Comparative Detoxication

9. THE METABOLISM OF SOME HALOGENATED COMPOUNDS BY CONJUGATION WITH GLUTATHIONE IN THE LOCUST*

BY A. J. COHEN AND J. N. SMITH
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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It has been shown that the metabolism of chlorobenzene in locusts and vertebrates is similar, and that the isomeric chlorophenylcysteines and chlorophenylmercapturic acids were present in locust excreta as acid-labile precursors (Gessner & Smith, 1960). Benzyl chloride and *p*-nitrobenzyl chloride are also metabolized to mercapturic acids in mammals but without the formation of intermediate acid-labile precursors. It is known, however, that in mammals *S*-substituted glutathione derivatives are intermediates in the formation of mercapturic acids from either aromatic compounds or arylalkyl halides (Knight & Young, 1958; Barnes, James & Wood, 1959; Bray, Franklin & James, 1959; Booth, Boyland & Sims, 1960).

We wished to see if cysteine or glutathione conjugates were formed in locusts from compounds, e.g. benzyl chloride, that contain labile or reactive halogen, since these simple compounds might serve as models for the more complex chlorinated insecticides. Little attention has been paid to detoxications of this type in insects, and the observation of Bettini & Boccacci (1958) that bromoacetic acid was metabolized by cockroaches to *S*-(carboxymethyl)glutathione appears to be an isolated case. Glutathione conjugates or cysteine conjugates have also been suspected as metabolites of Gammexane (γ-hexachlorocyclohexane) in flies (Bradbury & Standen, 1959), but the evidence is indirect and it is not known whether the sulphur-containing metabolites are aromatic or cycloaliphatic.

EXPERIMENTAL

Materials

Locusts and tissue preparations. Fifth-instar hoppers or adults of *Schistocerca gregaria* were obtained from the Anti-Locust Research Centre and kept on a grass diet as recommended by Hunter-Jones (1956).

Compounds were administered by injection into the thorax in acetone solution (10 μl. containing 100 μg.).

Locust 'fillets' were prepared by cutting off the terminal segment of the hoppers, drawing out the head and gut and dividing the carcass by ventral and dorsal cuts. The two half-carcasses (i.e. the 'fillets') consisted mainly of fat body, gonads and integument with a small amount of undeveloped flight muscle. These were incubated in Petri dishes or small conical flasks in air at room temperature in grasshopper saline (Carlson, 1946) to which was added the organic compound being investigated and 1% (w/v) of a commercial blood albumin. Sufficient saline was used to just cover the 'fillets'.

Homogenates of separate locust tissues were prepared by grinding for a few minutes with saline or buffer in a glass Potter–Elvehjem homogenizer.

Reference compounds. All melting points are uncorrected. *S*-Benzyl-L-cysteine, m.p. 216°, *S*-(*p*-chlorobenzyl)-L-cysteine, m.p. 204 (decomp.), *S*-(naphth-1-ylmethyl)-L-cysteine hydrochloride, m.p. 211°, and *S*-(*p*-nitrobenzyl)-L-cysteine, m.p. 193 (decomp.), were made by the method of Theodoropoulos (1959) and crystallized from aqueous ethanol or from 2N-HCl. *S*-(2,4-Dinitrophenyl)-L-cysteine, m.p. 145° (decomp.), was made by the method of Hansen, Kjaer & Schwimmer (1959).

S-Benzylmercapturic acid, m.p. 144°, and *S*-(*p*-nitrobenzyl)mercapturic acid, m.p. 132°, were made by acetylating the corresponding cysteine derivatives by the method of Zbarsky & Young (1943).

S-Benzylglutathione, m.p. 199° (decomp.), *S*-(*p*-chlorobenzyl)glutathione, m.p. 198° (decomp.), and *S*-(*p*-nitrobenzyl)glutathione, m.p. 202° (decomp.), were prepared, in poor yield, by using glutathione instead of cysteine in Theodoropoulos's (1959) method. Better yields were obtained by condensation in liquid ammonia.

Sodium (210 mg., 9 m-moles) was dissolved in liquid ammonia (50–100 ml.) in a Dewar flask, and glutathione (900 mg., 3 m-moles) was added. When this had dissolved, the chloro compound (3 m-moles), e.g. *p*-nitrobenzyl chloride, was added, and after 15 min. the solution was poured into a beaker and allowed to evaporate at room temperature. The residue was dissolved in 5 ml. of water, extracted with 25 ml. of ether and the glutathione conjugate precipitated from the aqueous solution by adjusting to pH 3 with 2N-HCl. The glutathione conjugates were crystallized from water or aq. 50% (v/v) ethanol.

S-(p-*Nitrobenzyl)glutathione* had m.p. 205° (decomp.), $[\alpha]_D^{25} -21 \pm 2°$ (*c* 1 in 0·1N-KOH), $\lambda_{max.}$ in water 280 mμ (ε9600) (Found: C, 45·8; H, 5·10; N, 12·6; S, 7·2. $C_{17}H_{22}N_4O_8S$ requires C, 46·1; H, 5·0; N, 12·7; S, 7·3%).

S-(*Naphth-1-ylmethyl)glutathione* had m.p. 199° (decomp.), $[\alpha]_D^{25} +14 \pm 2°$ (*c* 1 in N-HCl) (Found: C, 56·5; H, 5·7; N, 9·3; S, 7·4. $C_{21}H_{25}N_3O_6S$ requires C, 56·4; H, 5·6; N, 9·4; S, 7·2%).

S-*Phenethylglutathione* had m.p. 220° (decomp.), $[\alpha]_D^{25} -12 \pm 2°$ (*c* 1 in N-HCl) (Found: C, 52·9; H, 6·1; N, 9·6; S, 8·0. $C_{18}H_{25}N_3O_6S$ requires C, 52·6; H, 6·1; N, 10·2; S, 7·8%).

* Part 8: Gessner & Smith (1960).

S-(2-Chloro-4-nitrophenyl)glutathione formed needles from aqueous ethanol, m.p. 218° (decomp.), $[\alpha]_D^{22}+8\pm2°$ (*c* 1 in N-HCl) (Found: C, 41·1; H, 4·2; N, 12·0. Calc. for $C_{16}H_{19}ClN_4O_8S$: C, 41·5; H, 4·1; N, 12·1%).

Glutathione (3 g.), sodium (0·7 g.) and the disodium salt of phenoltetrabromophthaleindisulphonate were allowed to react in 300 ml. of liquid ammonia as described above. After 6 hr. the ammonia was allowed to evaporate and the residue dissolved in 50 ml. of water. The solution was adjusted to pH 3 with acetic acid and 500 ml. of ethanol was added. The precipitate was purified by dissolution in aqueous sodium acetate–acetic acid, pH 3, and precipitation with ethanol to give 9·4 g. of a white non-hygroscopic product. This was chromatographically identical with a sample of the 'BSP conjugate' from rat bile (Combes & Stakelum, 1961) provided by Dr W. H. H. Andrews and was homogeneous on paper chromatograms but was not obtained as crystals. The synthetic phenoltetrabromophthaleindisulphonate conjugate appeared to be a *disodium salt*, $[\alpha]_D^{25}+13\pm2°$ (*c* 0·5 in N-HCl), $\lambda_{max.}$ in 0·1N-NaOH 228, 280, 360 and 578 mμ (ϵ 28500, 12000, 7400 and 57500 respectively) (Found: C, 31·4; H, 3·7; N, 3·5; Na, 4·1; S, 8·2; loss of wt. at 100°, 7·9. $C_{30}H_{24}Br_3N_3Na_2O_{16}S_3$, $5H_2O$ requires C, 31·2; H, 3·0; N, 3·7; Na, 4·0; S, 8·3; H_2O, 7·8%).

S-(2,4-Dinitrophenyl)glutathione, m.p. 191° (decomp.), was prepared according to the method of Saunders (1934) and had $[\alpha]_D^{22}+45\pm4°$ (*c* 1 in N-HCl), $\lambda_{max.}$ in water 336 mμ (ϵ 10500).

p-Nitrobenzyl chloride had m.p. 72°; other compounds were commercial samples which were redistilled before use.

Methods

Paper chromatography and paper ionophoresis. These were carried out as described by Smith (1958). Approximate R_F values to show the degree of separation of metabolites are shown in Table 1. In the identification of metabolites by electrophoresis, all the reference compounds were run simultaneously and Table 2 shows the separations observed at the pH values stated. No precise significance is attached to the absolute directions of migration since no allowance was made for electro-osmotic effects. Migrations are therefore quoted relative to that of *p*-nitrobenzoic acid.

The detection of compounds on paper was carried out as follows:

(*a*) All nitro compounds quenched the fluorescence of paper in light from a filtered low-pressure mercury arc (Hanovia Chromatolite).

(*b*) Aromatic aminobenzoyl compounds sprayed with 0·5% *p*-dimethylaminocinnamaldehyde in ethanolic 50% (v/v) acetic acid gave a carmine-red colour. Aminobenzyl compounds gave pink colours. Nitro compounds were then detected on the same papers by reduction with a spray containing 1% (w/v) $TiCl_3$ in aq. 1% (w/v) HCl. When *p*-dimethylaminobenzaldehyde was used in this reaction both aminobenzyl and aminobenzoyl compounds gave identical yellow colours.

(*c*) Thio ether derivatives sprayed with the iodoplatinate reagent of Toennies & Kolb (1951) gave pale-yellow spots on a pink ground.

(*d*) α-Amino acids sprayed with 1% (w/v) ninhydrin in *n*-butanol gave purple spots on heating to 100°.

Table 1. *R_F values of some metabolites of benzyl chloride and related compounds*

Whatman no. 1 or 4 paper was used (except for solvents K and L) and run until the solvent front had moved 12–15 in. The solvent systems used were: A, butan-1-ol–acetic acid–water (4:1:5, by vol.); B, propan-1-ol–aq. NH_3 (sp.gr. 0·88) (7:3, v/v); C, ethanol–water (4:1, v/v); D, ethyl methyl ketone–2N-acetic acid–water (200:1:100, by vol.); E, butan-1-ol–2N-NH_3–water (4:1:5, by vol.); F, butan-1-ol–ethanol–water (4:1:1, by vol.); G, propan-1-ol–2N-acetic acid–water (10:1:5, by vol.); H, ethanol–butan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (12:4:1:3, by vol); I, propan-1-ol–water (4:1, v/v); J, benzene–acetic acid–water (1:1:1, by vol.); K, 0·1N-NH_3 on diethylaminoethylcellulose paper; L, 0·1N-NH_3 on aminoethylcellulose paper.

Solvent system	R_F A	B	C	D	E	F	G	H	I	J	K	L
S-Benzylcysteine	0·6	0·7	0·6	0·2	—	—	—	—	—	—	—	—
S-Benzylmercapturic acid	0·8	0·8	0·8	0·8	—	—	—	—	—	—	—	—
S-Benzylglutathione	0·3	0·3	0·4	0·1	—	—	—	—	—	—	—	—
S-(*p*-Chlorobenzyl)cysteine	0·6	0·6	0·6	—	—	—	—	—	—	—	—	—
S-(*p*-Chlorobenzyl)glutathione	0·6	0·4	0·3	—	—	—	—	—	—	—	—	—
S-(2,4-Dinitrophenyl)cysteine	0·9	0·6	0·5	—	—	—	0·9	—	—	—	—	—
S-(2,4-Dinitrophenyl)glutathione	0·5	0·2	0·2	—	—	—	0·7	—	—	—	—	—
S-(2-Chloro-4-nitrophenyl)glutathione	0·5	—	0·1	—	—	—	—	—	—	—	—	—
p-Nitrobenzyl chloride	1·0	0·9	0·8	1·0	—	1·0	0·9	—	—	—	0·7	0
S-(*p*-Nitrobenzyl)cysteine	0·7	0·6	0·6	0·4	0·5	0·6	0·8	0·7	0·5	0	0·5	0·4
S-(*p*-Nitrobenzyl)mercapturic acid	0·9	0·8	0·8	0·9	0·6	0·7	0·9	0·9	0·7	0·3	0·6	0·4
S-(*p*-Nitrobenzyl)glutathione	0·5	0·3	0·3	0·1	—	—	0·7	0·3	0·3	0	0·4	0·2
p-Nitrobenzoic acid	0·9	0·8	0·8	0·9	0·6	0·7	0·9	0·9	0·8	0·8	0·3	0·3
p-Nitrohippuric acid	0·9	0·7	0·7	0·8	0·4	0·7	0·9	0·7	0·7	0·2	0·5	0·5
Glycine	—	0·2	0·5	—	—	—	—	—	—	—	—	—
Glutamic acid	—	0·1	0·4	—	—	—	—	—	—	—	—	—
Cysteine	—	0	0·5	—	—	—	—	—	—	—	—	—
Glutathione	—	0	0·4	—	—	—	—	—	—	—	—	—
Phenoltetrabromophthaleindisulphonate–glutathione conjugate	0·2	—	0·5	0	—	—	0·4	—	—	—	—	—
Phenoltetrabromophthaleindisulphonate	0·5	—	0·8	0·6	—	—	0·9	—	—	—	—	—

Table 2. *Ionophoresis of some nitro compounds*

Separations are given in cm., relative to *p*-nitrobenzoic acid, towards anode (+) or cathode (−). Ionophoresis was carried out on Whatman no. 1 paper in Britton and Robinson buffer (British Drug Houses Ltd.) at approx. 10 v/cm. for 1 hr.

	Separation (cm.)							
pH	1·7*	3·0	4·8	6·0	7·0	8·2	10·0	12·3†
p-Nitrobenzoic acid	0	0	0	0	0	0	0	0
p-Nitrohippuric acid	0	−0·1	−0·6	−0·4	−0·7	−1·2	−0·6	−1·0
S-(*p*-Nitrobenzyl)glutathione	−1·7	−0·7	−1·8	−2·2	−1·7	−2·3	−2·5	0
S-(*p*-Nitrobenzyl)cysteine	−2·2	−1·3	−4·1	−3·6	−1·2	−1·7	−3·1	−2·0
S-(*p*-Nitrobenzyl)mercapturic acid	0	+0·1	−0·9	−1·3	−1·2	−1·6	−2·4	−2·3

* 0·02 N-HCl. † 0·02 N-NaOH.

(*e*) Phenoltetrabromophthaleindisulphonate and its glutathione derivative gave intense purple colours when exposed to NH_3 vapour.

Determination of p-*nitrobenzyl chloride.* Gas–liquid chromatography was used on a 6 ft. 20% silicone oil-on-Celite column at 175°, with electron-capture detection. A linear calibration curve relating peak recorder height to μg. of *p*-nitrobenzyl chloride was prepared over a range 0·02–0·1 μg., and injection samples in the range 5–20 μl. were used.

Locusts were homogenized in a M.S.E. top-drive homogenizer with 10 ml. of benzene and, after centrifuging, 5–20 μl. samples of the benzene were used for analysis.

Determination of other nitro compounds. Titanous chloride [1 ml. of a 1·5% (w/v) solution in 1·5% (w/v) HCl] was added to 4 ml. of an aqueous solution of the nitro compound. After 10 min., 4 ml. of 0·5% *p*-dimethylaminobenzaldehyde in ethanolic 50% (v/v) acetic acid was added and the yellow colour measured after 5 min. at 450 mμ, by using a reagent blank, in the Unicam SP. 500 spectrophotometer. Linear calibration curves were prepared by this method for *p*-nitrobenzoic acid, *S*-(*p*-nitrobenzyl)cysteine and *S*-(*p*-nitrobenzyl)glutathione over the range 10–100 μg.

Zones on paper chromatograms containing nitro compounds were located by the quenching of fluorescence and were cut out. The paper was placed in 4 ml. of water and 1 ml. of $TiCl_3$ solution, and the nitro compounds were estimated as described above. Known amounts of the above three nitro compounds mixed with locust excreta were recovered within ±5 μg. when chromatographed in solvent systems A, B or C.

Determination of acid-soluble nitro compounds in homogenates. Individual locusts that had been dosed with 100 μg. of *p*-nitrobenzyl chloride were disintegrated, in a M.S.E. top-drive homogenizer, in a mixture of 20 ml. of 3% (w/v) trichloroacetic acid and 5 ml. of $CHCl_3$. After centrifuging, 1·5–2·0 ml. of the aqueous layer was shaken vigorously for 15 sec. with 1 ml. of 1% (w/w) zinc amalgam. The reduced solution (1 ml.) was mixed with an equal volume of 0·3% *p*-dimethylaminobenzaldehyde in acetic acid and the yellow colour measured at 450 mμ in the Unicam SP. 500 spectrophotometer. A calibration curve constructed from solutions of *S*-(*p*-nitrobenzyl)glutathione in 3% trichloroacetic acid was linear over the range 0–50 μg./ml. and recoveries of this compound from pure solutions or from chromatograms were within 5% over this range. Recoveries from locust homogenates depended on the volume of trichloroacetic acid used. Experimental results were therefore calculated from calibration curves prepared from known amounts of *S*-(*p*-nitrobenzyl)glutathione added to a homogenate of one locust in 20 ml. of 3% trichloroacetic acid. Recoveries of *S*-(*p*-nitrobenzyl)glutathione calculated from this calibration curve were within ±5% over the range 0–20 μg./ml.

Colorimetric determination of S-*benzylcysteine.* *S*-Benzylcysteine was separated on Whatman no. 1 paper in solvent system A and the required zone cut out after comparison with a reference spot. *S*-Benzylcysteine was estimated on the paper by the ninhydrin method of Fowden (1951). Linear calibration curves were obtained for this compound and for *S*-(*p*-nitrobenzyl)cysteine, and recoveries of either compound added to locust excreta were within 5 μg. over the range 20–100 μg.

Measurement of radioactivity. Radioactive chromatograms were counted under a thin-end-window counter tube covered with a masking slot 1 cm. wide. The strips were moved by hand in 1 cm. steps and the results plotted as histograms. Solutions containing radioactive compounds were assayed as described by Gessner & Smith (1960).

RESULTS

Metabolism of benzyl chloride. Benzyl chloride (100 μg. in 10 μl. of acetone) was injected into hoppers which were then kept in separate beakers for 24 hr. The excreta were moistened with a drop of water and the paste was chromatographed in solvent system A. No mercapturic acid could be detected by colour reaction but weak spots corresponding to *S*-benzylcysteine were present.

Quantitative determinations of the excreted *S*-benzylcysteine, by the ninhydrin method, are summarized in Table 3.

Detection of other possible metabolites was complicated by the large amounts of amino acids present in normal locust excreta, and a search for other cysteine-containing metabolites was made in locusts dosed with DL-[^{35}S]cystine.

Hoppers were injected with 10 μl. of aqueous sodium [^{35}S]cystinate (containing 0·5 mg.; 50 μc). Benzyl chloride was then injected as described above, and the excreta were collected and chromatographed as described above but with the addition of 100 μg. each of *S*-benzylcysteine,

S-benzylglutathione and *S*-benzylmercapturic acid on each 2 cm. chromatographic strip.

Chromatograms were run in solvent systems A, B, C and D, and the strips scanned for radioactivity and also sprayed with the iodoplatinate reagent to reveal the positions of the added reference compounds. In each solvent system used, radioactive peaks corresponding to *S*-benzylcysteine were present.

Adult locusts were similarly treated with the same amount of [^{35}S]cystine and benzyl chloride, as described above, and in other experiments the benzyl chloride was given 3 hr. before the cystine, and 3, 24 and 48 hr. after dosing with cystine, and chromatographed as described above in solvent system A. Labelled metabolites of benzyl chloride were detected in the excreta collected 24 hr. after dosage, but only minor amounts were detectable in the next 24 hr. period. Assessments of the relative amounts of *S*-benzylcysteine, *S*-benzylmercapturic acid and *S*-benzylglutathione present in the excreta were made by measurement of the areas of the appropriate zones on the histograms and are summarized in Table 4.

In most experiments, only the zones corresponding to *S*-benzylcysteine were clearly recognizable over the background radioactivity, but zones corresponding to *S*-benzylglutathione, *S*-benzylmercapturic acid and *S*-benzylcysteine were eluted with water and portions rechromatographed with added non-labelled reference compounds on 2 cm. strips of Whatman no. 4 paper. Coincidence between radioactive peaks and zones detected with the iodoplatinate reagent was then obtained in solvents A, B, C and D for *S*-benzylcysteine (peak 250 counts/min. above background) and *S*-benzylglutathione (peaks 100 counts/min. above background), but the radioactivity in zones containing non-radioactive *S*-benzylmercapturic acid was not significant. In similar chromatograms prepared from the mercapturic acid fraction of Expt. 4 (Table 4), small radioactive peaks (approx. 50 counts/min. above background) coinciding with *S*-benzylmercapturic acid were found in the four solvent systems used.

Detection of metabolites of p*-nitrobenzyl chloride.* (*a*) In excreta of insects: locusts were each dosed with 100 μg. of *p*-nitrobenzyl chloride in 10 μl. of acetone, and excreta collected for 24 hr. The excreta were chromatographed in solvent systems A, B, C and D (Table 1), and in each solvent colour reactions revealed spots corresponding in R_F to the reference compounds *p*-nitrobenzoic acid, *S*-(*p*-nitrobenzyl)cysteine and *S*-(*p*-nitrobenzyl)glutathione. In other experiments where excreta or the contents of locust hind-gut were examined, only *p*-nitrobenzoic acid and *S*-(*p*-nitrobenzyl)cysteine could be detected.

Quantitative measurements of the three metabolites, after separation in solvent A, by the two colorimetric methods used are quoted in Table 5.

(*b*) In locust bodies: a total of 2 mg. of *p*-nitrobenzyl chloride was given to 20 locusts and after 5 hr. they were homogenized in an Ato-Mix blender

Table 3. *Estimations of* S*-benzylcysteine in locust excreta*

Results are given as means with the ranges in parentheses, with the numbers of experiments given as superscript.

Compound dosed or incubated	Percentage of administered compound recovered as *S*-benzylcysteine: After metabolism by locust in 24 hr.	After incubation with voided excreta for 24 hr.
Benzyl chloride (1 mg.)	24 (11–42)^{20}	—
S-Benzylglutathione (1 mg.)	12 (1–21)^{14}	20 (10–21)^{3}
S-Benzylcysteine (1 mg.)	12 (1–28)^{22}	13 (9–18)^{3}

Table 4. *Excretion of* ^{35}S*-labelled metabolites from locusts given* [^{35}S]*cystine and benzyl chloride*

The areas under chromatographic plots are given as arbitrary units.

Expt. no.	Delay between dosing with cystine and injection of benzyl chloride (hr.)	Areas under chromatographic plot corresponding to: *S*-Benzylcysteine	*S*-Benzylglutathione	*S*-Benzylmercapturic acid
1	−3	37	5	2
2	0	70	5	2
3	3	25	5	3
4	24	32	10	2
5	48	3	8	2

Table 5. *Metabolism of some nitro compounds by locusts*

Results are given as means with the ranges in parentheses, with the numbers of experiments given as superscript.

Compounds excreted	Percentage of dose in 24 hr. excreta after dosing with: *p*-Nitrobenzyl chloride (100 μg.)	*S*-(*p*-Nitrobenzyl)-glutathione (300 μg.)	*S*-(*p*-Nitrobenzyl)-cysteine (500 μg.)
S-(*p*-Nitrobenzyl)glutathione	7 (3–15)[13]	19 (6–29)[9]	—
S-(*p*-Nitrobenzyl)cysteine	17 (11–21)[13]	33 (5–55)[9]	34 (20–42)[8]
	22* (9–49)[5]	—	—
p-Nitrobenzoic acid	12 (0–26)[13]	—	6 (3–9)[8]
Unidentified nitro compounds	—	—	25† (10–42)[8]

* Estimated by Fowden's (1951) ninhydrin method. † Expressed as apparent *S*-(*p*-nitrobenzyl)cysteine.

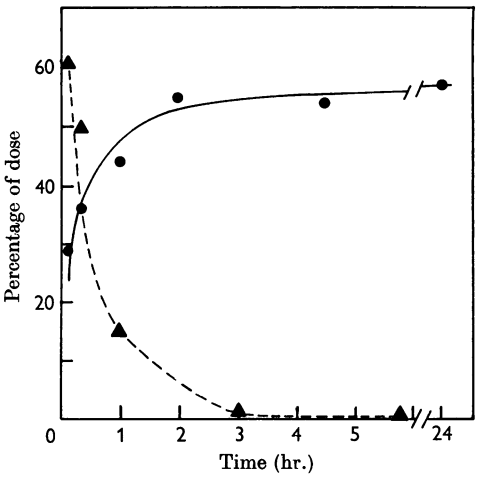


Fig. 1. Metabolism of *p*-nitrobenzyl chloride in locusts. The Figure shows: ▲, percentage of dose unchanged; ●, *S*-(*p*-nitrobenzyl)glutathione formed. Locusts were dosed with 100 μg. of *p*-nitrobenzyl chloride and pairs were analysed at intervals by the methods given in the text.

with 50 ml. of ethanol. The brei was made alkaline with 50 ml. of N-ammonia and, after centrifuging, the supernatant was concentrated *in vacuo* and portions were chromatographed as described above. The same metabolites were present as in excreta, but, in this and other similar experiments where bodies were extracted, the major metabolite was always *S*-(*p*-nitrobenzyl)glutathione.

Quantitative determinations of the acid-soluble nitro compounds present in locusts and residual *p*-nitrobenzyl chloride are shown in Fig. 1.

In other experiments, the trichloroacetic acid solution in the usual estimation procedure was replaced by water or by N-hydrochloric acid and the solution examined by paper chromatography. In extracts made 2 hr. after dosing, only *S*-(*p*-nitrobenzyl)glutathione was detected on paper chromatograms. No *p*-nitrobenzylated protein could be seen.

(*c*) In tissue preparations: fifth-instar locust 'fillets' were incubated in air with saline containing 1 % (w/v) of albumin and 40 mg. of *p*-nitrobenzyl chloride. After 2 hr. the liquid was filtered, boiled and centrifuged. The supernatant was evaporated to small volume *in vacuo* and chromatographed on a 30 cm. wide sheet of Whatman no. 1 paper in solvent system A. The major product was chromatographically identical with *S*-(*p*-nitrobenzyl)-glutathione, and after elution with water this band was found by the colorimetric procedure to contain the equivalent of 1·5 mg. of this metabolite. Small amounts of material corresponding in R_F and colour reactions to *S*-(*p*-nitrobenzyl)cysteine and *p*-nitrobenzoic acid were also present.

In similar experiments in which fat body, gut or malpighian tubes were incubated in saline, gut and malpighian tubes formed both the cysteine and glutathione metabolites but the fat-body preparations yielded predominantly *S*-(*p*-nitrobenzyl)-glutathione.

Further characterization of the metabolites. Extracts from locusts bodies [see (*b*) above] or saline from 'fillet' experiments [see (*c*) above] were separated on large-scale paper chromatograms in solvent system A, and the zones corresponding to *S*-(*p*-nitrobenzyl)glutathione, *S*-(*p*-nitrobenzyl)cysteine and *p*-nitrobenzoic acid were eluted with 0·1 N-ammonia and rechromatographed in solvent system B for further purification. Eluates of the second chromatograms were used for characterization of the metabolites.

S-(*p*-Nitrobenzyl)cysteine and *S*-(*p*-nitrobenzyl)-glutathione present in these eluates were identical in chromatographic behaviour with synthetic reference compounds in each of the 12 solvent systems of Table 1 and on ionophoresis at each of the pH values of Table 2.

The *p*-nitrobenzoic acid in the eluates was also identical with reference material in all the solvent and buffer systems. Though *S*-(*p*-nitrobenzyl)-mercapturic acid would have been associated with *p*-nitrobenzoic acid in the initial chromatography in solvents A and B no evidence of its presence in

Table 6. *Paper-chromatographic detection of glutathione derivatives and cysteine derivatives of some organic halogen compounds in locusts and tissue preparations*

The organic halogen compounds were either given as dose of 200 μg./locust or were present at concentration of 5 mM in tissue preparations. +, Derivative detected; −, derivative not detected.

	24 hr. excreta from intact locusts		Locust 'fillets'		Homogenized fat body and 5 mM-glutathione	
	Glutathione conjugate	Cysteine conjugate	Glutathione conjugate	Cysteine conjugate	Glutathione conjugate	Cysteine conjugate
p-Chlorobenzyl chloride	.	.	+	+	+	−
p-Nitrobenzyl bromide	.	.	+	+	+	−
1-Chloro-2,4-dinitrobenzene	+	+	+	+	+	−
1-Fluoro-2,4-dinitrobenzene	+	+	+	+	+	−
1,2-Dichloro-4-nitrobenzene	+	−	+	−	+	−
Phenoltetrabromophthaleindisulphonate	−	−	+	−	+	−

these extracts was obtained from the subsequent ionophoresis or chromatography.

A sample of the eluted *S*-(*p*-nitrobenzyl)glutathione, containing no other amino acids, was heated with 0·5 ml. of 6N-hydrochloric acid for 4 hr. at 100°. The volatile acid was removed by evaporation with water *in vacuo* and the residue examined chromatographically in solvent systems A, B and C together with reference samples of amino acids quoted in Table 1. Intense spots corresponding to glycine, glutamic acid and *S*-(*p*-nitrobenzyl)-cysteine were found on the chromatograms together with traces of other material giving a ninhydrin reaction.

A portion of the hydrolysate was treated with sodium hydrogen carbonate and 1-fluoro-2,4-dinitrobenzene (Sanger, 1945), and the DNP derivatives were chromatographed on Whatman no. 4 paper in the phosphate buffer system (Block & Weiss, 1956). Yellow spots were present at the same R_F values as those of the DNP derivatives prepared from the reference amino acids glycine (0·32), glutamic acid (0·57) and *S*-(*p*-nitrobenzyl)-cysteine (0·14).

Metabolism of some other organic halogen compounds. Acetone solutions (200 μg./10 μl.) of possible precursors of glutathione metabolites were injected into locusts and the excreta examined on paper chromatograms with the appropriate reference compounds. Metabolites were detected by the iodoplatinate reaction and fluorescence quenching.

The substrates were also incubated with four fifth-instar locust 'fillets' and the medium was worked-up and examined chromatographically as described above. The results are summarized in Table 6. Substrates (5 mM) were also incubated for 1 hr. in fat-body homogenates at pH 6 fortified with 5 mM-glutathione (see Cohen, Smith & Turbert, 1964).

Metabolism of the glutathione conjugates. *S*-(*p*-Nitrobenzyl)glutathione and *S*-benzylglutathione were injected into hoppers and the excreta examined next day by qualitative and quantitative paper chromatography. Considerable conversion into the corresponding cysteine derivatives was observed (Tables 3 and 5). Excreta were collected on subsequent days but no further significant excretion of metabolites was found.

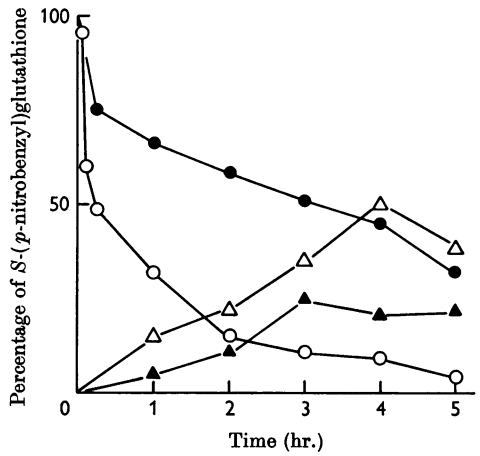


Fig. 2. Incubation of *S*-(*p*-nitrobenzyl)glutathione with homogenates of locust tissues. The conditions are given in the text. The Figure shows: percentage of initial *S*-(*p*-nitrobenzyl)glutathione present unchanged in gut homogenate (●) and in malpighian-tube homogenate (○); and *S*-(*p*-nitrobenzyl)cysteine formed in gut homogenate (▲) and in malpighian-tube homogenate (△).

This hydrolysis occurred readily in homogenates of gut and malpighian tubes (Fig. 2) but was slow in fat-body preparations, and was not detected in haemolymph or crop fluid. Enzyme solutions were prepared by homogenizing malpighian tubes from ten locusts and mid- and hind-guts from four locusts in 2·5 ml. of phosphate–citrate buffer (McIlvaine, 1921) at pH 6. *S*-(*p*-Nitrobenzyl)-glutathione was added to give a 5 mM solution and

the mixture was incubated at 37° in air. Samples (0·1 ml.) were withdrawn at intervals and chromatographed in solvent system B. The separated zones corresponding to *S*-(*p*-nitrobenzyl)glutathione were then estimated as described above. In three experiments similar to that of Fig. 2, 50 %, 60 % and 85 % of the glutathione was converted in 2 hr. by malpighian-tube homogenates. In equivalent experiments with gut dissected free from malpighian tubes, the corresponding values were 15 %, 40 % and 42 %.

The hydrolysis also occurred in excreta. Three freshly voided moist faecal pellets were ground with 1 mg. of *S*-(*p*-nitrobenzyl)glutathione and left in a stoppered tube for 24 hr. at room temperature. Chromatograms of the paste in solvent systems A, C and D showed the presence of *S*-(*p*-nitrobenzyl)-cysteine and some residual *S*-(*p*-nitrobenzyl)-glutathione. Similar experiments with *S*-benzylglutathione showed that this was also hydrolysed in voided excreta (Table 3).

Metabolism of the cysteine conjugates. *S*-(*p*-Nitrobenzyl)cysteine and *S*-benzylcysteine were also metabolized when given to locusts (Tables 3 and 5) or when incubated with freshly voided excreta. *S*-(*p*-Nitrobenzyl)cysteine administered to locusts gave, as well as some unchanged material, metabolites with R_F values 0·3 and 0·8 in solvent system C. Compounds with these R_F values were also obtained when *S*-(*p*-nitrobenzyl)cysteine was incubated with fresh moist locust excreta for 24 hr. No change was found when it was incubated for 2 hr. with crop fluid or homogenized gut, fat body, malpighian tubes or excreta.

The substance with R_F 0·8 in solvent C from excreta of locusts dosed with *S*-(*p*-nitrobenzyl)-cysteine was examined, after elution, by paper chromatography in solvent systems A, B, C and D, and ionophoresis at each pH value given in Table 2, and found to contain mainly *p*-nitrobenzoic acid together with smaller amounts of *S*-(*p*-nitrobenzyl)-mercapturic acid.

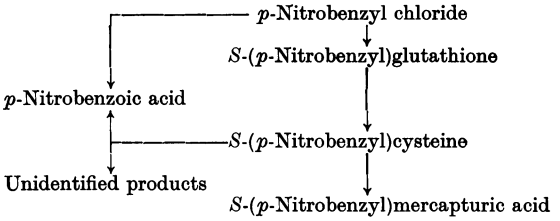
The material with R_F 0·3 in solvent C from excreta of locusts dosed with *S*-(*p*-nitrobenzyl)-cysteine was not identified, but on ionophoresis it separated into two components. One of these had ionophoretic properties identical with those of *S*-(*p*-nitrobenzyl)cysteine and the other behaved in ionophoretic experiments as an acid with pK_a 2–3.

DISCUSSION

In the initial stages of the present study attempts were made to label the sulphur-containing amino acids of locusts so that any metabolites containing cysteine might be easily detectable (see Knight & Young, 1958). The administration of $^{35}SO_4^{2-}$ ion, which is readily incorporated into sulphur-containing amino acids in cockroaches (Henry & Block, 1960), did not produce any significant labelling of the cysteine in locusts. When DL-[^{35}S]cystine was administered to locusts, the subsequent injection of benzyl chloride gave rise to much benzylcysteine in the excreta but little benzylglutathione could be found. These results could be interpreted in terms of a non-enzymic reaction between the free cysteine present in the blood or tissues with benzyl chloride at the neutral pH of the locust. The small yield of benzylglutathione could also have been derived non-enzymically from glutathione labelled with the [^{35}S]cystine administered since small amounts of free reduced glutathione are normally present in locust tissues (Hopkins & Morgan, 1945).

Experiments with *p*-nitrobenzyl chloride yielded more direct evidence that the glutathione conjugate was the primary metabolite that was subsequently broken down in the gut and excreta to a cysteine conjugate and other products. These reactions are summarized in Scheme 1, which also shows that *p*-nitrobenzoic acid is also present as a minor metabolite of *p*-nitrobenzyl chloride. The metabolism of *p*-nitrobenzyl chloride was rapid in *Schistocerca* (Fig. 1) and after 2 hr. almost no unchanged material was extractable with benzene. The only acid-soluble nitro compound detectable at this time was *S*-(*p*-nitrobenzyl)glutathione, and, though a reaction of *p*-nitrobenzyl chloride with tissue proteins is a possibility, no evidence of this was obtained. It is probable, therefore, that a rapid conjugation with glutathione is responsible for the detoxication of *p*-nitrobenzyl chloride. More than half of the dose was present as a glutathione conjugate 2 hr. after dosing and much of the remainder of the dose was converted into *p*-nitrobenzoic acid as in vertebrates (Bray, James & Thorpe, 1958). This was probably formed by hydrolysis and subsequent oxidation of the *p*-nitrobenzyl alcohol produced, but some may have also been a product of secondary reactions that could metabolize the glutathione conjugates or cysteine conjugates to *p*-nitrobenzoic acid.

Though the detoxication of the halogen compounds studied is fundamentally the same in locusts and vertebrates, secondary reactions in locusts complicate the situation. The initial glut-



Scheme 1

athione derivatives are excreted as mercapturic acids by vertebrates and the kidney does not, in most animals, allow the passage of unacetylated metabolites. In locusts, on the other hand, the mercapturic acids are insignificant metabolic products, and both glutathione derivatives and the cysteine derivatives formed by hydrolysis are excreted. Moreover, if elimination of the gut contents is delayed, as when the locust is not feeding freely, the action of gut contents on the cysteine conjugates may yield substantial quantities of further degradation products.

Since the initial step in the detoxication of reactive halogen compounds, e.g. benzyl chloride, involves a condensation with glutathione, it is possible that the effective toxicity of this type of compound may depend on the concentration of glutathione in the tissues. Resistance to arsenic in the cattle tick and some insects has been related to an increased content of glutathione in the resistant strains (Whitehead, 1961). It may be significant that the resistant ticks, having higher glutathione contents than normal, also showed an increased tolerance to reactive halogen compounds such as ω-chloroacetophenone.

SUMMARY

1. *S*-(*p*-Nitrobenzyl)glutathione, *S*-(naphth-1-ylmethyl)glutathione, *S*-(2-phenylethyl)glutathione and a glutathione conjugate of phenoltetrabromophthaleindisulphonate have been synthesized.

2. Locusts rapidly metabolized *p*-nitrobenzyl chloride to *S*-(*p*-nitrobenzyl)glutathione.

3. This glutathione conjugate was subsequently hydrolysed in locust gut, malpighian tubes and excreta to *S*-(*p*-nitrobenzyl)cysteine, which was in turn converted into unidentified products.

4. Glutathione conjugates and cysteine conjugates of phenoltetrabromophthaleindisulphonate, benzyl chloride, *p*-chlorobenzyl chloride, *p*-nitrobenzyl bromide, 1-chloro-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrobenzene and 3,4-dichloro-1-nitrobenzene have been identified chromatographically in locusts dosed with these compounds.

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