Conjugations with Glutathione

THE ENZYMIC CONJUGATION OF SOME CHLOROCYCLOHEXENES

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1. α -3,4,5,6-Tetrachlorocyclohex-1-ene and γ -2,3,4,5,6-pentachlorocyclohex-1-ene are conjugated with glutathione *in vitro* by a rat-liver enzyme that is probably glutathione *S*-aryltransferase. 2. Chlorocyclohexane and the α -, β -, γ - and δ -isomers of hexachlorocyclohexane were not substrates for rat-liver glutathione *S*-aryltransferase. 3. Glutathione-*S*-aryltransferase activity was present in tissue preparations of houseflies of insecticide-resistant and -susceptible strains. More activity was found in a dieldrin-resistant strain of houseflies fed on dieldrin than in either a dieldrin-resistant strain not fed on dieldrin or a control strain of dieldrin-susceptible houseflies. 4. Housefly soluble supernatant preparations converted *S*-(2-chloro-4-nitrophenyl)glutathione into the corresponding cysteine and mercapturic acid derivatives.

Booth, Boyland & Sims (1961) have described an enzyme, termed glutathione *S*-aryltransferase, in rat liver that catalyses the conjugations of glutathione with aromatic halogen-containing compounds and which is present in a variety of vertebrate species (Grover & Sims, 1964) and in some insects (Boyland, Grover, Sims & Williams, 1963; Cohen, Smith & Turbet, 1964). γ -Pentachlorocyclohexene is an intermediate in the metabolism of γ -hexachlorocyclohexane (Gammexane) by houseflies (Sternburg & Kearns, 1956), and Bradbury & Standen (1959) showed that water-soluble sulphur-containing metabolites are formed from Gammexane by this species. Bettini & Boccacci (1958) have reported the conversion of bromoacetic acid into *S*-(carboxymethyl)glutathione in locusts, and it is possible that the formation of water-soluble glutathione conjugates represents one stage of a common detoxication mechanism in insects. The present paper is concerned with the glutathione-*S*-aryltransferase activity present in houseflies of insecticide-resistant and -susceptible strains and describes the enzyme-catalysed conjugation of γ -2,3,4,5,6-pentachlorocyclohex-1-ene and α -3,4,5,6-tetrachlorocyclohex-1-ene by rat-liver preparations and by tissue fractions from houseflies.

EXPERIMENTAL

Materials. 1,2-Dichloro-4-nitrobenzene was recrystallized from ethanol. Glutathione was obtained from Schwarz Bio-Research Inc., Mount Vernon, N.Y., U.S.A., and γ -hexachlorocyclohexane (Gammexane) and chlorocyclo-

hexane from British Drug Houses Ltd., Poole, Dorset. Samples of α -, β - and δ -hexachlorocyclohexane were kindly given by Dr J. M. Barnes. γ -2,3,4,5,6-Pentachlorocyclohex-1-ene was prepared by the monodehydrochlorination of γ -hexachlorocyclohexane (Nakazima, Okubo & Katamura, 1949) as a colourless liquid, b.p. 86°/1 mm., n_D^{20} 1.5570. Kolka, Orloff & Griffing (1954) found n_D^{25} 1.5630. 3,4,5,6-Tetrachloro-1,2-epoxycyclohexane was prepared by the oxidation of α -3,4,5,6-tetrachlorocyclohex-1-ene with chromium trioxide (Taker, Nakajima & Tomida, 1956) as prisms from light petroleum (b.p. 60–80°), m.p. 89°.

Housefly pupae of dieldrin-resistant and -susceptible strains were obtained from the Pest Infestation Laboratory, Slough, Bucks., and puparia of Gammexane-resistant and -susceptible strains from the Jealots Hill Research Station of Imperial Chemical Industries Ltd., Bracknell, Berks.

Determination of glutathione-S-aryltransferase activity.

(a) With 1,2-dichloro-4-nitrobenzene. In experiments with rat-liver enzyme preparations, reaction mixtures containing 1,2-dichloro-4-nitrobenzene (1.25 mm), and glutathione (3.75 mm) in 0.1 M-pyrophosphate buffer, pH 8.0 (4 ml.), were incubated at 37° and the rate of increase in the extinction at 344 m μ (Booth *et al.* 1961) was measured with equipment described by Grover & Sims (1964). Reaction mixtures for use with housefly-tissue preparations contained 1,2-dichloro-4-nitrobenzene (0.5 mm) and were incubated at 25°.

(b) With chlorocycloaliphatic compounds. Reaction mixtures containing glutathione (1.0 mm), the dialysed enzyme preparation and the substrate (0.5 mm, added as 0.5 ml. of ethanolic solution) in 0.1 M-tris-acetate buffer, pH 8.0, in a total volume of 10 ml., were incubated anaerobically in Thunberg tubes for the times and at the temperatures indicated in Table 2. Reaction mixtures were incubated in triplicate and the control tubes used to estimate chemical reactions had the enzyme preparation added at the end of the incubations. Other control Thunberg tubes

containing enzyme, substrate and buffer in the tubes and glutathione in the side arms were evacuated and stored at 4° for up to 18 hr. before the contents were mixed. The amount of chloride liberated was measured by the formation of a ferric thiocyanate complex, as described by Al-Kassab, Boyland & Williams (1963), except that the saturated mercuric thiocyanate solution used contained 0.5% of NaNO₂. Glutathione loss was measured by titration with iodate (Al-Kassab *et al.* 1963).

Enzyme preparations. (a) Rat-liver homogenate, soluble supernatant fraction and partially purified preparations of glutathione *S*-aryltransferase were prepared as described by Booth *et al.* (1961) and stored at -15°.

(b) Freshly emerged *Musca domestica* were anaesthetized with carbon dioxide and killed by cooling to -15°. Whole flies were homogenized in 10 vol. of 0.1 M-phosphate buffer, pH 7.4, in a Potter & Elvehjem (1936) type homogenizer with a Teflon pestle, and the suspension was centrifuged for 30 min. at 2000g to remove debris. Some of the supernatant from this centrifugation, termed the homogenate, was ultracentrifuged at 95000g for 1 hr. in a Spinco model L preparative ultracentrifuge with rotor no. 40 to give the soluble supernatant fraction, which was dialysed against distilled water for 18 hr. before use in experiments involving chloride release.

All operations were carried out at 3-8° and preparations were stored at -15°. Protein was estimated by a modification of the biuret method (Fincham, 1954).

Identification of glutathione conjugates on paper chromatograms. Reaction mixtures contained glutathione (5 mm), rat-liver or housefly soluble supernatant fraction (1 ml.) in pyrophosphate buffer, pH 8.0, in a final volume of 5 ml. Substrates were added in ethanol (0.1 ml.) to give a concn. of 1 mM; a proportion of some of the substrates was precipitated but the excess was allowed to remain in suspension. A tube containing boiled enzyme preparation was also prepared

for each substrate. After being incubated for 3 hr. at 25° for housefly experiments or at 37° for rat-liver preparations, the mixtures were acidified with acetic acid (0.5 ml.), treated with activated charcoal and the products eluted from charcoal with methanol-benzene-aq. NH₃ (sp.gr. 0.88), as described by Booth *et al.* (1961). Concentrated eluates were applied to Whatman no. 1 chromatography paper and descending chromatograms were developed in butan-1-ol-propan-1-ol-aq. 2N-NH₃ (2:1:1, by vol.) or butan-1-ol-acetic acid-water (12:3:5, by vol.) for 18 hr. The dried chromatograms were examined under ultraviolet light and were then either sprayed with 0.1M-K₂Cr₂O₇-acetic acid (1:1, v/v), followed by 0.1M-AgNO₃ (Knight & Young, 1958), or dipped in ninhydrin (0.4%) in acetone, heated in an oven at 70° for 5 min. and then dipped in a solution of Cu(NO₃)₂ (0.2%) in acetone. On paper chromatograms glutathione conjugates gave yellow and pink colours respectively with the above reagents. The formation of glutathione conjugates in the incubation mixtures was indicated by the presence on chromatograms of spots with these characteristics that were not apparent when the substrates were omitted from the incubation mixtures.

RESULTS

Glutathione-*S*-aryltransferase activity in houseflies. The enzyme activities present in whole-fly homogenates and soluble supernatant fractions from insecticide-resistant and -susceptible strains of houseflies were measured with 1,2-dichloro-4-nitrobenzene as substrate (Table 1). Glutathione-*S*-aryltransferase activity was present in the soluble supernatant fraction of all strains of housefly examined; flies of a dieldrin-resistant strain fed

Table 1. *Glutathione-S-aryltransferase activity of houseflies*

Enzyme activities towards 1,2-dichloro-4-nitrobenzene were measured by recording the increase in extinction at 344 mμ (Booth *et al.* 1961) of reaction mixtures containing glutathione (3.75 mm), 1,2-dichloro-4-nitrobenzene (0.5 mm) and housefly tissue preparations in 0.1 M-pyrophosphate buffer, pH 8.0 (4 ml.), at 25°. One unit of enzyme is defined as the amount that will form 1 μmole of *S*-(2-chloro-4-nitrophenyl)glutathione/min.

Housefly type	10 ² × Specific activity (units/mg. of protein)							
	Wt. of flies homogenized (g.)		Units/g. of tissue homogenate		Soluble supernatant fraction		Dialysed soluble supernatant fraction	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
<i>M. domestica</i> *	6.7	9.2	0.25	0.48	0.50	0.49	0.77	—
<i>M. domestica</i> (Gammexane-resistant)*	6.7	9.3	0.25	0.36	0.52	0.44	0.96	—
<i>M. domestica</i> †	0.43	1.44	0.54	0.30	—	0.43	—	0.48
<i>M. domestica vicina</i> (dieldrin-resistant)†‡	1.05	0.38	0.38	0.22	—	0.30	—	0.28
<i>M. domestica vicina</i> (dieldrin-resistant)†§	0.90	0.35	1.13	1.33	—	1.07	—	1.18

* Obtained from Jealots Hill Research Station of Imperial Chemical Industries Ltd.

† Obtained from Pest Infestation Laboratory.

‡ P.I.L. strain, dieldrin-resistant but not given dieldrin.

§ P.I.L. strain, given dieldrin in the diet.

on dietary dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-4-endo,exo-5,8-dimethanonaphthalene) possessed more activity than either a dieldrin-resistant strain not fed on dieldrin or a susceptible strain of flies from the same source.

Glutathione conjugation with chlorocycloaliphatic compounds. The release of Cl^- and the loss of glutathione were measured after the anaerobic incubation of several chlorocycloaliphatic compounds with enzyme preparations from rat liver and from houseflies (Table 2), and these results were considered in conjunction with evidence obtained by paper chromatography. The amounts of Cl^- released enzymically from α -3,4,5,6-tetrachlorocyclohex-1-ene and from γ -2,3,4,5,6-pentachlorocyclohex-1-ene during the incubation with partially-purified rat-liver glutathione *S*-aryltransferase were equivalent to the amounts of glutathione lost enzymically. Glutathione conjugates were detected on paper chromatograms after both these compounds had been incubated with fresh rat-liver enzyme preparations, but not when boiled enzyme preparations were used.

The loss of glutathione and the amount of Cl^- released during the incubation of 3,4,5,6-tetrachloro-1,2-epoxycyclohexane with partially purified rat-liver glutathione *S*-aryltransferase were not equivalent, but the formation of a glutathione conjugate of this compound was detected by paper chromatography, in agreement with the report of Booth *et al.* (1961).

Glutathione conjugates of chlorocyclohexane and of the α -, β -, γ - and δ -isomers of hexachlorocyclohexane were not detected on paper chromatograms prepared from the incubation mixtures of these compounds with rat-liver enzyme preparations, and no detectable amounts of Cl^- were released during the incubations of these hexachlorocyclohexane isomers with rat-liver soluble supernatant fraction.

In contrast, appreciable amounts of Cl^- were released from γ -hexachlorocyclohexane, without any enzymic loss of glutathione, during the anaerobic incubations of this compound with soluble supernatant fractions prepared from Gammexane-resistant and -susceptible strains of houseflies.

The glutathione conjugate formed on incubating γ -2,3,4,5,6-pentachlorocyclohex-1-ene with these housefly preparations was indistinguishable on paper chromatograms from the conjugate formed in the incubation of the compound with rat-liver glutathione-*S*-aryltransferase preparations.

Enzyme-catalysed conjugation of γ -2,3,4,5,6-pentachlorocyclohex-1-ene with glutathione. Johnson (1963) has shown the presence of an enzyme in rat-liver soluble supernatant fraction that catalyses the conjugation of some alkyl halides with gluta-

Table 2. Glutathione conjugation with chlorocycloaliphatic compounds

Compound	Enzyme preparation	Incubation time (hr.)	Incubation temp.	Cl ⁻ released (mm)		Glutathione loss (mm)		Conjugation observed on paper chromatograms*
				Total	Enzymic	Total	Enzymic	
				0.08	0.01	0.31	0.10	
Chlorocyclohexane	(b)	18	37°	0.20	0.18	0.29	0.17	—
α -3,4,5,6-Tetrachlorocyclohex-1-ene	(b)	18	37	0.22	0.10	0.33	0.14	+
3,4,5,6-Tetrachloro-1,2-epoxycyclohexane	(b)	18	37	0.29	0.21	0.36	0.19	+
γ -2,3,4,5,6-Pentachlorocyclohex-1-ene	(b)	18	37	0	0	0.07	0	+
α -Hexachlorocyclohexane	(a)	4	37	0	0	0	0	—
β -Hexachlorocyclohexane	(a)	4	37	0	0	0.05	0	—
γ -Hexachlorocyclohexane (Gammexane)	(a)	4	37	0	0	0.10	0	—
γ -Hexachlorocyclohexane (Gammexane)	(b)	18	37	0.08	0.03	0.26	0.12	—
γ -Hexachlorocyclohexane (Gammexane)	(c)	18	25	0.47	0.35	0	0	—
γ -Hexachlorocyclohexane (Gammexane)	(d)	18	25	0.46	0.38	0.08	0	—
δ -Hexachlorocyclohexane	(a)	4	37	0	0	0.12	0	—

* Conjugate formation was not detected when boiled enzyme preparations were used.

The composition of the reaction mixtures, the details of the enzyme preparations and the methods of determination of Cl^- and glutathione were those described in the text. Enzyme preparations used were: (a) rat-liver soluble supernatant fraction; (b) partially purified rat-liver glutathione *S*-aryltransferase; (c) *M. domestica* (Gammexane-resistant strain) soluble supernatant fraction; (d) *M. domestica* (Gammexane-susceptible strain) soluble supernatant fraction. The enzymic loss of glutathione and release of Cl^- are calculated from the activity found in the presence of enzyme preparations less the activity in the absence of enzyme. All conjugates formed moved in butan-1-ol-propan-1-ol-aq. 2*N*- NH_3 (2:1:1, by vol.) at R_f 0.23-0.25, gave pink colours with ninhydrin- $\text{Cu}(\text{NO}_3)_2$ reagent and a positive reaction with the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent of Knight & Young (1958).

thione and is distinct from glutathione *S*-aryltransferase. Boyland & Williams (1964) have described a third enzyme in rat-liver soluble supernatant fraction that catalyses the conjugation of glutathione with some epoxides. The effect of γ -2,3,4,5,6-pentachlorocyclohex-1-ene on the rate of conjugation of 1-dichloro-4-nitrobenzene with glutathione catalysed by rat-liver enzyme preparations was investigated. The addition of γ -2,3,4,5,6-pentachlorocyclohex-1,2-ene (0.5 mm) did not inhibit the rate of 1,2-dichloro-4-nitrobenzene conjugation in reaction mixtures containing 1,2-dichloro-4-nitrobenzene (0.125 mm), glutathione (3.75 mm) and rat-liver soluble supernatant fraction in 0.1 M-pyrophosphate buffer, pH 8.0, in a total volume of 4 ml. The low solubility of γ -2,3,4,5,6-pentachlorocyclohex-1-ene in water prevented the use of higher concentrations of this compound for inhibition studies. However, no change in the ratio of the activities towards these substrates was found during the purification of the rat-liver enzyme; this is evidence that glutathione *S*-aryltransferase was responsible for catalysing the conjugation of glutathione with both 1,2-dichloro-4-nitrobenzene and γ -2,3,4,5,6-pentachlorocyclohex-1-ene.

Hydrolysis of S-(2-chloro-4-nitrophenyl)glutathione. Incubation of *S*-(2-chloro-4-nitrophenyl)-glutathione with soluble supernatant fractions prepared from Gammexane-resistant and -susceptible strains of houseflies and examination of the products on paper chromatograms showed that fractions from both strains were able to hydrolyse the conjugate to a compound chromatographically indistinguishable from authentic *S*-(2-chloro-4-nitrophenyl)cysteine and to acetylate this to give 2-chloro-4-nitrophenylmercapturic acid.

DISCUSSION

The conjugations of α -3,4,5,6-tetrachlorocyclohex-1-ene and of γ -2,3,4,5,6-pentachlorocyclohex-1-ene with glutathione *in vitro* are catalysed by a rat-liver enzyme that is probably glutathione *S*-aryltransferase. Both of the compounds possess chlorine atoms activated by adjacent double bonds; enzymic condensation of glutathione with either of these chlorocyclohexenes resulted in the release of one Cl⁻ ion/molecule of glutathione conjugated. The fully saturated chlorocycloaliphatic compounds studied, chlorocyclohexane and the hexachlorocyclohexane isomers, were not substrates for this enzyme. This is in agreement with previous work, which has shown that a variety of aromatic compounds with activated halogen or nitro groups act as substrates for rat-liver glutathione *S*-aryltransferase (Booth *et al.* 1961; Al-Kassab *et al.* 1963); similar results have

been obtained with a glutathione *S*-aryltransferase present in locusts (Cohen, 1963).

The identity of the enzyme responsible for the formation of a glutathione conjugate of 3,4,5,6-tetrachloro-1,2-epoxycyclohexane is not clear. Although Cl⁻ was released during the incubation of this compound with partially purified glutathione *S*-aryltransferase, these rat-liver preparations are now known to contain an additional enzyme that catalyses glutathione conjugation with some epoxides (Boyland & Williams, 1964).

Sternburg, Kearns & Moorefield (1954) have shown the presence of a glutathione-dependent enzyme in strains of houseflies resistant to DDT [1,1,1-trichloro-2,2-di-(*p*-chlorophenyl)ethane], but this enzyme, DDT dehydrochlorinase, which dehydrochlorinates DDT to the non-toxic DDE [1,1-dichloro-2,2-di-(*p*-chlorophenyl)ethane], is not involved in Gammexane detoxication by houseflies (Sternburg & Kearns, 1956). No conjugation of glutathione with γ -hexachlorocyclohexane was detected in the present work, although experiments showed that soluble supernatant fractions from both Gammexane-resistant and -susceptible strains of houseflies released appreciable amounts of Cl⁻ from this compound. This may be due to an enzymic dehydrochlorination system distinct from DDT dehydrochlorinase; Bradbury (1957) has reported that the metabolism of Gammexane in houseflies to water-soluble metabolites results in the release of Cl⁻.

Equivalent activities of a glutathione *S*-aryltransferase were found in soluble supernatant fractions prepared from Gammexane-resistant and -susceptible strains of houseflies, but the activity of this enzyme present in a strain of dieldrin-resistant flies fed on dietary dieldrin was higher than that present in either a dieldrin-resistant strain not fed on dieldrin or a susceptible strain of flies obtained from the same source. It is unlikely that rapid dieldrin detoxication is responsible for the insecticide resistance of these dieldrin-fed houseflies, since Bridges & Cox (1959) have shown that high concentrations of apparently unchanged dieldrin persisted in these flies for long periods. Gammexane has been shown to be more rapidly metabolized by insecticide-resistant strains of houseflies than by susceptible flies (Bradbury & Standen, 1955; Sternburg & Kearns, 1956). Busvine & Townsend (1963) have put forward evidence to support the view that the mechanism of housefly resistance to Gammexane may consist of two factors, one being a common factor in insects responsible for cross-resistance to a range of chlorinated insecticides and not involving Gammexane metabolism, and the other being increased Gammexane detoxication, present in resistant houseflies but not in other insect species. In the present work no

appreciable difference was found between enzyme preparations from Gammexane-resistant and -susceptible strains of houseflies when the amount of Cl^- released from Gammexane was measured, nor was there any difference in glutathione-*S*-aryltransferase activities. In rats the main pathway for Gammexane metabolism seems to involve dehydrochlorination to trichlorobenzene followed by hydroxylation to phenols, which are excreted free and as their glucuronic acid and sulphuric acid conjugates (P. L. Grover & P. Sims, unpublished work). If Gammexane metabolism in houseflies follows a similar pathway it is possible that it is the activity of the microsomal hydroxylating systems that is enhanced in Gammexane-resistant strains of housefly. Increases in the activity of the hepatic microsomal hydroxylating systems of some mammalian species are known to occur after the administration of a variety of foreign compounds (Conney & Burns, 1961) including Chlordane (1,2,4,5,6,7,8,8-octachloro-4,7-methano-3a,4,7,7a-tetrahydroindane), a chlorinated cyclodiene insecticide (Hart, Shultice & Fouts, 1963).

S-(2-Chloro-4-nitrophenyl)glutathione is hydrolysed by housefly soluble supernatant fractions to give the corresponding cysteine derivative, which is acetylated to the mercapturic acid, and the presence of a glutathione *S*-aryltransferase indicates that houseflies detoxicate foreign compounds via mercapturic acid synthesis. This supports the suggestion of Bradbury & Standen (1959) that the dichlorothiophenols isolated after the alkaline hydrolysis of extracts of Gammexane-treated houseflies were derived from the corresponding dichlorophenylmercapturic acids.

The formation of dichlorophenylmercapturic acids from Gammexane in houseflies can be explained if dehydrochlorination to γ -2,3,4,5,6-pentachlorocyclohex-1-ene is followed first by glutathione conjugation and then by aromatization of the cyclohexane ring by further dehydrochlorination, a process that would involve the release of four chlorine atoms/molecule of Gammexane metabolized. Bradbury (1957) has in fact found that four chlorine atoms are liberated/molecule of this insecticide metabolized by houseflies.

Glutathione-*S*-aryltransferase activity has been found in other species of insects (Cohen *et al.* 1964), and mercapturic acid formation may be a common detoxication mechanism in these organisms.

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