-iodobutane. No definite catalysis of the reaction of the other compounds in Table 3 could be detected, and the enzyme probably plays no part in the detoxication of the cancer chemotherapeutic agents HN2 [di-(β -chloroethyl)methylamine], chlorambucil [*p*-*NN*-di-(β -chloroethyl)aminophenylbutyric acid] or melfalan [L-*p*-di-(β -chloroethyl)amino-phenylalanine].

Glutathione *S*-aryltransferase may also have some activity towards iodomethane as dialysis of rat-liver supernatant against distilled water removed only 90–95% of the activity towards iodomethane. Further, the ammonium sulphate-fractionated preparation of glutathione *S*-aryltransferase (Booth *et al.* 1961) and preparations free of 'glutathione *S*-epoxidetransferase' all contain some residual activity towards iodomethane.

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REFERENCES

- Al-Kassab, S., Boyland, E. & Williams, K. (1963). *Biochem. J.* **87**, 4.
- Barnes, M. M., James, S. P. & Wood, P. R. (1959). *Biochem. J.* **71**, 680.
- Booth, J., Boyland, E., Sato, T. & Sims, P. (1960b). *Biochem. J.* **77**, 182.
- Booth, J., Boyland, E. & Sims, P. (1960a). *Biochem. J.* **74**, 117.
- Booth, J., Boyland, E. & Sims, P. (1961). *Biochem. J.* **79**, 516.
- Bray, H. G., Franklin, T. J. & James, S. P. (1959). *Biochem. J.* **71**, 690.
- Brooks, C. J. W. & Young, L. (1956). *Biochem. J.* **63**, 264.
- Cook, E. S. & Hill, A. J. (1940). *J. Amer. chem. Soc.* **62**, 1995.
- Cunningham, B., Brooks, S. C. & Kirk, P. L. (1941). *J. biol. Chem.* **139**, 11.
- Everett, J. L. & Kon, G. A. R. (1950). *J. chem. Soc.* p. 3131.
- Feit, P. W. (1960). *Chem. Ber.* **93**, 116.
- Fincham, J. R. S. (1954). *J. gen. Microbiol.* **11**, 236.
- Friedman, L. & Logullo, F. M. (1963). *J. Amer. chem. Soc.* **85**, 1549.
- Grover, P. L. & Sims, P. (1964). *Biochem. J.* **90**, 603.
- Heppel, L. A. & Hilmoe, R. J. (1950). *J. biol. Chem.* **183**, 129.
- Johnson, M. K. (1963). *Biochem. J.* **87**, 9 p.
- Straus, F. & Rohrbacher, A. (1921). *Ber. dtsch. chem. Ges.* **54**, 40.
- Tiseleus, A. (1954). *Ark. Kemi*, **7**, 443.
- Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 465.