In acid solution, premercapturic acids which probably have structures like (IV) (cf. Boyland & Sims, 1958), readily undergo a reaction similar to this postulated decysteinylation and, if this reaction were accomplished by an enzyme (cf. Knight & Young, 1958), it could be a source of the phenols (e.g. V) found as metabolites of chlorobenzene in locusts (Gessner & Smith, 1960). In this case the *m*-chlorophenol would arise from the same precursor as the *p*-chlorophenylcysteine.

SUMMARY

1. The excretion and metabolism of [¹⁴C]chlorobenzene has been studied in the locust *Schistocerca gregaria*.

2. About half of the dose was excreted unchanged.

3. The probable presence, in excreta, of *o*-, *m*- and *p*-chlorophenyl-L-cysteine and smaller amounts of *m*- and *p*-chlorophenylmercapturic acid was shown by isotopic-dilution techniques.

4. These compounds were excreted as acid-labile precursors.

5. A weak acylase is present in crop fluid and excreta which slowly hydrolyses L-*p*-chlorophenylmercapturic acid to *p*-chlorophenyl-L-cysteine.

6. *p*-Chlorophenyl-L-cysteine is enzymically degraded to unidentified products by crop fluid and excreta.

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Comparative Detoxication

8. THE METABOLISM OF CHLOROBENZENE IN LOCUSTS: PHENOLIC METABOLITES, A COMPARISON WITH SOME VERTEBRATE SPECIES

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Oxidation of foreign aromatic compounds is one of the most common detoxication mechanisms found in vertebrates, but whether this process occurs to any significant extent in insects is not yet clear. Some examples of the hydroxylation of aromatic rings in insects are known but these mainly concern the oxidation of naturally occurring

substrates involved in amino acid metabolism or cuticle tanning (Richards, 1951; Smith, 1955; Dennell, 1958). There is also some evidence that some insecticides may be oxidized to phenols in insects (Terriere & Schonbrod, 1955; Bradbury & Standen, 1958), but the identity of the metabolites is not known.

In vertebrates the hydroxylation of natural aromatic substrates appears to be carried out by specific enzyme systems which differ from that which carries out the detoxication oxidations (Mitoma, Posner, Reitz & Udenfriend, 1956). Brodie & Hogben (1957) have suggested that the development of this detoxication system by terrestrial vertebrates is connected with their need to produce polar, water-soluble metabolites capable of excretion by the kidney.

It was of interest to see whether an insect had developed a similar oxidation system producing a similar pattern of phenolic metabolites to those known in vertebrates. Chlorobenzene was chosen for testing for the oxidation system in the locust as its metabolism has been well studied in vertebrates (Smith, Spencer & Williams, 1950; Azouz, Parke & Williams, 1952, 1953) and because of its relation to a number of widely used insecticides.

EXPERIMENTAL

Materials

[¹⁴C]Chlorobenzene. This was obtained (0.01 mc/mg.) from The Radiochemical Centre (Amersham, Bucks) and was normally dissolved in a suitable volume of arachis oil before use, as described by Gessner & Smith (1960).

Reference compounds. *m*- and *p*-Chlorophenol, 2-chloroquinol and 4-chlororesorcinol were commercial samples. Commercial samples of *o*-chlorophenol contained phenol and were purified according to Wohlleben (1909). 4-Chlorocatechol was prepared according to Peratoner (1898) and purified from 3-chlorocatechol via its toluene-*p*-sulphonate (Smith *et al.* 1950). The 4-chlorocatechol obtained by alkaline hydrolysis of this derivative had m.p. 92° and was chromatographically homogeneous. 2-Chlororesorcinol (m.p. 97°) was prepared according to Milligan & Hope (1941).

The potassium salts of *o*-, *m*- and *p*-chlorophenyl hydrogen sulphate were prepared according to Burkhardt & Lapworth (1926). *o*-Chlorophenyl β -glucoside (m.p. 170°; [α]_D²⁵ -90°), *m*-chlorophenyl β -glucoside (m.p. 179°; [α]_D²⁵ -36°) and *p*-chlorophenyl β -glucoside (m.p. 175°; [α]_D²⁵ -87°) were prepared by the method of Glazer & Wulwek, 1924. L-Chlorophenylmercapturic acids and chlorophenyl-L-cysteines were samples used previously (Gessner & Smith, 1960).

Methods

Animals and dosing. Locusts (*Schistocerca gregaria*) were obtained and kept, and excreta and locust bodies were homogenized and stored as an ethanolic suspension as previously described (Gessner & Smith, 1960).

Rabbits, cats, rats and ferrets were kept in cages over metal funnels to collect urine and chlorobenzene was given orally by stomach tube (rabbits and rats) or in gelatin capsules (cats and ferrets). Urines from each species were collected until they appeared to be free from chlorophenols and were pooled. With cats it was necessary to collect the urine for at least 4 days.

Measurement of radioactivity. This was carried out as described previously (Gessner & Smith, 1960).

Paper chromatography. This was carried out as described by Smith, Smithies & Williams (1953) and approximate *R_F* values are quoted in Table 1. Satisfactory separations of the three isomeric chlorophenols or their conjugates were obtained only in systems A and B. In separations in solvent system A, the Whatman no. 4 filter paper was soaked in 0.2N-Na₂CO₃ and squeezed between sheets of blotting paper with a hard-rubber roller to press out as much liquid as possible. Solutions of chlorophenols were then applied in ether or methanol, and the paper was dried in air for a few minutes before developing in *n*-hexane containing 10% (v/v) of isopropyl ether. If the aqueous phase was sprayed on to the paper before use it was more difficult to obtain level fronts.

For separations in system B, the paper was soaked in a 20% (v/v) solution of formamide in methanol and pressed between blotting paper as described above. Solutions were

Table 1. *R_F* values of some possible metabolites of chlorobenzene

Solvent mixtures, with time of run in parentheses, were: A, hexane-isopropyl ether (10:1, v/v) on paper treated with 0.2N-Na₂CO₃ (1.5 hr.); B, hexane on paper treated with formamide (1.5 hr.); C, butan-1-ol-acetic acid-water (4:1:5, by vol.) (7 hr.); D, butan-1-ol saturated with water (7 hr.); E, benzene-acetic acid-water (1:1:2, by vol.) (3 hr.); F, pyridine-benzene-acetic acid-water (3:1:5:3, by vol.) (7 hr.); G, butan-1-ol-benzene-acetic acid-water (1:1:1:5, by vol.) (7 hr.); H, hexane-isopropyl ether (5:1, v/v) on paper treated with glycerol (1.5 hr.). Whatman no. 4 paper and the descending method were used.

Solvent mixture	<i>R_F</i>							
	A	B	C	D	E	F	G	H
<i>o</i> -Chlorophenol	0.3	0.5	1.0	0.9	0.8	1.0	1.0	0.9
<i>m</i> -Chlorophenol	0.5	0.7	1.0	0.9	0.8	1.0	1.0	0.9
<i>p</i> -Chlorophenol	0.6	0.6	1.0	0.9	0.8	1.0	1.0	0.9
<i>o</i> -, <i>m</i> - or <i>p</i> -Chlorophenylglucoside	0	—	0.8	0.8	0	0.8	0.6	—
<i>o</i> -, <i>m</i> - or <i>p</i> -Chlorophenylsulphate	0	—	0.6	0.4	0	0.6	0.2	—
<i>o</i> -, <i>m</i> - or <i>p</i> -Chlorophenylcysteine	0	—	0.7	0.6	0	0.6	0.5	—
<i>o</i> -, <i>m</i> - or <i>p</i> -Chlorophenylmercapturic acid	—	—	0.9	0.5	0.7	0.6	1.0	—
4-Chlorocatechol	0	0.1	1.0	—	0.3	0.9	1.0	0.2
4-Chlororesorcinol	0	—	0.9	—	0.2	0.9	0.9	—
2-Chlororesorcinol	0	—	0.9	—	0.25	0.9	—	0.03
2-Chloroquinol	0	—	0.9	—	0.2	0.9	0.9	0.03
Phenol	0.4	0.3	—	—	0.9	—	—	0.6

applied in ether or methanol and the papers dried in air until the smell of methanol was no longer detectable (about 10–15 min.). Papers were then developed in *n*-hexane. Separations were satisfactory with this system only when Whatman no. 4 paper was used. No separation resulted when methanol was omitted and papers were treated with pure formamide. System H was prepared in the same way as B except that glycerol replaced formamide and *n*-hexane-isopropyl ether (5:1, v/v) was used as the mobile phase.

Detection of compounds on chromatograms. Phenols were detected by spraying with ethanolic 0.01% dichloroquinone-chloroimide followed by a spray of saturated aq. NaHCO₃. Chloromonophenols and 4-chlorocatechol gave blue colours and 2- and 4-chlororesorcinol gave purple colours. 2-Chloroquinol gave a green-brown spot which soon faded to brown. 4-Chlorocatechol and 2-chloroquinol gave immediate black spots when sprayed with 0.1N-AgNO₃ containing 1% (v/v) of aq. NH₃ soln. (sp.gr. 0.88). Other polyphenols reacted in a few minutes with this reagent to give brown spots. Chloroquinol and 4-chlororesorcinol, which had the same *R_F*, could be distinguished by these two reactions.

Glucosides were located by spraying the papers with a 1:5 (v/v) dilution of *Helix pomatia* gastric juice, which is rich in β -glucosidase, followed 5 min. later by the dichloroquinonechloroimide reagent to detect liberated phenols. Ethereal sulphates were detected by hydrolysis with a spray of 0.1N-HCl followed by heating to 50–60° for 10 min. and detection of the liberated phenols. Chlorophenylcysteines and chlorophenylmercapturic acids were detected as described by Gessner & Smith (1960).

Chromatography of [¹⁴C]compounds. Paper chromatograms were run in the usual way on Whatman no. 4 paper and reference compounds were normally mixed with about 0.1 μ C of the radioactive extracts so that radioautographs could be compared with the colorimetric location of reference spots. Radioautographs were obtained by leaving the developed chromatograms in contact with X-ray film in light-proof cassettes for 14 days. When volatile radioactive phenolic material was present on the chromatograms the whole film was lightly fogged on development, and though attempts were made to minimize this effect by spraying the chromatograms with 2N-Na₂CO₃ before radioautography, it was apparent that some diffusion and loss of phenols still occurred.

The radioactive chromatograms were also scanned under an end-window counter. A thin aluminium mask was made to cover all but a 1 cm. slot across the window of the counter tube and the paper was passed across this, each centimetre being counted for 1 min. Results were plotted graphically and compared with the spots produced by colorimetric detection of reference compounds incorporated into the mixture and with radioautographs. With the procedures used it was observed that a peak scanning at 20 counts/min. over background did not darken the X-ray film after 14 days.

Separation of chlorophenols on partition columns. Hyflo Supercel (Johns-Manville Co.) was stirred with an equal weight of aq. 0.2N-Na₂CO₃ until an even dry mix was obtained. This was made into a slurry with *n*-hexane and packed into a column 2.5 cm. $\times$ 100 cm. by lightly consolidating the filling with a wooden pestle. Columns usually allowed 2–3 drops of hexane/sec. to flow through and no better separation was found when tighter packing was used. Mixtures of *o*-, *m*- and *p*-chlorophenol (1–20 mg.) were put

on the column in *n*-hexane (10–100 ml.) and development was carried out with *n*-hexane-isopropyl ether (15:1, v/v). Fractions of eluate (10 ml.) were collected and the u.v. absorption of each was measured at 280 m μ against a hexane blank to detect the presence of *m*- or *p*-chlorophenol. When *o*-chlorophenol was being eluted, 50 ml. fractions were taken, and since these contained much less phenol it was more convenient to extract the hexane eluates with 4 ml. of 0.1N-NaOH and to measure the absorption of this at 294 m μ against a 0.1N-NaOH blank. With columns of the dimensions quoted, *m*- and *p*-chlorophenol appeared as two overlapping bands in the elution chromatogram from 400 to 800 ml. and *o*-chlorophenol from 1200 to 2500 ml. of eluate. Fractions containing *m*- and *p*-chlorophenol or *o*-chlorophenol were pooled and extracted with 6 $\times$ 15 ml. of 0.1N-NaOH; the solutions were diluted to 100 ml. and the extracted phenols estimated colorimetrically as described below.

Estimation of chlorophenols

***o*-Chlorophenol.** The u.v. absorption of solutions in 0.1N-NaOH was measured at 294 m μ in a Unicam SP. 500 spectrophotometer and the amount of *o*-chlorophenol present was calculated by reference to a calibration curve of similar solutions of *o*-chlorophenol in 0.1N-NaOH. Recoveries of *o*-chlorophenol (0.5–5.0 mg.) mixed with 10–20 mg. amounts of *m*- and *p*-chlorophenol and separated on partition columns were within the range 80–95%.

***m*- and *p*-Chlorophenol in mixtures.** The total concentration of *meta* or *para* isomer was obtained by measurement of the u.v. absorption in the Unicam SP. 500 spectrophotometer in 0.1N-NaOH at 299 m μ , where the two isomers have the same extinction coefficient (ϵ 2400), and by reference to a calibration curve prepared from either *m*- or *p*-chlorophenol. The sum of the concentrations of *meta* and *para* isomers in mixtures was recovered by this method within $\pm 2\%$ after passage through a partition column.

***m*-Chlorophenol in the presence of *p*-chlorophenol.** A suitable volume, usually 0.5–2.0 ml. of the solution of *m*- and *p*-chlorophenol in 0.1N-NaOH, was neutralized with 0.1N-HCl and diluted to 10 ml. with water. Saturated aq. NaHCO₃ (1 ml.) and aq. 0.1% Brentamine Fast Red (a stabilized diazotized 5-nitro-*o*-anisidine) were added and the red colour was measured at 500 m μ against a reagent blank after 15 min. in the Unicam SP. 500 spectrophotometer. The reagent was unstable in solution and was prepared immediately before use, but the red colour was stable, when formed, for at least 1 hr. *p*-Chlorophenol also reacted with this reagent but the intensity of the colour was less than 2% of that given by the *meta* isomer and the concentration of *p*-chlorophenol in mixtures was satisfactorily determined by difference between the sum of *meta* and *para* isomers estimated as above and the colorimetric estimations of *m*-chlorophenol. Recoveries of the individual phenols in mixtures depended on the relative amounts present, but if the ratio of *para* isomer:*meta* isomer was less than 5:1 recoveries of either were within $\pm 10\%$ after passage through the chromatographic column and extraction from the eluate.

Extraction of monochlorophenols from excreta

Vertebrate urine, containing the equivalent of 0.5 g. of chlorobenzene, was diluted to 200 ml. with water and made 4N with H₂SO₄. Locust excreta were suspended in 4N-

H₂SO₄. After refluxing for 4 hr. the volatile phenols were steam-distilled until 500 ml. of distillate was collected. This was made alkaline with a few drops of 40% (w/v) NaOH and evaporated to a syrup *in vacuo*. The small residue was acidified with a few drops of 20N-H₂SO₄ and the liberated phenols were taken up in 100 ml. of *n*-hexane. A portion of this solution containing 5–10 mg. of mixed phenols was then separated on the partition column as described above.

Chromatography of chlorobenzene excreta

Paper chromatography. (a) With radioactive excreta. An ethanolic homogenate of excreta from locusts dosed with [¹⁴C]chlorobenzene was chromatographed on Whatman no. 4 paper in solvent-system C. Much of the radioactivity located by radioautography and scanning was associated with unidentified material (Table 2), but no unconjugated chloromonophenols (*R_F* 1.0) were detected on this chromatogram. When excreta containing 0.03 μC of radioactivity were separated in solvent-system D, bands were detected by radioautography which coincided with the chlorophenylglucosides and the chlorophenylcysteines (Table 3). When a similar sample of excreta containing 0.03 μC was hydrolysed by heating at 100° with 2N-HCl for 6 hr. in a sealed tube before chromatography, no

glucoside band was detected but the chloromonophenol band was considerably intensified. It was concluded from these chromatographic experiments that the chloromonophenols were excreted mainly as their *O*-conjugates.

(b) With non-radioactive excreta. Locusts (200) were each given 0.5 mg. of non-radioactive chlorobenzene and the excreta were collected for 2 days. The excreta were refluxed with 4N-H₂SO₄ for 3 hr. and the cooled solution was continuously extracted with ether for 6 hr. Paper chromatograms

Table 2. *Paper chromatography of unhydrolysed excreta from locusts dosed with [¹⁴C]chlorobenzene*

Solvent system C was run until the front had moved 12 in.

<i>R_F</i>	Counts/min. at peak activity of zone	Coincidence with reference compounds
0.1	45	.
0.35	40	.
0.50	620	.
0.60	350	Chlorophenylsulphates
0.72	300	Chlorophenylcysteines
0.89	200	Chlorophenylglucosides, chloropolyphenols, chlorophenylmercapturic acids

Table 3. *Paper chromatography of hydrolysed and unhydrolysed excreta from locusts dosed with [¹⁴C]chlorobenzene*

Solvent system D was run until the front had moved 12 in. Intensity of spots: ++, strong; +, weak; -, nil.

<i>R_F</i>	Intensity of radioautograph spots		Coincidence with reference compounds
	Before hydrolysis	After hydrolysis	
0.58	-	+	.
0.61	++	++	Chlorophenylcysteines
0.68	++	-	Chlorophenylglucosides
0.89	+	++	Chlorophenols

Table 4. *Spectrophotometric estimation of chloromonophenols present in hydrolysed urines of animals dosed with chlorobenzene*

	Expt. no.	Percentage of dose as chlorophenols			Ratio (<i>o</i> -isomer: <i>m</i> -isomer: <i>p</i> -isomer)
		<i>o</i> -	<i>m</i> -	<i>p</i> -	
Locusts	1	2.6	2.0	4.2	1.3:1:2.1
	2*	—	4.4	9.4	—:1:2.1
Rabbit	1	1.1	2.8	4.6	0.4:1:1.6
	2	1.2	2.8	6.1	0.4:1:2.2
Rat	1	1.8	2.9	4.9	0.6:1:1.7
	2	2.5	1.8	3.7	1.4:1:2.1
	3	2.8	3.6	6.0	0.8:1:1.7
Ferret	1	1.2	0.8	5.9	1.5:1:7.4
	2	0.4	0.3	2.7	1.3:1:9.0
Cat	1	1.4	3.1	14.9	0.5:1:4.8
	2	1.6	3.1	19.8	0.5:1:6.4

* Estimated by isotopic dilution.

of the extract in the solvents of Table 1 showed the presence of *o*-, *m*- and *p*-chlorophenol and phenol together with a number of apparently polyphenolic products, among which an intense spot of 4-chlorocatechol and a weak spot of 2-chloroquinol were recognized. Similar results were also obtained by paper chromatography of urines of the vertebrates quoted in Table 4, and in the urine of frogs (*Rana temporaria*) and toads (*Bufo bufo*) dosed with chlorobenzene, except that in experiments with frogs only traces of *o*-chlorophenol were found and 4-chlororesorcinol was present in place of 2-chloroquinol.

Quantitative estimation of chlorophenols in locust excreta. Locusts (200) were each dosed with 0.5 mg. of non-radioactive chlorobenzene and excreta were collected for 2 days. The monophenols were separated after hydrolysis and steam-distillation (see above). Analysis by the partition-column method showed that the hydrolysed excreta contained 1.3 mg. of *o*-, 1.0 mg. of *m*- and 2.1 mg. of *p*-chlorophenol. The separated phenols were chromatographed in solvent A and only *m*- and *p*-chlorophenol or *o*-chlorophenol were found in the appropriate extract. Phenol, which was detectable in ether extracts of the hydrolysed excreta, did not appear in the column extract. Similar estimates of the chloromonophenols were made in the urine of vertebrates after dosing with chlorobenzene (0.25 g./kg.) and the results are summarized in Table 4.

Isotopic-dilution procedures

Excreta were collected for 24 or 48 hr. from locusts dosed with [^{14}C]chlorobenzene and the excreta and the locusts were homogenized separately in ethanol. Isotopic dilutions were carried out on both homogenates as described earlier (Gessner & Smith, 1960). Sufficient homogenate was

normally taken to give a count rate of at least 5 counts/min. when a compound representing 1% of the dose was being estimated. Re-isolated derivatives were converted into a second derivative which was shown to have the same equivalent specific activity. All derivatives used in the re-isolation procedures were prepared according to the directions of Huntress (1948) or Huntress & Mulliken (1946). Results of the isotopic-dilution analysis are quoted in Table 5.

***p*-Chlorophenol.** About 0.5 g. of *p*-chlorophenol was added to the radioactive homogenate and the mixture refluxed with 50 ml. of 5*N*-NaOH for 24 hr. After cooling and washing with 2 $\times$ 50 ml. of ether, the alkaline solution was shaken with benzoyl chloride and the *p*-chlorophenyl benzoate formed was crystallized from ethanol to constant specific activity and m.p. 87°. The purified compound was diluted with a weighed amount of non-radioactive *p*-chlorophenyl benzoate and 1 g. was refluxed for 3 hr. with 3 ml. of triethylene glycol containing 0.5 g. of KOH. After cooling the solution was acidified with conc. HCl and extracted with 5 $\times$ 100 ml. of ether. The ether was washed with NaHCO₃ to remove HCl and benzoic acid and the chlorophenol was then extracted into 5 ml. of 2*N*-NaOH. This solution was treated with *m*-nitrobenzoyl chloride and the *p*-chlorophenyl *m*-nitrobenzoate was recrystallized from ethanol until of constant m.p. (124°) and specific activity, which was equivalent to that of the *p*-chlorophenyl benzoate.

***m*-Chlorophenol.** The *m*-chlorophenyl benzoate (m.p. 71°) was prepared and crystallized to constant specific activity as described for the *p*-isomer. The derivative was refluxed for 4 hr. with 10 ml. of 10% NaOH for 4 hr., cooled and the solution of *m*-chlorophenol was converted into its *m*-nitrobenzoyl ester. The *m*-chlorophenyl *m*-nitrobenzoate (m.p. 92°) was purified as described above to constant specific activity equivalent to that of the *m*-chlorophenyl benzoate.

***o*-Chlorophenol.** The *o*-chlorophenol was mixed with the radioactive homogenate and refluxed in 5*N*-NaOH as described above, and the phenol was extracted continuously with ether after acidification with 10*N*-HCl. After removal of the ether the residue was converted into

Table 5. *Metabolites of [^{14}C]chlorobenzene*

Results are expressed as percentages of radioactivity in excreta or remaining in locust. —: Not examined.

Dose/locust (mg.)	...	...	0.35		0.05	0.45		0.3	0.6
			Excreta						
	First day	Second day	Locust	Locust	Excreta	Locust	Excreta	Excreta	
Chlorobenzene*	6.6	10.0	47.5	—	—	—	13.9	—	—
Phenol	—	—	—	—	—	—	—	—	2.4
<i>o</i> -Chlorophenol	—	—	—	3.0	1.0	—	—	—	6.9
<i>m</i> -Chlorophenol	—	—	—	18.1	11.8	8.4	—	—	—
<i>p</i> -Chlorophenol	25.3	2.18	5.8	—	25	8.4	—	—	—
4-Chlorocatechol	21.9	8.7	6.4	10.0	—	—	—	—	—
<i>p</i> -Chlorophenylcysteine*	14.7	4.8	<1.0	8.9	6.1	—	—	—	4.6
<i>p</i> -Chlorophenylmercapturic acid*	2.6	<3.0	<1.0	—	2.2	—	—	—	1.1
<i>m</i> -Chlorophenylcysteine*	—	—	—	—	7.9	—	1.1	—	2.0
<i>m</i> -Chlorophenylmercapturic acid*	—	—	—	—	—	—	—	—	0.3
<i>o</i> -Chlorophenylcysteine*	—	—	—	—	—	—	—	—	1.5
Percentage of dose in excreta or locust	25.0	5.5	20.6	50	37.6	17.8	28.5	30.1	
	(excreta)		(locust)	(locust)	(excreta)	(locust)	(excreta)	(excreta)	

* Calculated from figures quoted by Gessner & Smith (1960).

o-chlorophenyl *m'*-nitrobenzoate, which was crystallized to constant specific activity and m.p. (99°) from ethanol.

4-Chlorocatechol. A portion (0.5 g.) was added to the radioactive sample and the mixture made 15N with H₂SO₄ and kept at 100° for 30 min. The cooled solution was extracted with 4 × 30 ml. of benzene. This solution was dried (CaCl₂) and concentrated until crystallization occurred. The 4-chlorocatechol was recrystallized from benzene and light petroleum (b.p. 90–100) until of constant specific activity and m.p. (91°). The purified 4-chlorocatechol was benzoylated with benzoyl chloride and pyridine and the 4-chlorocatechol dibenzoate (m.p. 96°) crystallized from ethanol and from ether until of constant specific activity, which was equivalent to that of the 4-chlorocatechol.

Phenol. The mixture of phenol and radioactive homogenate was refluxed with 2N-NaOH for 6 hr. and the phenol was continuously extracted with ether after acidification with 10N-HCl. After removal of the ether, the residue was dissolved in conc. H₂SO₄ and converted into picric acid as described by Huntress & Mulliken (1946). The picric acid was crystallized to constant specific activity and m.p. (122°) from 2N-HCl. The picric acid (50 mg.) was treated with an equal weight of *p*-toluidine in 2 ml. of water and boiled. The *p*-toluidine picrate, which formed on cooling, was recrystallized (m.p. 170°) from water and had the same equivalent specific activity as the picric acid.

Hydroxylation of chlorobenzene by the 'model' system

Chlorobenzene was suspended in 500 ml. of water and oxidized by the 'model' ascorbic acid-O₂-ferrous iron system (Udenfriend, Clark, Axelrod & Brodie, 1954), as described by Mead, Smith & Williams (1958). After the droplets of chlorobenzene had disappeared (about 0.5 hr.) the solution was acidified and extracted with ether. The ether was evaporated on the water bath and the residue examined by paper chromatography. Spots corresponding to *o*-, *m*- and *p*-chlorophenol, phenol, 4-chlorocatechol and 2-chloroquinol were detected, but the relative amounts of mono- and poly-phenols varied in different experiments. Chloroquinol was always present in much larger amounts than were 4-chlorocatechol or the monophenols, in the 'model' system than in the urines examined; when the experiment was carried on for longer periods chloroquinol appeared to be the major product, along with much unidentified polyphenolic material.

DISCUSSION

The results obtained show that hydroxylation of chlorobenzene in the locust yields a mixture of phenols similar to that produced by a number of vertebrate species. A large proportion of the phenolic material is 4-chlorocatechol and small amounts of 2-chloroquinol were detected in the excreta of each species. In locust excreta the latter compound was detectable on paper chromatograms from experiments with 200 locusts, but in one attempt to measure it by isotopic dilution it was not present in amounts greater than 1% of the excreted activity. In addition, the three isomeric

chloromonophenols and smaller amounts of phenol were formed by all species examined. The similarity between the phenolic metabolites of chlorobenzene in locusts and vertebrates, especially the presence of *m*- as well as *o*- and *p*-chlorophenol, suggests that similar hydroxylation systems are present in all the species examined. The mixture of phenols found in the excreta was also qualitatively similar to that formed in the 'model' oxidation system, as has been found with other aromatic compounds (Mead *et al.* 1958; Mason, 1957).

Previous observations of the isomer ratios of phenolic metabolites in different species have been confined to compounds which give overwhelmingly *ortho*- or *para*-substituted phenols (Parke & Williams, 1956), and the considerable variation in the *ortho* isomer:*para* isomer ratio found in different animals could have been due, among other possibilities, to steric hindrance or special *ortho*-substitution effects in particular species. Such effects would not operate at the *meta* or *para* positions and, in a compound like chlorobenzene, which gives rise to significant amounts of the *meta*-substituted metabolite, the relative degree of substitution at these two positions should depend on the intimate mechanism of the substitution process *in vivo*. Three mechanisms which have been suggested for this process are the breakdown of premercapturic acids or similar compounds (Knight & Young, 1958; Gessner & Smith, 1960), the dehydration of a dihydrodiol (Boyland, 1950) and a direct hydroxylation following free-radical-substitution rules (Smith, 1950).

It is not thought likely that much of the phenolic material estimated in locust excreta was derived from cysteine-containing precursors since the naphthylpremercapturic acid studied by Knight & Young (1958) and Boyland & Simms (1958) gave only small amounts of naphthols when decomposed in strongly acid solution. The amounts of the isomers of the chlorophenols found in locust excreta were roughly the same as those of various cysteine derivatives present, and paper chromatography suggested that the phenols were excreted as their *O*-conjugates. The presence of *m*-chlorophenol in all the vertebrate urines could be attributed to the decomposition of the premercapturic acid, which gives rise to *p*-chlorophenylmercapturic acid (cf. Gessner & Smith, 1960), since no information is available about the presence or relative amount of any *m*-chlorophenylmercapturic acid in any of the vertebrate species.

The phenols might also be derived from the acid dehydration of a dihydrodiol, such as (I) → (II), or a glucoside conjugate of this (cf. Robinson, Smith & Williams, 1951). The diols do not appear to be biochemical precursors of the phenols in rats since attempts to convert them into phenols in liver

preparations have been unsuccessful (Booth & Boyland, 1957), and it is probable that phenols and dihydrodiols are formed by parallel processes and both are excreted in the urine. The dihydrodiol (I) excreted by the rabbit after dosing with chlorobenzene is known to be converted by acid into *p*-chlorophenol only (Smith *et al.* 1950), and any other possible structure of this type would be expected to give only *o*- or *p*-chlorophenol. The presence therefore of considerable amounts of *m*-chlorophenol in the monophenol fractions from the excreta of all the species examined makes it unlikely that the monophenols are produced solely from the decomposition of dihydrodiols present in the excreta.

m-Chlorophenol, together with the *ortho* and *para* isomers, is produced from chlorobenzene by chemical hydroxylating systems which probably depend on the generation of free OH radicals, e.g. Fenton's reagent, X-irradiation (Johnson, Stein & Weiss, 1951) and the ascorbic acid-ferrous iron system, so that if the biochemical hydroxylation functions by a free-radical process, *m*-chlorophenol would be an expected metabolite. If this were the case, approximately the same ratio of *o*-, *m*- and *p*-chlorophenol would be expected in each species, provided that it is assumed that the phenols are not further metabolized except by conjugation. When different species were examined it was found that considerable variation occurred in the ratio of the yields of *m*- and *p*-chlorophenol, the two mainly carnivorous species, cat and ferret, excreting considerably more *p*- than *m*-chlorophenol (Table 4). This may be an indication that all the species possess at least two hydroxylation systems, one of which follows the orientation rules of free-radical substitution and gives *o*-, *m*- and *p*-chlorophenol and a second system producing dihydrodiols which, on acid treatment, give the extra *p*-chlorophenol found in the urine. It is also possible that the cat and ferret have an enzyme system capable of converting dihydrodiols into phenols before excretion, since this would also give rise to a relative increase in the amount of *p*-chlorophenol in the urine.

A further point of similarity between the locust and the vertebrate species is the excretion of phenol as a metabolite of chlorobenzene. Chlorine is removed from chlorobenzene by the three chemical hydroxylating systems quoted above and the presence of phenol as a metabolite may be a further

indication of an analogous biochemical process. It is equally possible, however, that a 1:2-dihydrodiol (III) is formed, which might be converted either into *o*-chlorophenol or into phenol.

SUMMARY

1. Phenol, *o*-, *m*- and *p*-chlorophenol and 4-chlorocatechol have been detected in the excreta of locusts dosed with chlorobenzene.
2. The amounts of these phenols present have been determined by isotopic-dilution procedures after dosing locusts with [^{14}C]chlorobenzene.
3. Determination of *o*-, *m*- and *p*-chlorophenol in locust excreta has also been carried out by a chromatographic-colorimetric method and the results have been compared with similar estimations on the urines of cats, ferrets, rats and rabbits dosed with chlorobenzene.
4. The significance of the ratio of *o*-chlorophenol: *m*-chlorophenol: *p*-chlorophenol excreted by different species has been discussed.

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The Isolation and Characterization of D-Serine Ethanolamine Phosphodiester from Earthworms

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It has been suggested that the biological precursor of lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) is serine ethanolamine phosphodiester (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, II) and that this may give rise to lombricine by a transamidination reaction involving arginine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959).

This suggestion received some support since it was shown that serine ethanolamine phosphodiester did in fact exist in small amounts in earthworm tissue (Rosenberg & Ennor, 1959). The evidence for its presence was based on chromatographic data obtained with the compound itself, its hydrolysis products and its guanylated derivative, for it was not possible, at that time, to obtain it in amounts large enough to permit identification by more conventional means. However, the report of the occurrence of D-serine in lombricine (Beatty, Magrath & Ennor, 1959) suggested that it would be of interest to isolate larger amounts of serine ethanolamine phosphodiester and to determine the configuration of the serine present in this molecule.

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This paper describes a procedure for the isolation of serine ethanolamine phosphodiester, and provides both a complete characterization of the compound and a comparison with synthetic D-serine ethanolamine phosphodiester. The specific rotation of the natural compound was found to be the same as that of the synthetic one, indicating that the serine moiety has the D-configuration. The preparation

procedure has also yielded lombricine in amounts considerably greater than those reported previously (Rosenberg & Ennor, 1959).

MATERIALS AND METHODS

All reagents were analytical grade and solvents used for chromatography were purified by fractional distillation. Synthetic serine ethanolamine phosphodiester (D-SEP) was prepared by Beatty & Magrath (1959) from D-serine.

The earthworms used were as previously described (Rosenberg & Ennor, 1959) and were removed from cold storage (-70°) just before use.

Ion-exchange resins and chromatography. The following ion-exchange resins were used: Zeo-Karb 225 (mesh 20–50, H^+ form); Dowex-50 (mesh 200–400, NH_4^+ form); Amberlite CG-120 (mesh 100–200, H^+ form). All resins before use were converted into the H^+ and NH_4^+ forms several times by treatment with 2N-HCl or aq. 2.5N- NH_3 soln. respectively, and when finally in the form required were