which was left in an open tube at 4–7°. The solution gradually became turbid and had a silky sheen on being stirred. Fig. 7 shows the appearance of the crystalline material suspended in saturated $(NH_4)_2SO_4$.

DISCUSSION

Immunological analysis of the F and S fractions of the leucocidin preparation showed more than one antigen in each fraction (Woodin, 1959). The present work establishes that the leucocidin activity results from synergism between one constituent of each fraction, the F and S components of leucocidin. It is probable that neither component is greatly contaminated with other immunologically active substances or with material of greatly different charge or sedimentation properties. A calculation of the degree of polydispersity has not been attempted as it has been found that both components can be easily denatured and it is likely that some denatured material is present in the most highly purified preparation of each component.

SUMMARY

1. An antibody-combining-power method has been developed for the determination of the concentration of the two components of leucocidin.

2. Both the F and the S fractions of the leucocidin preparation contain deoxyribonuclease.

3. The F and the S components of leucocidin have been purified with Dowex-2 (×8) and Amberlite CG-50.

4. The F component of leucocidin crystallizes from 0·2 M-phosphate buffer, pH 6·7, in the form of needles or as hexagonal plates.

5. The slow component of leucocidin has been crystallized by salting out with ammonium sulphate from 0·05 M-phosphate buffer, pH 6·7, in the form of very fine needles.

6. Both the F and S components of leucocidin behave immunologically as single substances and on electrophoresis or ultracentrifuging show a single boundary.

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Comparative Detoxication

7. THE METABOLISM OF CHLOROBENZENE IN LOCUSTS: EXCRETION OF UNCHANGED CHLOROBENZENE AND CYSTEINE CONJUGATES*

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Chlorinated cyclic hydrocarbons are among the most widely used insecticides but sometimes their value is reduced if the insects can metabolize the poison to inactive products. The importance of detoxication mechanisms in strains of some insects resistant to 2:2-bis-(*p*-chlorophenyl)-1:1:1-trichloroethane (DDT) is now well known (cf. Metcalf, 1955), and the natural resistance of the grasshopper family probably has a similar metabolic basis (Sternburg & Kearns, 1952).

* Part 6: Kikal & Smith (1959).

The metabolites of DDT in some insects are still not known and, as well as 2:2-bis-(*p*-chlorophenyl)-1:1-dichloroethylene (DDE), cockroaches excrete five unidentified metabolites which account for most of the dose (Robbins & Dahm, 1955; Lindquist & Dahm, 1956). Flies (Terriere & Schonbrod, 1955) and lice (Perry & Buckner, 1958) also excrete much of a dose of DDT as unidentified water-soluble material, which with flies appeared to be mainly phenolic in nature although part was not extractable from acid solution.

Detoxication mechanisms are also important in the resistance of some insects to γ-hexachlorocyclohexane (Gammexane) (Openoorth, 1955), and a complex mixture of water-soluble metabolites is formed by the degradation of this insecticide in flies (Bradbury, 1957). It is possible that some of these water-soluble products are cysteine derivatives like the mercapturic acids formed in the metabolism of chlorinated compounds in vertebrates (Williams, 1947). Mercapturic acid or arylcysteine formation in insects appears to have been recorded only by Kikal & Smith (1958), but the identification of a number of derivatives of dichlorothiophenols from flies dosed with isomers of benzene hexachloride suggests that a cysteine conjugation may be involved in the metabolism of these compounds (Bradbury & Standen, 1959). We have therefore tested for the presence of a detoxication mechanism involving cysteine conjugation by dosing locusts with [^{14}C]chlorobenzene.

EXPERIMENTAL

Materials

[^{14}C]*Chlorobenzene.* This was obtained (0·01 mc/mg.) from The Radiochemical Centre (Amersham, Bucks.) and was diluted either with inactive chlorobenzene or with arachis oil to give convenient volumes for injection. Its identity and purity (> 99·5%) were checked by comparing the activity of such solutions with that of the *p*-chlorobenzenesulphonamide prepared from them after dilution with additional inactive chlorobenzene (see below).

Reference compounds. L-*p*-Chlorophenylmercapturic acid, m.p. 153°, *p*-chlorophenyl-L-cysteine, m.p. 191° (decomp.), L-*m*-chlorophenylmercapturic acid, 165°, *m*-chlorophenyl-L-cysteine, 183° (decomp.), and *o*-chlorophenyl-L-cysteine, m.p. 180° (decomp.), were prepared according to Parke (1955) and Parke & Williams (1951). The cysteine derivatives were more conveniently purified from cystine via their hydrochlorides by crystallizing crude chlorophenyl-L-cysteines from the minimum amount of 2N-HCl and drying over KOH *in vacuo*. p-*Chlorophenyl-L-cysteine hydrochloride* formed colourless plates, m.p. 205° (decomp.); $[\alpha]_D^{30}$ +15 in 0·1N-NaOH (*c*, 1) (Found: HCl, 13·6%). m-*Chlorophenyl-L-cysteine hydrochloride* formed colourless needles, m.p. 180° (decomp.); $[\alpha]_D^{30}$ +13 in 0·1N-NaOH (*c*, 1) (Found: HCl, 13·1%). o-*Chlorophenyl-L-cysteine hydrochloride* formed colourless plates, m.p. 190° (decomp.); $[\alpha]_D^{30}$ +8 in 0·1N-NaOH (*c*, 1) (Found HCl, 13·6. $C_9H_{10}O_2NClS \cdot HCl$ requires HCl, 13·6%). All isomers were moderately soluble in cold water and very soluble in ethanol.

Methods

Locusts and dosing. Schistocerca gregaria were obtained from the Anti-Locust Research Centre as immature adults and were stored under the conditions recommended by Hunter-Jones (1956). Compounds were administered by injection into the thorax with an Agla micrometer syringe as solutions (0·1–0·05 ml.) in arachis oil or water. Unless the respired air was to be collected, the dosed locusts were kept singly in paper-covered 500 ml. beakers at 25–30°. Dissection of the insects was carried out on a wax block sprinkled with solid CO_2 to minimize loss of any volatile metabolite and the dissected tissues were weighed and stored in glass-stoppered bottles at −40°.

Measurement of radioactivity. All measurements were carried out with thin end-window-counter tubes, the background of which was 15–18 counts/min., and specific activities were determined by comparison with a 0·1 μc/g. polymer reference sample (The Radiochemical Centre) which counted at about 100 counts/min. over background. Solid samples were counted on 1·5 cm.^{2} planchettes in 'infinitely thick' layers, and liquids and homogenates in inverted planchettes, which were referred to similarly prepared plastic standards. Counts were made for times sufficient to give S.E. ±5% (cf. Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). Tissues and excreta were usually mixed with a small known volume of water or ethanol in a glass Potter homogenizer and counted as thick homogenates, but in some cases 10–20 mg. of tissue samples were oxidized to CO_2 by the Van Slyke & Folch (1940) procedure and counted as $BaCO_3$. No significant difference was found in results obtained by the two techniques.

Paper chromatography. This was carried out as described earlier (Smith, Smithies & Williams, 1953) and approximate R_F values are quoted in Table 1. No solvents were found to

Table 1. *R_F values of chlorophenyl-mercapturic acids and -cysteines*

Whatman no. 4 paper and the descending method were used. Solvent mixtures, with times of run in parentheses, were: A, butan-1-ol–acetic acid–water (4:1:5, by vol.) (7 hr.); B, methyl ethyl ketone–water–2N-acetic acid (200:100:1, by vol.) (2 hr.); C, methyl ethyl ketone saturated with water (2 hr.); D, benzene–butan-1-ol–acetic acid–water (1:1:1:5, by vol.) (7 hr.); E, methyl ethyl ketone–aq. 2N-NH_3 soln. (1:1, v/v) (2 hr.).

	R_F				
Solvent mixture	A	B	C	D*	E*
o-, *m*- and *p*-Chlorophenylmercapturic acid	0·9	0·8	0·5	0·8	0·2
o-, *m*- and *p*-Chlorophenyl-L-cysteine	0·7	0·3	0·3	0·5	0·2

* *para*Isomer only.

separate satisfactorily the three isomers of either chlorophenylcysteine or chlorophenylmercapturic acid. The chlorophenylcysteines were detected by the purple colour formed when the paper was sprayed with 0·1% (w/v) butan-1-ol solution of ninhydrin and heated to 100° for 10 min. They also quenched the blue fluorescence of filter paper under illumination with a filtered low-pressure Hg arc (Hanovia Chromatolite). Chlorophenyl-cysteines and -mercapturic acids were both detected by the $Ag_2Cr_2O_7$ reagent of Knight & Young (1958). Normal crop fluid and excreta of locusts contained large amounts of ninhydrin-reacting materials but these had R_F values smaller than those of any of the cysteine conjugates in the solvents used.

Spectrophotometric estimation of p-*chlorophenylmercapturic acid.* Samples of the ethanolic suspension of excreta containing 50–300 μg. of *p*-chlorophenylmercapturic acid were separated by paper chromatography in solvent system B (Table 1). The zone containing the mercapturic acid, located on a reference strip by the $Ag_2Cr_2O_7$ reagent, was cut out and extracted by keeping with occasional shaking in 10 ml. of ethanol for 1 hr. The *p*-chlorophenylmercapturic acid in the extract was estimated by measurement of the absorption at 260 mμ in a Unicam SP. 500 spectrophotometer and comparison with a calibration curve prepared from known amounts of *p*-chlorophenylmercapturic acid similarly separated and extracted. Extracts of paper blanks showed no absorption at this wavelength and recoveries of *p*-chlorophenylmercapturic acid (50–300 μg.) added to normal locust excreta were within ±10 μg.

Estimation of p-*chlorophenyl-*L-*cysteine.* (*a*) Ninhydrin method. The *p*-chlorophenyl-L-cysteine (40–100 μg.) was separated from other ninhydrin-reacting material in excreta by chromatography in solvent A or B as described for the mercapturic acid above. Zones containing *p*-chlorophenylcysteine, located by the fluorescence-quenching effect, were cut out and treated with 1 ml. of 0·1 N-NaOH. After drying over H_2SO_4 *in vacuo* overnight to remove NH_3, the excess of alkali was neutralized (Fowden, 1951) and the amino acid on the paper estimated by the procedure of Yemm & Cocking (1953), the paper being immersed in the reagent solution. Amounts of *p*-chlorophenyl-L-cysteine (40–160 μg.) added to normal locust excreta were recovered within 5 μg. when estimated by this procedure. *m*-Chlorophenylcysteine gave a calibration curve identical with that of the *p*-isomer. (*b*) Fluorimetric method. *p*-Chlorophenyl-L-cysteine fluoresced in ultraviolet light (see Table 2). The relation between fluorescence and concentration in 0·1 N-NaOH was linear from 1 to 6 μg./ml., the lower limit being governed by the background light scatter of the solutions. Solutions of higher concentration, where concentration quenching occurred, were measured after dilution to bring the concentration within the linear range. No fluorescence of the *o*- or *m*-isomers was detected in the concentration range 1–100 μg./ml. at the instrument settings used to measure the *p*-isomers. The *p*-chlorophenylcysteine (5–30 μg.) was separated from mercapturic acids and other fluorescing substances by paper chromatography in solvent A or B and the papers were dried in a current of air for at least 4 hr. The appropriate zones were located and cut out and the chlorophenylcysteine was extracted from the paper by shaking for 30 min. in 5 ml. of 0·1 N-NaOH. The solution was filtered through washed glass wool to remove paper fibres, and the fluorescence of the solution was compared with a standard solution (5 μg./ml.) of *p*-chlorophenylcysteine in 0·1 N-NaOH at 360 mμ in an Aminco–Bowman spectrophotofluorimeter with exciting wavelength set to 280 mμ. Recoveries of *p*-chlorophenyl-L-cysteine (10–100 μg.) added to normal locust excreta were within ±2 μg.

Distribution of radioactivity in locust

Excretion of unchanged chlorobenzene. Locusts were injected with [^{14}C]chlorobenzene (0·05–0·5 mg.) and placed in a 500 ml. flask through which a slow stream of air, washed through 2 N-NaOH, was drawn. The air leaving the flask was drawn through a drying tube containing magnesium perchlorate, through two tubes, each containing 10 ml. of ethanol, cooled to −50°, and finally through 2 N-NaOH.

Table 2. *Instrumental values of fluorescence of some metabolites of chlorobenzene*

Readings are the product of meter multiplier and deflexion in the Aminco–Bowman spectrophotofluorimeter. Solutions contained 5 μg./ml.

	In 0·1 N-NaOH			In 0·1 N-HCl		
	Exciting wavelength (mμ)	Fluorescence wavelength (mμ)	Reading	Exciting wavelength (mμ)	Fluorescence wavelength (mμ)	Reading
p-Chlorophenylcysteine	280	360	1·35	280	340	0·81
p-Chlorophenylmercapturic acid	270	350	0·51	270	360	0·90

Table 3. *Radioactivity recovered after injection of* [^{14}C]*chlorobenzene into locusts*

No. of locusts	Chlorobenzene injected (mg./locust)	Percentage of radioactivity found after 48 hr. in		
		Expired air	Excreta	Locust
1*	0·05	51	No excreta	49
1*	0·05	49	1	50
2	0·05	40	29	31
1	0·35	53	33	14
4	0·45	35	44	21
6	0·6	18	30	52

* 24 hr. experiment.

The ethanol tubes were removed periodically and the ^{14}C was assayed by liquid counting. At no time was more than a trace of activity found in the second ethanol tube. Control experiments showed that 0·2–1·0 mg. of [^{14}C]-chlorobenzene was quantitatively recovered in this apparatus in 6 hr.

After 48 hr. the excreta were removed and the flask was rinsed out with a small volume of ethanol; the excreta and washings were mixed in an Ato-Mix homogenizer to form a thick suspension which was assayed for ^{14}C (see Table 3). The radioactivity collected in the cold ethanol trap was pooled and, by dilution analysis (see below), was shown to be all in the form of chlorobenzene. The CO_2 trapped in the final NaOH wash-bottle was precipitated as $BaCO_3$ and assayed for ^{14}C. In two experiments with high-activity [^{14}C]chlorobenzene less than 0·1 % of the dose was found as CO_2.

Radioactivity in locust organs. Immature adult locusts were each dosed with 2 mg. (0·2 μc) of [^{14}C]chlorobenzene and kept at 25–30°. At intervals one locust was removed and dissected and the radioactivity measured in the fat, muscle, foregut, hindgut, heart, integument and excreta. A small accumulation of radioactivity appeared to take place in the fat which was at a maximum 8 hr. after dosing, when 15 % of the total dose was found in this site. No significant activity appeared to accumulate in any other sample tested except the hindgut and excreta, where the radioactivity increased progressively. In other experiments some variation was found in the rate at which the radioactivity appeared in the excreta (Table 3).

Radioactivity in excreta. A sample of freshly voided excreta, containing 0·66 μc, from locusts dosed with [^{14}C]chlorobenzene was heated for 12 hr. at 100° in a sealed tube with 10 ml. of N-HCl. After cooling, the solution was partitioned into neutral (15·9 %), alkali-soluble (61·7 %) and acid-soluble (20·4 %) fractions as shown in Table 4. The neutral fraction (15·9 %) was shown by dilution with inactive chlorobenzene and counting as *p*-chlorobenzenesulphonamide (see below) to consist only of chlorobenzene. The alkali-soluble fraction appeared to consist mainly of phenols (Gessner & Smith, 1960). Further continuous extraction of the acid-soluble fraction with ether removed no more radioactivity.

Estimations by isotopic dilution

The radioactivity in excreta was partly present in an insoluble form which could be removed from the fibrous food residues only by repeated washing with ethanol or N-NaOH. The thick homogenate of the excreta, after the radioactivity had been counted, was therefore diluted with ethanol to give about a 1–5 % (w/v) suspension and samples for dilution analysis were withdrawn from this after shaking well. Inactive compounds were usually dissolved in a volume of this suspension such that, after isolation again, a metabolite accounting for 1 % of the activity counted at not less than 5 counts/min. After purification to constant specific activity the isolated material was normally converted into a second derivative, and this, after purification, was shown to have the same equivalent specific activity as the first, Results quoted in Table 5 are calculated from two such concordant values.

Chlorobenzene. The ethanolic solution containing [^{14}C]-chlorobenzene was mixed with five times its volume of water and 1 g. of inactive chlorobenzene. The mixture was then extracted twice with an equal volume of ether. After drying over $CaCl_2$ the ether was evaporated and chlorosulphonated as described by Huntress & Carten (1940). The *p*-chlorobenzenesulphonyl chloride, m.p. 48°, was counted after crystallization from dry ether and converted into *p*-chlorobenzenesulphonamide, m.p. 144° (Huntress & Carten, 1940). This was crystallized to constant activity from aqueous ethanol.

p-*Chlorophenyl*-L-*cysteine.* The non-radioactive compound (0·2–0·5 g.) was added to a suitable volume of radioactive ethanolic suspension or homogenate and heated with 20–50 ml. of 0·5 N-H_2SO_4 until solution was complete. After filtration from grass or tissue debris, the filtrate was washed with 2 × 25 ml. of ether and 2 × 25 ml. of $CHCl_3$ and adjusted to pH 4·0–5·0. The precipitated *p*-chlorophenylcysteine was recrystallized from ethanol–water (1 : 1, v/v) to constant m.p. (191°, decomp.) and constant specific activity. The purified product was recrystallized from N-HCl to give *p*-chlorophenyl-L-cysteine hydrochloride, m.p. 205° (decomp.), with the same equivalent specific activity.

L-p-*Chlorophenylmercapturic acid.* The radioactive solution or ethanolic homogenate was mixed with non-radioactive L-*p*-chlorophenylmercapturic acid (0·2 g.), 25 ml. of 2 N-HCl and 25 ml. of ethanol and warmed until solution was complete. After removal of any grass residues from the excreta by filtration, the solution was extracted with 5 × 100 ml. of $CHCl_3$ and the mercapturic acid re-extracted from the pooled $CHCl_3$ layers with 10 ml. of 2 N-NaOH. The alkaline solution was acidified to pH 3·0 and the precipi-

Table 4. *Partition of radioactivity of hydrolysed excreta from locusts dosed with* [^{14}C]*chlorobenzene*

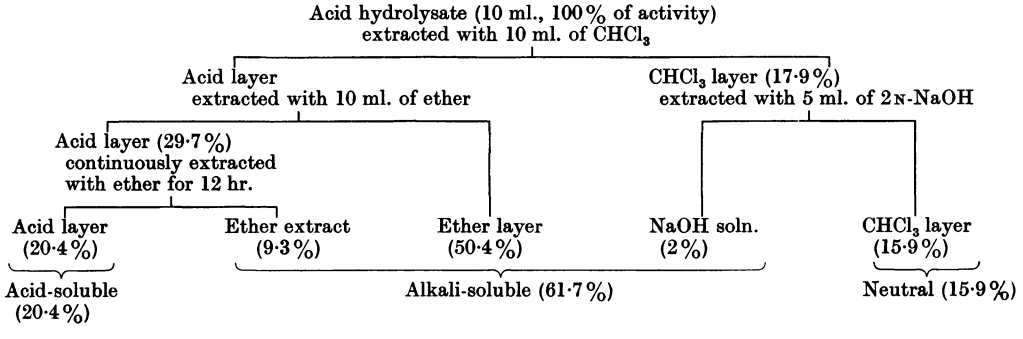


Table 5. *Excretion in 48 hr. of some metabolites of* [^{14}C]*chlorobenzene by locusts*

—, Not examined.

No. of locusts used	1	5	1	4	6
Dose of chlorobenzene (mg./locust)	0·05*	0·3	0·35	0·45	0·6
Metabolite excreted	Percentage of dose				
Chlorobenzene					
In expired air	49·5	—	48·7	30·1	18·0
In excreta (first day)	—	3·9	1·7	—	—
In excreta (second day)	—	—	0·6	—	—
Left in locust	—	—	9·8	—	29·0
p-Chlorophenylcysteine					
In excreta (first day)	—	—	3·7	2·3	1·4
In excreta (second day)	—	—	0·3		
Left in locust	4·5	—	<1·0	.	.
p-Chlorophenylmercapturic acid					
In excreta (first day)	—	—	0·7	0·8	0·3
In excreta (second day)	—	—	<0·2		
Left in locust	—	—	<0·1	.	.
m-Chlorophenylcysteine in excreta	—	0·3	—	3·0	0·6
m-Chlorophenylmercapturic acid in excreta	—	—	—	—	0·1
o-Chlorophenylcysteine in excreta	—	—	—	—	0·4

* This locust provided no excreta.

Table 6. *Cysteine conjugates in excreta of locusts dosed with* [^{14}C]*chlorobenzene*

Pooled excreta from six locusts given 0·6 mg. of [^{14}C]-chlorobenzene each were used for each experiment.

Metabolite	Percentage of total radioactivity of excreta: Without acidification	After heating with N-HCl
m-Chlorophenyl-L-cysteine	<0·5	2·2
L-*m*-Chlorophenylmercapturic acid	<0·2	0·3
p-Chlorophenyl-L-cysteine	1·0	5·0
L-*p*-Chlorophenylmercapturic acid	<0·2	1·2

tated mercapturic acid recrystallized from ethanol–water (1:1, v/v) to constant m.p. (152°) and specific activity. The purified mercapturic acid (0·1 g.) was boiled with 20 ml. of N-NaOH for 2 hr. A portion (10 ml.) of 2N-H_2SO_4 was then added and the solution titrated with 0·05N-I_2 solution until precipitation of *pp'*-dichlorodiphenyl sulphide was complete (cf. Parke, 1955). The disulphide (m.p. 71°) after washing with water and drying had the same equivalent specific activity as the mercapturic acid.

m-*Chlorophenyl-L-cysteine.* A suitable volume of the ethanolic suspension of excreta was diluted with water and 0·5 g. of non-radioactive *m*-chlorophenyl-L-cysteine added. The solution was made N to HCl, heated to 80–90° and cooled immediately. The precipitate formed on neutralization with 2N-NaOH was reprecipitated three times by solution in N-NaOH and acidification with 2N-acetic acid and then crystallized from 5N-HCl to constant activity. The *m*-chlorophenyl-L-cysteine hydrochloride was converted into the free base, m.p. 183° (decomp.), and had the same equivalent specific activity as the hydrochloride.

L-m-*Chlorophenylmercapturic acid.* The non-radioactive material (0·5 g.) was added to the ethanolic suspension of excreta with water and the solution made N to HCl. The solution was heated until all the added material was dissolved, and cooled. The precipitate was purified by solution in N-NaOH and reprecipitation with 2N-acetic acid and then crystallized to constant specific activity and m.p. (165°) from 50% (v/v) ethanol.

o-*Chlorophenyl-L-cysteine.* This was isolated again as described for the *m*-isomer and counted as the hydrochloride, m.p. 190° (decomp.), and as *o*-chlorophenylcysteine, m.p. 180° (decomp.).

Precursors of the chlorophenyl-cysteines and -mercapturic acids

Excreta were collected for 2 days after dosing locusts with [^{14}C]chlorobenzene and were homogenized in ethanol. Suitable volumes of this suspension were added to non-radioactive samples (0·5 g.) of *m*- and *p*-chlorophenyl-L-cysteine and *m*- and *p*-chlorophenylmercapturic acid which had been dissolved in 1 mol.prop. of NaOH and diluted to 20 ml. with water. The solutions were immediately acidified with 2N-acetic acid and the precipitates collected. These were purified by twice precipitating from solution in 0·1N-NaOH with 2N-acetic acid and then crystallized as described above until the specific activity was constant or not significantly different from the background.

A second set of estimations on this sample of excreta was carried out as described above except that the solutions were heated for a few seconds at 80° after making N to HCl, and quickly cooled before isolation of the compounds. The results of these analyses, quoted in Table 6, show that only small amounts of mercapturic acid or cysteines are present in excreta before acid treatment. It was concluded from these results that both the mercapturic acids and chlorophenylcysteines estimated in locust excreta are excreted as precursors similar to the premercapturic acids found in vertebrates (Knight & Young, 1958; Boyland & Sims, 1958).

Absence of a chlorobenzene precursor. An aqueous suspension of freshly voided excreta, from locusts dosed with [^{14}C]chlorobenzene, which contained 0·58 μC, was divided into two equal parts and non-radioactive chlorobenzene (0·5 g.) was added to each. One part was immediately extracted with ether and the chlorobenzene recovered and converted into *p*-chlorobenzenesulphonamide as described above. The second portion was heated in a sealed tube at 100° for 12 hr. with N-HCl and the chlorobenzene was extracted and converted into *p*-chlorobenzenesulphonamide. After crystallization to constant activity the first preparation carried 0·068 μC and the second 0·069 μC, and it was concluded that an acid-labile precursor (cf. Bourne & Young, 1934) of chlorobenzene was not a significant metabolite of chlorobenzene in the locust.

Fate of the cysteine conjugates in locusts

In most species, mercapturic acids appear to be the main cysteine conjugate excreted and it was thought possible that the preponderance of arylcysteines in locust excreta might be due to the action of an acylase in the gut which hydrolysed the initial detoxication product (cf. Kikal & Smith, 1959). We have therefore attempted to measure the apparent acylase activity in gut and crop fluid with *p*-chlorophenylmercapturic acid as substrate.

Enzymic hydrolysis of L-p-*chlorophenylmercapturic acid.* The sources of enzyme were crop fluid (cf. Robinson, Smith & Williams, 1953) diluted tenfold with water, or excreta ground in a mortar with four times the weight of water and squeezed through cheesecloth. In the estimation of enzyme activity 0·5 ml. of enzyme, 0·25 ml. of substrate (*p*-chlorophenylmercapturic acid and 1 mol.prop. of NaOH in aqueous solution) and 0·25 ml. of buffer, pH 3·0–8·0 (prepared from 0·2 M-Na_2HPO_4–0·1 M-citric acid) were incubated at 37° for 18–36 hr. Control tubes were prepared (*a*) in which the substrate was added at the end of the incubation period, and (*b*) in which the enzyme was added at the end of the incubation period. Controls (*b*) showed that no hydrolysis of mercapturic acid occurred at any pH in the absence of enzyme, but in controls (*a*) some changes, visible on the paper chromatograms, occurred in the large amounts of ninhydrin-reacting compounds present in the enzyme extracts. After incubation and completion of the control tubes, 0·1 ml. of mixture was withdrawn and separated on Whatman no. 4 paper in solvent system B and the amount of chlorophenylcysteine present was determined by one of the procedures described above. Optimum enzymic hydrolysis of mercapturic acid to *p*-chlorophenylcysteine was found at pH 6·0 in both crop fluid and excreta. The degree of hydrolysis increased with increasing substrate concentration up to a limit set by the solubility of the substrate at pH 6·0 (0·025 M).

When freshly voided excreta or crop fluid were assayed under the optimum conditions of pH and substrate concentration the activity found was of the order of 100 μg./hr./g. of excreta or crop fluid. Control experiments showed, however, that *p*-chlorophenyl-L-cysteine was itself destroyed under these conditions. When 1–2 mg. of *p*-chlorophenylcysteine was incubated at pH 6·0 with 0·5 ml. of the enzyme extracts used for acylase activity, 70–100 % was destroyed in 48 hr.

Fate of injected p-*chlorophenylmercapturic acid and* p-*chlorophenyl-*L-*cysteine. p*-Chlorophenylmercapturic acid (2 mg.) was injected into locusts as its sodium salt in 0·1 ml. of water and excreta were collected for 48 hr. The excreta were homogenized with 2 ml. of 50 % (v/v) aqueous ethanolic 0·05 N-HCl and 0·2 ml. of the liquid was chromatographed in solvent system B; the *p*-chlorophenyl-cysteine and -mercapturic acid were estimated as described above. No *p*-chlorophenylcysteine could be detected and 66–79 % of the dose of the mercapturic acid was recovered. No further mercapturic acid was found in the excreta collected over the next 3 days.

When *p*-chlorophenyl-L-cysteine (0·5–1·0 mg.) was similarly injected and the 48 hr. excreta were extracted as described above less than 2 % of the dose appeared as the mercapturic acid, and recoveries of the *p*-chlorophenylcysteine ranged from 9·5 to 12·5 % of the dose. No further material was found when excreta were collected for the next 4 days and no *p*-chlorophenylcysteine could be detected chromatographically in the haemolymph.

Nature of the degradation product. p-Chlorophenylcysteine (1 mg.) was incubated for 48 hr. with 0·5 ml. of the excreta enzyme at pH 6·0 as described above and the reaction mixture was examined chromatographically. No *p*-chlorophenyl-cysteine or -mercapturic acid was found, but a spot was present with R_F 0·9 in system D and R_F 0·4 in system E, which reacted strongly with $Ag_2Cr_2O_7$ but gave no ninhydrin reaction. Similar spots from unsprayed chromatograms were eluted

with ethanol and examined by paper electrophoresis as described by Smith (1958). The unknown material migrated to the anode at pH values from 3·0 to 12·0, but, unlike *p*-chlorophenylcysteine, it did not migrate at pH 1·0.

DISCUSSION

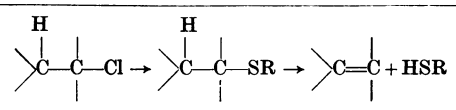
About half of the chlorobenzene injected into the locusts was excreted unchanged, presumably by spiracular respiration, since in one experiment (Table 3) where no excreta were produced 51 % of the dose was recovered by aeration. In other experiments some of the chlorobenzene recovered by aeration may have been voided with the excreta, since, when these were examined after being kept in the respiration chamber for 48 hr., up to 14 % of the radioactivity in the excreta was present as unchanged chlorobenzene.

Non-polar fat-soluble substances tend to be retained by the vertebrate kidney and it has been suggested (Brodie & Hogben, 1957) that the detoxication mechanisms of terrestrial vertebrates have developed to change such lipid-soluble material into highly polar, easily excreted products. The orthopteran excretory system, which involves simple diffusion through the Malpighian tubules and water reabsorption in the rectum (Ramsay, 1958), appears, from the excretion of chlorobenzene, to retain lipid-soluble material less effectively. The necessity for locusts to form polar detoxication products may therefore be less pressing than for vertebrates. Formation of the detoxication products was slow and after 48 hr. much of the administered radioactivity still remained in the locust, much of it still as unchanged material.

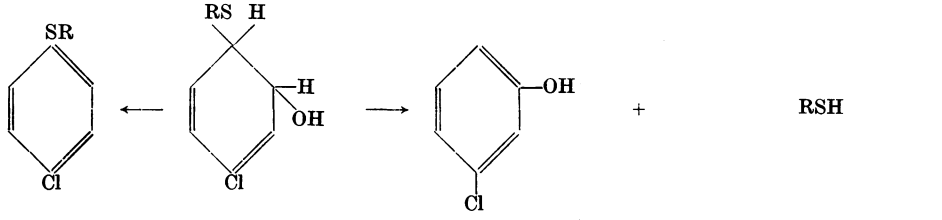
The cysteine conjugates found in locust excreta were present as acid-labile precursors of chlorophenyl-L-cysteines and -mercapturic acids. This situation is essentially similar to that found in vertebrates. In the vertebrates, however, only the *para*isomer of chlorophenylmercapturic acid has been found as a metabolite of chlorobenzene and a search for other isomers is called for since the presence of both *meta* and *para* isomers in the locust is of interest in the discussion of possible mechanisms for the formation of the premercapturic acids. The absence of any pronounced directive effects of the chlorine in the formation of the cysteine conjugates of chlorobenzene recalls the similar lack of directive effects in some biochemical hydroxylations (Smith, 1950; Gessner & Smith, 1960).

A further difference between the metabolism of chlorobenzene in the locust and in vertebrates is that the chlorophenylcysteines formed are mainly found in the non-acetylated form in the locust, whereas in vertebrates the cysteine conjugates are isolated as their acetyl derivatives, the mercapturic acids. It is not thought likely that the *p*-chlorophenylcysteine found in locust excreta was derived from an acetylated precursor by hydrolysis in the gut (cf. Kikal & Smith, 1959), since the acylase activity in crop fluid and excreta was very low. Moreover, when *p*-chlorophenylmercapturic acid was injected into the locust most of it could be recovered unchanged in the excreta. It is probable that the amounts of chlorophenylcysteines estimated in excreta should be somewhat larger than those actually found since some decomposition occurred in the gut.

As well as the aromatic mercapturic acids and premercapturic acids found in locusts and vertebrates, the latter can also form mercapturic acids by replacement of active halogen atoms such as the chlorine in benzyl chloride (cf. Knight & Young, 1958). It is possible that the sulphur-containing metabolite of Gammexane observed by Bradbury & Standen (1959) is of this type and it is interesting to consider whether a similar cysteine derivative is involved in metabolism of DDT. Glutathione, which could be the source of the cysteine of the mercapturic acids (Bray, Franklin & James, 1959), is required for the metabolism of both Gammexane and DDT *in vitro*, and the former is largely converted into sulphur conjugates *in vivo*. If DDT (I) formed a similar cysteine conjugate (II), a decysteinylation reaction would give the known metabolites (III), DDE from DDT and pentachloro*cyclo*hexene (Bradbury & Standen, 1958) from Gammexane.



(I) (II) (III)



(IV) (V)

R = $-CH_2 \cdot CH \cdot NH_2 \cdot CO_2H$

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 [^{14}C]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.