The Metabolism of γ-2,3,4,5,6-Pentachlorocyclohex-1-ene and γ-Hexachlorocyclohexane in Rats

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1. After intraperitoneal administration, γ-hexachlorocyclohexane (Gammexane) and γ-2,3,4,5,6-pentachlorocyclohex-1-ene were converted by rats into 2,3,5- and 2,4,5-trichlorophenol, which were excreted as free phenols and as sulphuric acid and glucuronic acid conjugates. 2. Derivatives of 2,4,5-trichlorophenol and 2,4,5-trichlorophenyl glucosiduronic acid and 2,4-dichlorophenylmercapturic acid were isolated from the urine as metabolites of γ-2,3,4,5,6-pentachlorocyclohex-1-ene. 3. The phenolic metabolites of γ-hexachlorocyclohexane and γ-2,3,4,5,6-pentachlorocyclohex-1-ene isolated from urine were similar to those of 1,2,4-trichlorobenzene, which indicates that the two latter compounds are intermediates in γ-hexachlorocyclohexane metabolism in rats.

Koransky & Portig (1963) have reported that both α- and γ-hexachlorocyclohexane are dechlorinated by rats to yield water-soluble compounds that are excreted in the urine. γ-2,3,4,5,6-Pentachlorocyclohex-1-ene, a monodehydrochlorination product of γ-hexachlorocyclohexane (Gammexane), has been shown to be an intermediate in Gammexane metabolism in houseflies (Sternberg & Kearns, 1956) and forms a conjugate with glutathione in the presence of glutathione *S*-aryltransferase preparations from rat liver or from houseflies (Sims & Grover, 1965).

The present paper describes the isolation of derivatives of 2,4,5-trichlorophenol and 2,4,5-trichlorophenyl glucosiduronic acid and of 2,4-dichlorophenylmercapturic acid from the urine of rats that had been injected with γ-2,3,4,5,6-pentachlorocyclohex-1-ene, and the detection of excreted 2,3,5-trichlorophenyl derivatives. Evidence was also obtained for the urinary excretion of these compounds by rats that had been injected with γ-hexachlorocyclohexane.

EXPERIMENTAL

Spectra. Ultraviolet-absorption spectra were measured in ethanol in a Unicam SP. 500 spectrometer and infrared-absorption spectra with a Perkin–Elmer model 137 Infracord.

Melting points. These are uncorrected.

Chromatography. Except where stated paper chromatography was carried out on Whatman no. 1 paper by downward development for 18hr. in one of the following: 1, butan-1-ol–propan-1-ol–aq. 2N-NH_3 (2:1:1, by vol.); 2, butan-1-ol–acetic acid–water (12:3:5, by vol.); 3, the organic phase of butan-1-ol–benzene–aq. 3N-NH_3 (1:1:2, by vol.). The dried chromatograms were examined under u.v. light and were then sprayed with (a) 0·1M-$K_2Cr_2O_7$–acetic acid (1:1, v/v), followed by 0·1M-$AgNO_3$ (Knight & Young, 1958), or with (b) aq. 1% solution of Brentamine Fast Red B salt, followed by 2N-NH_3, or with (c) 2,6-dichloroquinonechloroimide (0·5% in ethanol), followed by 2N-Na_2CO_3.

Thin-layer chromatograms were prepared by coating glass plates with a film of silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0·25mm. thickness. The chromatograms were developed for 10cm. with either benzene or benzene–ethanol (19:1, v/v) and after drying were sprayed with one of the colour reagents referred to above.

Two-dimensional thin-layer chromatograms were developed with benzene followed by benzene–ethanol (19:1, v/v).

Hydrolyses. Acid hydrolyses were carried out by heating with 5N-HCl for 15min. at 100°. Hydrolyses with sulphatase (Taka-diastase; Parke, Davis and Co. Ltd., Hounslow, Middlesex) were carried out in 0·1M-sodium acetate–acetic acid buffer, pH5·7, and with β-glucuronidase (Ketodase; Warner–Chilcott Laboratories, Morris Plain, N.J., U.S.A.) in 0·1M-sodium acetate–acetic acid buffer, pH5·0, the solutions being incubated overnight at 37°. The products of the hydrolyses were extracted with ether for examination by chromatography.

Materials. γ-Hexachlorocyclohexane (Gammexane), 2,4-dichloroaniline and 2,4,5-trichlorophenol were obtained from British Drug Houses Ltd. (Poole, Dorset) and 2,4,6-trichlorophenol from Hopkin and Williams Ltd. (Chadwell Heath, Essex). γ-2,3,4,5,6-Pentachlorocyclohex-1-ene was prepared as described by Sims & Grover (1965). 2,3,6- and 3,4,5-Trichlorophenol were kindly given by Dr J. A. Silk of the Jealots Hill Research Station of Imperial Chemical Industries Ltd., Bracknell, Berks. 2,3,5-Trichlorophenol was prepared from 2,4-dichloroaniline as described

by Hodgson & Kershaw (1929). 2,3,5-Trichlorophenyl benzoate was obtained as needles from light petroleum (b.p. 60–80°), m.p. 94° (Found: C, 51·5; H, 2·4; Cl, 34·7. Calc. for $C_{13}H_7Cl_3O_2$: C, 51·8; H, 2·3; Cl, 35·3%). Hodgson & Kershaw (1929) found m.p. 103°. *S*-(2,4-Dichlorophenyl)-L-cysteine was prepared from 2,4-dichloroaniline by the method of Parke & Williams (1951), except that the product was removed from the reaction mixture by adsorption on activated charcoal followed by elution with methanol–aq. NH_3 (sp.gr. 0·88) (19:1, v/v). The product remaining after the evaporation of the solvent was dissolved in aq. 2N-NH_3 and the solution was filtered and the cysteine derivative precipitated by the addition of 2N-acetic acid. *S*-(2,4-Dichlorophenyl)-L-cysteine was crystallized from aq. ethanol as plates, m.p. 182°. Parke (1955) found m.p. 180°. Acetylation of this compound with acetic anhydride in 2N-NaOH yielded 2,4-dichlorophenylmercapturic acid in needles from water, m.p. 157°. Parke (1955) found m.p. 160°. The dicyclohexylammonium salt of 2,4-dichlorophenylmercapturic acid prepared as described by Thomson, Barnsley & Young (1963) was crystallized from acetone–ethanol (9:1, v/v) as needles, m.p. 194° (decomp.).

Animal experiments. (*a*) Metabolism of γ-2,3,4,5,6-pentachlorocyclohex-1-ene. 16 rats of the Chester Beatty strain, housed in all-glass metabolism cages arranged for the separate collection of urine and faeces, were each given γ-2,3,4,5,6-pentachlorocyclohex-1-ene (250 mg./kg.) by daily intraperitoneal injection until a total of 11 g. had been administered. The urines were collected daily, combined and stored at 0°. After acidification to pH 4·0 with acetic acid, the urine was treated with activated charcoal (100 g.; British Drug Houses Ltd.), the mixture filtered, the charcoal washed with water (2 l.) and the absorbed material eluted with methanol–aq. NH_3 (sp.gr. 0·88) (19:1, v/v; 14 l.). The eluate was evaporated under reduced pressure to yield a brown gum, which was chromatographed on a cellulose-powder column prepared from Whatman standard grade cellulose powder (700 g.). The column was developed with butan-1-ol–cyclohexane–aq. 2N-NH_3 (9:2:1, by vol.; 4 l.) followed by butan-1-ol–cyclohexane–aq. 2N-NH_3 (18:2:3, by vol.; 2 l.) and then by butan-1-ol saturated with aq. 2N-NH_3 (4 l.). Fractions (150 ml.) were collected and were evaporated under reduced pressure; the residues were examined by paper and thin-layer chromatography as described above. Those containing the same components were combined.

Fractions 7–9 formed a dark gum that contained free phenolic compounds. Examination by two-dimensional thin-layer chromatography showed that the phenolic compounds present included two substances indistinguishable in their colour reactions (Table 1) and chromatographic behaviour from 2,4,5- and 2,3,5-trichlorophenol respectively, the major metabolite in this fraction being the 2,4,5-isomer. When other thin-layer chromatograms were developed in one direction with benzene, sprayed with HCl, heated at 80° for 10 min. and then developed in the other direction with benzene–ethanol (19:1, v/v), 2,4,5- and 2,3,5-trichlorophenol were detected as free phenols together with substances that did not move from the origin in the first solvent but which gave rise to the two phenols after the chromatogram was treated with acid. When the products resulting from the treatment of other portions of the gum with sulphatase were examined in the same way no phenols (liberated by acid treatment of the paper chromatograms) were detected at the origin; this indicates that 2,4,5- and 2,3,5-trichlorophenol were excreted free and, in part, as sulphuric acid conjugates.

The remainder of the gum was treated with benzoyl chloride in 2N-NaOH and the product recrystallized twice from ethanol to give 2,4,5-trichlorophenyl benzoate as needles (200 mg., 1·5% of dose; m.p. and mixed m.p. 87–88°) (Found: C, 51·9; H, 2·6. Calc. for $C_{13}H_7Cl_3O_2$: C, 51·8; H, 2·3%). The infrared-absorption spectrum of this benzoate was indistinguishable from that of the authentic compound.

Paper chromatography of portions of the pale-brown gum obtained from fractions 10–28 showed that it contained a substance of R_F 0·66 in solvent 1 that was detected as a dark area when the dried chromatograms were examined under u.v. light and which gave a positive reaction with reagent (a). The gum was extracted with aq. 2N-NH_3 and the filtered extract acidified with conc. HCl. The oily precipitate solidified on standing to give an amorphous material that could not be crystallized. The material was dissolved in acetone (50 ml.) and treated with dicyclohexylamine (1 ml.) (Thomson *et al.* 1963). The crystalline salt that formed was filtered off and recrystallized three times from acetone–ethanol (9:1, v/v) to give the *dicyclohexylammonium salt* of 2,4-dichlorophenylmercapturic acid as needles (510 mg., 2·5%; m.p. 194°, decomp., undepressed when mixed with the synthetic salt) (Found: Cl, 14·6; N, 6·0; S, 6·7; $C_{23}H_{34}Cl_2N_2O_3S$ requires Cl, 14·5; N, 5·7; S, 6·5%). A solution of this salt (300 mg.) in hot water (80 ml.) was acidified with N-HCl (5 ml.) and the 2,4-dichlorophenylmercapturic acid that crystallized on cooling was recrystallized from hot water as needles (175 mg.), m.p. and mixed m.p. 157–158° (Found: C, 42·4; H, 3·7; Cl, 22·9;

Table 1. *Thin-layer chromatography of trichlorophenols*

Chromatograms were developed as described in the text.

	R_F		Colour reactions	
Phenol	Benzene	Benzene–ethanol (19:1, v/v)	2,6-Dichloroquinone-chloroimide–Na_2CO_3	Brentamine Fast Red B salt
2,3,5-Trichlorophenol	0·55	0·69	Blue	Orange
2,3,6-Trichlorophenol	0·72	0·82	Blue	Orange
2,4,5-Trichlorophenol	0·53	0·62	Blue–green*	Purple–brown
2,4,6-Trichlorophenol	0·68	0·92	Blue–green*	Pink
3,4,5-Trichlorophenol	0·34	0·62	Pale grey–blue*	Violet

* Delayed colour formation.

N, 4·4; S, 10·3. Calc. for $C_{11}H_{11}Cl_2NO_3S$: C, 42·9; H, 3·6; Cl, 23·0; N, 4·5; S, 10·4%). The u.v.-absorption spectrum in ethanol showed $\lambda_{max.}$ 262mμ (ε 12000) [Parke (1955) found $\lambda_{max.}$ 261–262mμ (ε 13800)] and the infrared-absorption spectrum was identical with that of the authentic compound.

Fractions 34–37 formed a dark gum that gave a positive test for glucuronic acid with naphtharesorcinol. Hydrolysis of portions with 5N-HCl or with β-glucuronidase gave two products that were identical in their chromatographic behaviour with 2,4,5- and 2,3,5-trichlorophenol respectively, the phenol present in the largest amount being the 2,4,5-isomer. The remainder of the gum was dissolved in methanol (500ml.) and treated with an excess of a solution of diazomethane in ether. The solvent was evaporated and the residue, in pyridine (50ml.), was treated with acetic anhydride (20ml.). The mixture was kept at room temperature overnight, poured into water (500ml.) and the solid that separated was recrystallized twice from ethanol. (−)-*Methyl (2,4,5-trichlorophenyl tri-O-acetyl-D-glucosid)uronate* was obtained as needles (2·3g., 10%), m.p. 164–165°, $[\alpha]_D^{24}$–48° (c 0·25 in ethanol) (Found: C, 44·8; H, 3·8; Cl, 20·4. $C_{19}H_{19}Cl_3O_{10}$ requires C, 44·4; H, 3·7; Cl, 20·7%). Hydrolysis of this compound (400mg.) with HCl for 4hr. at 100° yielded ether-soluble material that was treated with benzoyl chloride in 2N-NaOH. The product was recrystallized from ethanol to give 2,4,5-trichlorophenyl benzoate, m.p. and mixed m.p. 87°. The infrared-absorption spectrum was indistinguishable from that of the authentic compound.

No metabolites were detected in subsequent fractions collected from this column.

(*b*) Metabolism of γ-hexachlorocyclohexane. 36 rats of the Chester Beatty strain housed in metabolism cages were each given γ-hexachlorocyclohexane (40mg./kg.) as a solution in arachis oil by intraperitoneal injection every other day until a total of 4g. of the insecticide had been administered. The urine was collected and combined and stored at 0°, and, after acidification with HCl, was continuously extracted with ether. The ether extracts were evaporated under reduced pressure and the dark gum was chromatographed on a cellulose powder column as described above.

The earlier fractions collected from this column were shown by two-dimensional thin-layer chromatography to contain two phenolic metabolites, indistinguishable from authentic 2,4,5- and 2,3,5-trichlorophenol respectively. The fractions also contained a substance that gave a positive reaction with reagent (a) and that was identical in its behaviour, on paper chromatograms developed with solvents 1, 2 and 3, with 2,4-dichlorophenylmercapturic acid.

The aqueous layer remaining after the continuous extractions of the acidified urine from the rats injected with γ-hexachlorocyclohexane was treated with activated charcoal, in the manner described above, to yield a dark gum that gave a positive naphtharesorcinol reaction. Portions of the gum were hydrolysed with acid and with β-glucuronidase and the products examined on two-dimensional thin-layer chromatograms. Both products were found to contain compounds indistinguishable from 2,3,5- and 2,4,5-trichlorophenol.

An unacidified portion of the urine collected from the γ-hexachlorocyclohexane-treated rats was also examined. The material obtained by continuous ether-extraction of the urine was shown by thin-layer chromatography to contain compounds indistinguishable from 2,3,5- and 2,4,5-trichlorophenol. The aqueous layer remaining after ether-extraction was treated with activated charcoal; hydrolysis of portions of the concentrated charcoal eluate, with acid and with sulphatase, yielded products which also contained 2,3,5- and 2,4,5-trichlorophenol.

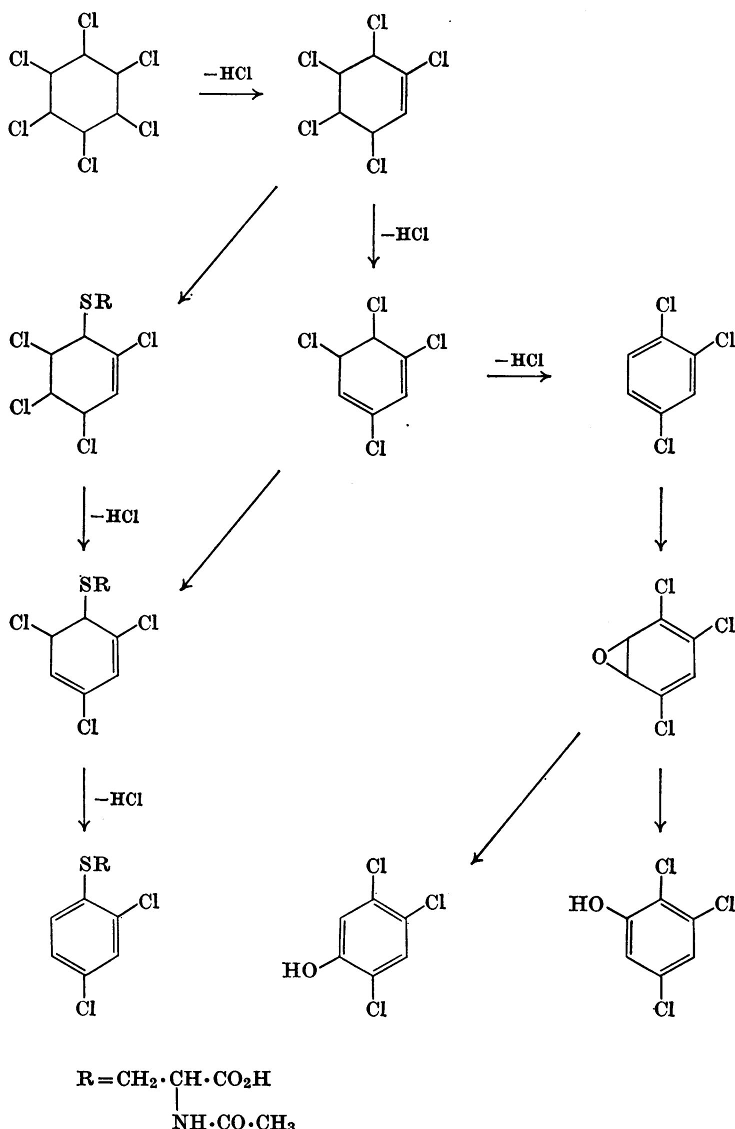
RESULTS AND DISCUSSION

The intraperitoneal administration of γ-2,3,4,5,6-pentachlorocyclohex-1-ene or γ-hexachlorocyclohexane to rats has resulted in the urinary excretion of trichlorophenols. 2,4,5-Trichlorophenol, as its benzoate, and 2,4,5-trichlorophenyl glucosiduronic acid, as a triacetyl methyl ester, have been isolated from the urine of rats that had received γ-2,3,4,5,6-pentachlorocyclohex-1-ene. In addition, smaller amounts of a second phenolic metabolite, identified as 2,3,5-trichlorophenol, were also excreted, both free and conjugated with glucuronic acid. Examination of extracts of urine which had not been acidified below pH 4·0 showed that both these phenols were also excreted as sulphuric acid conjugates.

The phenolic metabolites excreted by rats that had received γ-hexachlorocyclohexane were the same as those produced from γ-2,3,4,5,6-pentachlorohex-1-ene. 2,4,5- and 2,3,5-Trichlorophenol were excreted and were detected in the urine as free phenols and as sulphuric acid and glucuronic acid conjugates, but it was not possible to isolate any of these metabolites, largely because γ-hexachlorocyclohexane was tolerated only at much lower doses than were employed in the administration of γ-2,3,4,5,6-pentachlorocyclohex-1-ene to rats.

In addition, 2,4-dichlorophenylmercapturic acid has been isolated as a metabolite of γ-2,3,4,5,6-pentachlorocyclohex-1-ene, and a compound having similar chromatographic characteristics was also present in the urine of rats treated with γ-hexachlorocyclohexane. No evidence was obtained for the excretion of acid-labile mercapturic acids, of the type described by Knight & Young (1958), as metabolites of either γ-2,3,4,5,6-pentachlorocyclohex-1-ene or γ-hexachlorocyclohexane.

In many respects the later stages of the conversion *in vivo* of γ-2,3,4,5,6-pentachlorocyclohex-1-ene and γ-hexachlorocyclohexane into trichlorophenols by rats resemble the metabolism of 1,2,4-trichlorobenzene, which Jondorf, Parke & Williams (1955) have studied in rabbits. These authors found that 2,4,5-trichlorophenol and its glucuronic acid and sulphuric acid conjugates were the major metabolites of orally administered 1,2,4-trichlorobenzene found in urine, but that smaller amounts of 2,3,5-trichlorophenol were also excreted. As part of an investigation of the mechanism of alkaline dehydrochlorination of hexachlorocyclohexane isomers, Hughes, Ingold & Pasternak (1953) found that 70–80% of the resulting mixtures of trichlorobenzenes was



Scheme 1. Possible pathways in γ-hexachlorocyclohexane and γ-2,3,4,5,6-pentachlorocyclohex-1-ene metabolism.

1,2,4-trichlorobenzene. The urinary excretion of the same isomeric trichlorophenols from γ-hexachlorocyclohexane, γ-2,3,4,5,6-pentachlorocyclohex-1-ene and 1,2,4-trichlorobenzene suggests that the dehydrochlorination *in vivo* of γ-hexachlorocyclohexane in rats proceeds via γ-2,3,4,5,6-pentachlorocyclohex-1-ene to 1,2,4-trichlorobenzene. Koransky & Portig (1963) have found that both α- and γ-hexachlorocyclohexane are dechlorinated by rats to give water-soluble products, and James & Jeffery (1964) have obtained evidence to suggest that, in rabbits, part of a dose of bromocyclohexane is dehydrobrominated to cyclohexene, which is excreted as a mercapturic acid by a process that presumably involves epoxidation and conjugation with glutathione. Dehydrochlorination of γ-hexachlorocyclohexane may be the rate-limiting step in the metabolism of this compound in rats; long-term feeding experiments have shown that γ-hexachlorocyclohexane accumulates unchanged in the tissues and particularly in the fat (Davidow & Frawley, 1951).

γ-2,3,4,5,6-Pentachlorocyclohex-1-ene is a known intermediate in the breakdown of γ-hexachloro-

cyclohexane by houseflies (Sternberg & Kearns, 1956), and housefly tissue preparations are active in γ-hexachlorocyclohexane dehydrochlorination (Sims & Grover, 1965). If the metabolism of γ-hexachlorocyclohexane in rats and in houseflies proceeds via a similar mechanism it is possible that some of the unidentified water-soluble products of γ-hexachlorocyclohexane metabolism by houseflies, described by Bradbury & Standen (1960), were in fact trichlorophenols and their conjugates.

Jondorf *et al.* (1955) have suggested that 3,4,6-trichloro-1,2-dihydro-1,2-dihydroxybenzene is an intermediate in the metabolic conversion of 1,2,4-trichlorobenzene into 2,3,5- and 2,4,5-trichlorophenol. It is possible that the first stage of 1,2,4-trichlorobenzene metabolism is the formation of an epoxide of the type proposed by Boyland (1950) (see Scheme 1), which could rearrange to the isomeric phenols or react either with water to form the dihydrodihydroxy derivative or with glutathione to give, ultimately, the two isomeric trichlorophenylmercapturic acids which Jondorf *et al.* (1955) detected. These authors estimated that, in rabbits, only 0·3% of the dose of trichlorobenzene appeared as urinary trichlorophenylmercapturic acid derivatives. In the present work, although the amounts of trichlorophenols excreted were lower than those found by Jondorf *et al.* (1955), the proportion of the dose which appeared as a mercapturic acid was considerably higher and the metabolite isolated was a dichlorophenylmercapturic acid.

It seems likely that this dichlorophenylmercapturic acid was formed by a mechanism different from that giving rise to trichlorophenols. It has been shown that *in vitro* the conjugation of γ-2,3,4,5,6-pentachlorocyclohex-1-ene with glutathione is catalysed by a rat-liver enzyme that is probably glutathione *S*-aryltransferase, and that, in this case, a chlorine atom is replaced by glutathione (Sims & Grover, 1965) to give, presumably, a tetrachlorocyclohexene glutathione conjugate. Further dehydrochlorination of a conjugate of this type could result in the excretion of 2,4-dichlorophenylmercapturic acid (Scheme 1). In the experiments *in vitro* the rate of conjugation of γ-2,3,4,5,6-pentachlorocyclohex-1-ene with glutathione, catalysed by glutathione *S*-aryltransferase and measured by glutathione loss and Cl^- release, was comparatively slow (Sims & Grover, 1965) and the possibility of an alternative mechanism exists. This is that the chlorine atoms in positions 3 and 4 of 2,3,4,6-tetrachlorocyclohexa-1,5-diene (Scheme 1), formed by dehydrochlorination of γ-2,3,4,5,6-pentachlorocyclohex-1-ene, may be more readily replaced than the corresponding chlorine atom adjacent to the lone double bond in the pentachloro compound. In this way dehydrochlorination of γ-2,3,4,5,6-pentachlorocyclohex-1-ene could take place before enzymic conjugation with glutathione. Either of these two possible pathways could give rise, ultimately, to the excretion of 2,4-dichlorophenylmercapturic acid.

Bradbury & Standen (1959) have found that alkaline hydrolysis of the water-soluble metabolites formed by houseflies from γ-hexachlorocyclohexane yields dichlorothiophenols. These derivatives represented 60% of the total water-soluble metabolites formed by houseflies from γ-hexachlorocyclohexane and, whilst several isomeric dichlorophenols were present, 2,4-dichlorothiophenol was the major product. In view of the evidence obtained for the presence in houseflies of a dehydrochlorinating system and of glutathione-*S*-aryltransferase activity (Sims & Grover, 1965) it seems probable that the dichlorothiophenols detected by Bradbury & Standen (1959) were produced by alkaline hydrolysis of the corresponding mercapturic acid and that, as in rats, 2,4-dichlorophenylmercapturic acid was a metabolite.

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