The Conjugation of Phenols with Phosphate in Grass Grubs and Flies

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1. Houseflies, blowflies and New Zealand grass grubs were dosed with 1-naphthol, 2-naphthol or *p*-nitrophenol. 2. The corresponding monoaryl phosphates were identified in extracts of insects or excreta along with the β -glucosides and ethereal sulphates of the phenols. 3. No diaryl phosphates or glucosiduronates were detected but an unidentified metabolite of [^{14}C]1-naphthol was present in extracts of flies dosed with [^{14}C]1-naphthol.

The recognition of degradation products of a pesticide in insects is valuable in assessing the importance of metabolic breakdown in its toxicology and, where phenols are possible metabolites, phenolic conjugates are to be expected. In common with most animals, insects detoxicate phenols by formation of sulphate conjugates and most species are also capable of synthesizing phenolic β -glucosides (Smith, 1964). It has been suggested that some insects can also use glucuronic acid in the sugar conjugation (Terriere, Boose & Roubal, 1961; Inagami, 1955; Hassan, Zayed & Abdel-Hamid, 1965; Zayed, Hassan & Hussein, 1965), and Smith & Turbert (1964) detected an acidic conjugate in the excreta of flies dosed with 1-naphthol that had properties similar to 1-naphthyl dihydrogen phosphate. This latter type of phenolic conjugate had not previously been observed as a detoxication product in insects and the occurrence of monoaryl phosphates is not common in biochemistry.

We have now examined the behaviour of a number of phenols in flies and in the New Zealand grass grub (*Costelytra zealandica*), and it is shown below that substantial amounts of monoaryl phosphates are present in the bodies or excreta of all these insects after dosing with phenols.

MATERIALS AND METHODS

Reference compounds. (*p*-Nitrophenyl glucosid)uronic acid was prepared by catalytic oxidation of 1-*p*-nitrophenyl β -D-glucoside by the method of Marsh & Levvy (1958). After the calculated amount of alkali had been taken up and the solution had been acidified the product remained soluble but was isolated as its sparingly soluble *p*-toluidine salt. The *p*-toluidine salt of (*p*-nitrophenyl β -D-glucosid)uronic acid crystallized from water in plates, m.p. 176°, [α]_D²⁵ -77° (c 1 in 0.1 N-HCl) (Found C, 54.2; H, 5.6; N, 7.1; C₁₉H₂₂N₂O₉ requires C, 54.0; H, 5.3; N, 6.6%). (1-Naphthyl glucosid)uronic acid, (2-naphthyl glucosid)uronic acid and

their *p*-toluidine salts were prepared biosynthetically by the procedure of Berenbom & Young (1951) or by catalytic oxidation of the β -glucosides as above. Sodium (1-naphthyl glucosid)uronate, prepared by neutralization of the free acid, crystallized from water as colourless needles (Found: Na, 6.1; H₂O, 9.8; C₁₆H₁₅NaO₇·2H₂O requires Na, 6.1; H₂O, 9.5%). The *p*-toluidine salt of 1-naphthyl sulphate was prepared as described by Whitehead, Morrison & Young (1952) (Found: S, 9.9; Calc. for C₁₇H₁₇NO₄S: S, 9.7%). When an aqueous solution of 5-aminoacridine hydrochloride was added to a dilute aqueous solution of the toluidine salt, a crystalline precipitate of the very sparingly soluble 5-aminoacridine salt of 1-naphthyl hydrogen sulphate formed (Found: S, 7.8; C₂₃H₁₈N₂O₄S requires S, 7.7%). Other phenolic conjugates with sulphuric acid or glucose were prepared as described previously (Smith & Turbert, 1964; Smith, 1953).

Di-1-naphthyl hydrogen phosphate, m.p. 137°, was prepared by the method of Friedman & Seligman (1950). Di-*p*-nitrophenyl hydrogen phosphate and the sodium salts of the phosphoric acid monoesters with 1-naphthol, 2-naphthol and *p*-nitrophenol were commercial samples that were shown to be homogeneous by paper chromatography. The cyclohexylamine salt of 1-naphthyl dihydrogen phosphate, m.p. 193°, was sparingly soluble in water (Found: equiv. wt. 338; C₁₆H₂₂NO₄P requires equiv. wt. 323). The cyclohexylamine salt of di-1-naphthyl hydrogen phosphate, m.p. 216°, was also sparingly soluble in water (Found: equiv. wt. 445; C₂₆H₂₆NO₄P requires equiv. wt. 449).

[^{14}C]1-Naphthol (1mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Solutions were stored in the dark and the purity was checked by dilution analysis.

Insects and dosing. Houseflies (*Musca domestica*) were an insecticide-susceptible strain obtained from the Wallaceville Animal Research Station, New Zealand, and larvae were reared in the laboratory on an agar gel containing 100g. of yeast, 100g. of full-cream dried milk and 20g. of agar/l. Adults had access to sucrose and water and laid eggs on wet cottonwool rolls. Insecticide-susceptible blowflies (*Lucilia sericata*) were reared in the same way except that fresh liver was provided for egg laying. New Zealand grass grubs (*Costelytra zealandica*, weight about 100mg.) were collected

by the Department of Scientific and Industrial Research Entomology Division from an area never exposed to insecticides.

Phenols were administered to grass grubs by injection into the thorax and to flies by topical application as 5% (w/v) solutions in corn oil at a dosage of 0.5 μ g./mg. Insects were normally left for 2–8 hr. at room temperature before homogenization and extraction. Flies were dosed with non-radioactive compounds by providing adults with a 1% (w/v) suspension of the phenols in milk. Batches of 300–1000 flies were allowed to feed on this solution and sucrose crystals for a week or until all had died. [14 C]1-Naphthol was dissolved in aq. 50% (v/v) acetone (5mg./ml.) and applied topically to the thorax of flies or injected into grass grubs at a dosage of 0.25 μ g./mg.

Extraction of insects and excreta. In experiments with non-radioactive phenols 3–5 g. of insects were dosed and the bodies of the survivors ground in a mortar with ethanol (5ml./g. of insects) and rinsed into a Potter-Elvehjem homogenizer with sufficient water to give an aq. 80% (v/v) ethanol mixture. After homogenization the mixture was centrifuged to remove protein and debris, and the supernatant concentrated to small bulk *in vacuo* and extracted with ether. The aqueous layer was banded on to a large-scale chromatogram and separated in solvent A (see Table 1) on Whatman no. 1 paper. Bands of metabolites detected on these chromatograms were eluted in 0.1N-NH $_3$ and examined in other chromatographic and ionophoretic systems.

In fly experiments where excreta was examined, this was rubbed off the containers with aq. 80% (v/v) ethanol, centrifuged and the supernatant concentrated and examined as above.

Paper chromatography and ionophoresis. These were carried out as described by Smith (1958), and R_F values and relative migrations are quoted in Tables 1 and 2, to indicate

the degree of separation. A reversed-phase system of phenoxyethanol–phosphate buffer was also used to separate diaryl phosphates from the free naphthols. Whatman no. 4 paper was dipped in 10% (v/v) phenoxyethanol in ether and allowed to dry overnight. Compounds were applied to this paper and allowed to dry and the chromatogram was developed with a phosphate buffer, pH 5, prepared from 0.1M-H $_3$ PO $_4$ and 0.1M-Na $_2$ HPO $_4$. Mixed chromatograms and ionophoretic preparations of unknown and reference material were always used in identification experiments and in non-radioactive experiments compounds were normally run in all systems of Table 1 and at each pH of Table 2.

Detection of metabolites on paper. Phenols, glucosides, glucosiduronates or ethereal sulphates of 1-naphthol and *p*-nitrophenol were detected as described by Smith & Turbert (1964). 2-Naphthol conjugates were detected by their characteristic blue fluorescence under u.v. light (254 or 360 m μ max.) and could be distinguished from the light-blue fluorescence of free 2-naphthol.

Conjugates were also identified by spraying with appropriate enzyme solutions followed by identification of the phenols liberated.

Six enzyme preparations were used at different stages of this work. *Helix pomatia* gastric contents diluted 1:10 with water hydrolysed ethereal sulphates, glucosides, glucosiduronates and phosphates. A ‘mollusc glucuronidase’ (Sigma Chemical Co., St Louis, Mo., U.S.A.) (0.1% in water) behaved similarly, but a ‘bovine glucuronidase’ (Sigma Chemical Co.) of the same concentration hydrolysed only glucosiduronates and glucosides. A 5% extract of dried bovine ileum in M-Na $_2$ CO $_3$ hydrolysed only aryl phosphates; a purified alkaline phosphatase (Sigma Chemical Co.) (0.1% solution in M-Na $_2$ CO $_3$) hydrolysed only aryl phosphates; an aq. 1% (w/v) solution of emulsin (L. Light and Co. Ltd., Colnbrook, Bucks.) hydrolysed β -glucosides and had a feeble phosphatase activity.

Table 1. R_F values of some phenols and their conjugates

Solvents were run on Whatman no. 1 or no. 4 paper until the front had moved about 35 cm. Solvents were: A, butan-1-ol–acetic acid–water (4:1:5, by vol.); B, butan-1-ol–ethanol–acetic acid–water (10:4:1:5, by vol.); C, propan-1-ol–aq. NH $_3$ (sp.gr. 0.88) (7:3, v/v); D, butan-1-ol–ethanol–water (17:3:20, by vol.); E, butan-1-ol–pyridine–water (5:3:4, by vol.); F, pyridine–2-methylbutan-2-ol–water (7:7:6, by vol.); G, butan-1-ol–aq. NH $_3$ (sp.gr. 0.88)–water (4:1:5, by vol.).

Solvent ...	A	B	C	D	E	F	G
1-Naphthol	0.95	0.95	0.90	0.90	0.95	0.90	0.95
1-Naphthyl glucoside	0.85	0.80	0.80	0.85	0.75	0.85	0.80
(1-Naphthyl glucosid)uronic acid	0.75	0.75	0.60	0.55	0.65	0.45	0.25
1-Naphthyl hydrogen sulphate	0.65	0.75	0.80	0.75	0.75	0.75	0.70
1-Naphthyl dihydrogen phosphate	0.55	0.65	0.30	0.45	0.40	0.10	0.05
Di-1-naphthyl hydrogen phosphate	0.85	0.80	0.90	0.90	0.90	0.90	0.80
2-Naphthol	0.95	0.95	0.95	0.95	0.95	0.95	—
2-Naphthyl glucoside	0.80	0.80	0.85	0.85	0.90	0.80	—
(2-Naphthyl glucosid)uronic acid	0.75	0.75	0.60	0.35	0.65	0.50	—
2-Naphthyl hydrogen sulphate	0.60	0.80	0.90	0.25	0.75	0.95	—
2-Naphthyl dihydrogen phosphate	0.50	0.70	0.25	0.25	0.55	0.50	—
<i>p</i> -Nitrophenol	0.95	0.95	0.90	0.90	0.95	0.90	—
<i>p</i> -Nitrophenyl glucoside	0.65	0.75	0.80	0.55	0.85	0.75	—
(<i>p</i> -Nitrophenyl glucosid)uronic acid	0.65	0.70	0.55	0.30	0.65	0.50	—
<i>p</i> -Nitrophenyl hydrogen sulphate	0.55	0.65	0.85	0.60	0.70	0.70	—
<i>p</i> -Nitrophenyl dihydrogen phosphate	0.45	0.65	0.25	0.10	0.35	0.20	—
Di- <i>p</i> -nitrophenyl hydrogen phosphate	0.85	0.85	0.90	0.90	0.95	0.70	—

Table 2. *Ionophoresis on paper of some phenols and their conjugates*

Apparent migrations to anode (+) or to cathode (−) were measured in cm. relative to the glucoside of each phenol. Ionophoresis was in 0.1 M-citrate buffer, pH 3, or in 0.02 N-HCl or 0.02 N-NaOH at 10 v/cm. for 2 hr.

	pH	...	...	1.3	3.0	12.7
1-Naphthol				0	0	+3.5
1-Naphthyl glucoside				0	0	0
(1-Naphthyl glucosid)uronic acid				0	+1.0	+1.5
1-Naphthyl hydrogen sulphate				+7.5	+6.0	+4.5
1-Naphthyl dihydrogen phosphate				+5.5	+4.5	+5.5
Di-1-naphthyl hydrogen phosphate				+4.5	+3.5	+3.0
2-Naphthol				0	0	+2.5
2-Naphthyl glucoside				0	0	0
(2-Naphthyl glucosid)uronic acid				0	+2.5	+2.0
2-Naphthyl hydrogen sulphate				+6.5	+6.0	+4.5
2-Naphthyl dihydrogen phosphate				+4.5	+4.5	+5.5
<i>p</i> -Nitrophenol				0	+0.5	+4.5
<i>p</i> -Nitrophenyl glucoside				0	0	0
(<i>p</i> -Nitrophenyl glucosid)uronic acid				0	+2.5	+3.0
<i>p</i> -Nitrophenyl hydrogen sulphate				+8.5	+6.0	+5.5
<i>p</i> -Nitrophenyl hydrogen sulphate				+8.5	+6.0	+5.5
<i>p</i> -Nitrophenyl dihydrogen phosphate				+6.5	+5.0	+7.0
Di- <i>p</i> -nitrophenyl hydrogen phosphate				+5.0	+4.0	+4.0

p-Nitrophenyl phosphate gave a blue colour with the Hanes & Isherwood (1949) phosphate reagent but this was not effective on the other aryl phosphates studied.

Measurement of radioactivity. Solid samples in dilution analyses were counted in aluminium planchets with a thin end-window counter tube as described by Gessner & Smith (1960a) and radioautography was carried out as described by Gessner & Smith (1960b).

Paper chromatograms on 2 cm. strips of Whatman no. 4 paper were cut into 1 cm. sections and counted in the vials of a Packard 4312 scintillation spectrometer. A mixture containing 5 g. of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene was used as scintillator and 5 ml. was used in each vial. Control chromatograms prepared from undosed insects with the addition of 50 µg. of each of the 1-naphthol reference conjugates were also counted to confirm the absence of phosphorescent material. Quenching by other material on the chromatograms was shown to be absent by comparing the count rate of 0.001 µg of sodium [¹⁴C]benzoate on fresh paper with the same quantity on sections of chromatograms from control groups of insects.

Counting efficiency of sodium [¹⁴C]benzoate on Whatman no. 1 or no. 4 paper was 52% at the discriminator settings used (50–500 and 5% gain) and this value was used to calculate the recovery of 1-naphthol metabolites. Since 1-naphthol was slowly eluted from chromatograms by the scintillation mixture, samples of homogenate equivalent to one insect containing added 1-naphthol (1 g.) were extracted with benzene before paper chromatography and a portion of the extracted naphthol was counted in solution at 78% efficiency.

RESULTS

Metabolism of non-radioactive phenols

1-Naphthol. Insects were dosed as described above and extracts prepared from bodies or excreta.

Paper chromatography in solvent *A* showed the presence of three fluorescent zones of *R_F* 0.5, 0.7 and 0.8 in addition to the lighter fluorescence of 1-naphthol at the front. Large-scale paper chromatograms of extracts from 50–100 grass grubs, 100–300 blowflies or 300–1000 houseflies were cut into sections of *R_F* 0.4–0.6 (I), 0.6–0.75 (II) and 0.75–1.0 (III), and eluted with 0.1 N-ammonia. These eluates were rechromatographed with solvents *A–G* (see Table 1) and separated ionophoretically (at the pH values shown in Table 2) together with reference conjugates and mixtures of reference conjugates with the eluates.

Eluate I from each insect species used contained material identical in chromatographic and ionophoretic properties with 1-naphthyl dihydrogen phosphate. This material was hydrolysed by phosphatases (see above) to liberate a product having the colour reactions and fluorescence properties of 1-naphthol. This eluate also gave the reaction for inorganic phosphate when boiled with ammonium molybdate and nitric acid. Prolonged boiling with 2 N-hydrochloric acid liberated 1-naphthol, which was identified on paper chromatograms. Samples of this eluate from experiments with 500 houseflies or 60 grass grubs were rechromatographed in solvent *G* and the fluorescent zone was eluted in 0.1 N-sodium hydroxide. This eluate had a u.v. spectrum with $\lambda_{\max}$ 284 mµ similar to that of 1-naphthyl dihydrogen phosphate.

In most experiments this metabolite was a major product of 1-naphthol metabolism in the three species studied, and was the only product found as a trace metabolite in blowfly maggots that had been kept for 4 hr. in water saturated with 1-naphthol.

Eluate II from each insect species was also examined with the colour reactions described above and in each of the systems of Tables 1 and 2 behaved identically with 1-naphthyl hydrogen sulphate.

Eluate III was similarly examined and, in addition to 1-naphthol, contained a substance that behaved identically with 1-naphthyl glucoside. No spots corresponding to (1-naphthyl glucosid)uronic acid were found in eluate II or III.

The absence of di-1-naphthyl hydrogen phosphate from eluate III was demonstrated by chromatography in the reversed-phase system with phenoxy-ethanol-phosphate buffer, pH 5. In this system di-1-naphthyl hydrogen phosphate had a low R_F value (0.1) and 1-naphthol did not move. Other conjugates had high R_F values and by allowing buffer to drip from the bottom of the paper any di-1-naphthyl hydrogen phosphate was obtained in a zone clearly separated from other metabolites. No di-1-naphthyl hydrogen phosphate was found in any eluate, though added reference material could be readily recovered after addition to faeces or to homogenates.

2-Naphthol. Grass grubs, blowflies and houseflies were dosed with 2-naphthol as described above and extracts of bodies or excreta prepared and separated on paper chromatograms in solvent A. Three zones were located by fluorescence and eluates of the initial chromatograms made at R_F 0.45–0.55 (IV), 0.55–0.75 (V) and 0.75–1.0 (VI).

Eluate IV from each species contained material that was identified as 2-naphthyl dihydrogen phosphate by chromatography and ionophoresis, as above, by the ammonium molybdate reaction, by its response to the enzymes used and by hydrolysis to 2-naphthol enzymically and by prolonged boiling with hydrochloric acid. An eluate from an experiment with 60 grass grubs was purified by rechromatographing in solvent C and its u.v. spectrum in 0.1N-sodium hydroxide examined. This was similar between 260 and 300 $m\mu$ to that of an authentic sample of disodium 2-naphthyl phosphate and had λ_{max} 265, 274 and 283 $m\mu$.

Other phenols. Extracts from grass grubs, houseflies and blowflies dosed with *p*-nitrophenol contained material that when chromatographed in solvent A gave zones with R_F 0.5, 0.6 and 0.7, which were identified by chromatography and ionophoresis as described above as *p*-nitrophenyl dihydrogen phosphate, *p*-nitrophenyl hydrogen sulphate and *p*-nitrophenyl glucoside. Eluates of the region R_F 0.7–1.0 were examined for di-*p*-nitrophenyl hydrogen phosphate with the reversed-phase system, but none was found in any of the insect species used.

Extracts from both fly species, however, contained a fourth product with R_F 0.4 in solvent A that was not identified. This gave the colour

reactions of an aromatic nitro compound, but no nitrophenol could be detected after treatment with alkaline phosphatase or β -glucosidase. This metabolite was not present in grass-grub extracts.

Flies and grass grubs were also dosed with phenolphthalein, *m*-aminophenol, 8-quinolinol and 4-methylumbelliferone, and extracts were found to contain products that liberated these phenols again when treated with the purified alkaline phosphatase. These metabolites were not investigated further.

Metabolism of [^{14}C]1-naphthol

Dilution analyses. Ten grass grubs that had each been injected with 20 μg . (0.5 μc) of [^{14}C]1-naphthol were homogenized in ice-water after 8 hr. and the mixture was diluted to 10 ml. with ice-water. Portions of the suspension (1 ml.) were diluted with 10 ml. of acetone and refluxed for 4 hr. with the addition of 300 mg. of a non-radioactive diluent compound. 1-Naphthol, potassium 1-naphthyl sulphate, 1-naphthyl glucoside, (1-naphthyl glucosid)uronic acid, 1-naphthyl dihydrogen phosphate and di-1-naphthyl hydrogen phosphate were used as diluents and refluxing for 4 hr. was necessary to remove radioactivity from the insoluble debris.

The extracts were centrifuged to remove debris and concentrated to small bulk in a rotary evaporator. In analyses for 1-naphthol the residue was extracted into ether, which was evaporated to dryness and the residual naphthol, m.p. 97°, crystallized from *n*-heptane to constant specific activity. The evaporated residues from the other dilutions were transferred to 40 cm. sheets of Whatman no. 31 paper and the large-scale paper chromatograms run in solvent C, except for the recovery of 1-naphthyl glucoside, which was chromatographed in solvent D. The zones of diluent on each paper were located by observation under u.v. light and chromatographically eluted from the paper with 25 ml. of 0.1N-ammonia.

These eluates were concentrated in a rotary evaporator and the residue was crystallized to constant specific activity either directly or after conversion into a suitable derivative. After crystallization to constant specific activity, the products were converted into second derivatives that were shown to have equivalent specific activity.

1-Naphthyl hydrogen sulphate was crystallized first as its *p*-toluidine salt and then converted into its 5-aminoacridine salt.

1-Naphthyl glucoside, m.p. 170°, was crystallized from benzene-ethanol and then converted into its tetra-acetate, m.p. 173°.

1-Naphthyl dihydrogen phosphate was crystallized from water as its cyclohexylamine salt, m.p. 193°, and then converted into the free acid, m.p. 155°, which was crystallized from water.

Di-1-naphthyl hydrogen phosphate was crystallized from ethanol as its cyclohexylamine salt, m.p. 216°, but the equivalent specific activity decreased on conversion into the free acid, m.p. 135°, and when 10mg. of this was chromatographed on an 8cm.-wide strip of Whatman no. 4 paper in the upper layer of butan-2-one-water (4:1, v/v) the zone containing residual radioactivity separated from the di-1-naphthyl hydrogen phosphate. Assay of the activity on the paper showed only a background level of counts in the di-1-naphthyl hydrogen phosphate zone and it was calculated that the amount of di-1-naphthyl hydrogen phosphate produced by the grass grub did not exceed 0.1% of the dose of 1-naphthol.

(1-Naphthyl glucosid)uronate was first crystallized from aqueous ethanol as its sodium salt and also decreased in specific activity on conversion into the free acid. When 10mg. of this was chromatographed on 8cm.-wide strips of Whatman no. 4 paper in the upper layer of solvent system *D*, *E* or *F*, or separated ionophoretically at pH 4, the residual radioactivity was separated from the zones containing glucuronide and it was calculated that the amount of this metabolite present could not exceed 0.01% of the dose.

Portions of acetone-water extracts of grass grubs prepared as above after dosing with [^{14}C]1-naphthol were chromatographed in solvents *A*, *C* and *D* with the addition of 100 μg . of each of the reference conjugate and radioautographs were prepared. 1-Naphthol and its conjugates with sulphate,

glucose and phosphoric acid were detected by coincidence of fluorescent zones and radioautograph spots. No glucuronide conjugate or di-1-naphthyl hydrogen phosphate was detected. Results of two dilution analyses are quoted in Table 3.

Quantitative paper chromatography. Extracts were also made from groups of five grass grubs or flies after dosing with [^{14}C]1-naphthol, except that 5g. of additional non-radioactive 1-naphthol was added to the acetone extract before evaporation as described above. After evaporation to dryness *in vacuo* the 1-naphthol was extracted from the residue with 5 $\times$ 5ml. of benzene and the residue dissolved in water before being transferred quantitatively to 2cm.-wide strips of Whatman no. 4 paper. These were chromatographed in solvent *D* with 100 μg . of each reference conjugate and the papers cut into 1cm. sections and counted in the scintillation spectrometer. Histograms prepared from these scans of grass-grub extracts showed peak radioactivity corresponding to 1-naphthyl dihydrogen phosphate, 1-naphthyl glucoside and 1-naphthyl hydrogen sulphate together with a small amount of radioactivity near the origin (Fig. 1). Extracts made from insects that had been homogenized and extracted immediately after dosing contained only 1-naphthol.

Histograms of chromatograms of fly extracts (Fig. 1) showed an additional zone of radioactivity lying between the reference phosphate and glucosiduronate that was not present in experiments with grass grubs. This product (metabolite X;

Table 3. *Formation of metabolites of [^{14}C]1-naphthol by insects*

Groups of five insects were dosed at room temperature and later analysed chromatographically for metabolites as described in the text. Values for grass grubs show means and ranges of the numbers of experiments given in square brackets.

	Dose (μg .)	% of dose recovered				Unchanged
		As 1-naphthyl dihydrogen phosphate	As 1-naphthyl hydrogen sulphate	As 1-naphthyl glucoside	As metabolite X	
Houseflies (20mg.)	5					
2hr.		10.4, 4.5	1.0, 0.5	3.2, 5.0	5.3, 11.5	79, 68
4hr.		19.6, 6.7	1.7, 1.0	5.7, 5.5	4.7, 17.0	65, 62
6hr.		29.0, 7.2	1.8, 1.5	3.7, 6.2	4.4, 22.0	56, 65
Blowflies (50mg.)	10					
2hr.		4.3, 1.1	2.9, 0.6	4.5, 4.9	17.1, 4.0	63, 88
4hr.		4.0, 1.6	7.5, 0.8	9.5, 4.2	16.0, 7.5	50, 84
6hr.		6.0, 3.5	3.9, 1.6	6.5, 5.4	25.0, 13.0	46, 78
Grass grubs (100mg.)	20					
8hr.		8.0 (4.0-13.0) [9]	5.0 (3.0-8.0) [8]	3.5 (2.0-6.0) [8]	—	78 (72-83) [7]
8hr. (at 5°)		4.1, 4.9	2.5, 2.5	2.0, 2.0	—	87, 88
*8hr.		14.5, 7.5	6.0, 7.0	3.5, 5.0	—	78

* Results of dilution analysis.

Fig. 1. Chromatography of $[^{14}\text{C}]1$ -naphthol conjugates from a housefly (a) and grass grub (b). Insects were dosed (a) with $0.1\text{ }\mu\text{C}$ ($5\text{ }\mu\text{g.}$) and (b) with $0.005\text{ }\mu\text{C}$ ($20\text{ }\mu\text{g.}$) of $[^{14}\text{C}]1$ -naphthol and extracted and chromatographed in solvent *D* (see Table 1); 1 cm. strips of the chromatogram were counted in a scintillation spectrometer. Bars indicate the position of co-chromatographed non-radioactive conjugates of 1-naphthol with: 1, phosphate; 2, glucuronic acid; 3, sulphate; 4, glucose.

Table 3) also moved between the phosphate and glucosiduronate reference compounds in solvent *G* and was separated from the slower phosphate by running overnight in this solvent on Whatman no. 4 paper and allowing solvent to drip from the end of the paper. Quantitative measurements quoted in Table 3 for the phosphate and unknown metabolite were taken from chromatograms run in this way and other metabolites from those run in solvent *D*.

The unidentified metabolite often contained more radioactivity than the phosphate conjugate, but was not fluorescent and did not give 1-naphthol when treated with alkaline phosphatase or emulsin.

Zones of chromatograms of fly extracts in solvent *G* that contained added reference (1-naphthyl glucosid)uronate were eluted with 0.2N -ammonia and the eluate was further purified by ionophoresis in 0.05N -sodium hydroxide. Only background levels of radioactivity were associated with the fluorescent (1-naphthyl glucosid)uronate on the ionophoresis papers, and it was calculated that the amount of this metabolite formed by *Musca* or

Lucilia did not exceed 0.01% of the dose of $[^{14}\text{C}]1$ -naphthol.

DISCUSSION

These results show that phosphate conjugates may be found in insect extracts when phenols have been absorbed, although the importance of these compounds in the detoxication of the phenols may vary with insect species and mode of dosage. At the dosages used here in feeding experiments, aryl phosphates were minor constituents of fly excreta or extracts. No phosphate conjugation could be detected in locusts (Smith & Turbert, 1964), but houseflies dosed topically with sublethal amounts of 1-naphthol usually formed conjugates with phosphate more extensively than with either glucose or sulphate (Table 3). In previous studies of phenol conjugations in a variety of insects (Smith, 1955; Myers & Smith, 1954), either glucosides or sulphates, or both, were detected as conjugates and other metabolites were not significant. Phosphate conjugates have been found in some experiments with spiders and scorpions (Hitchcock, 1965), but when present were only found in trace amounts. Grass grubs, flies and the onychophoran *Peripatus* (Jordan, 1966) seem exceptional in that the phosphate conjugates are often the major phenolic conjugates present although the sulphate and glucoside conjugates of phenols are usually found as well.

No glucuronic acid conjugates were detected in the present study. This is in agreement with the work of Dutton & Ko (1964). This work, and now the detection of phosphate conjugates of phenols, raises the question whether previous reports of glucuronide conjugation in insects may have been based on the non-recognition of phosphates. The R_f values of phosphate conjugates, and of the unidentified metabolite of 1-naphthol in flies, in some solvents are similar to those of glucuronides. Some samples of 'glucuronidase', an enzyme that has been used to characterize glucosiduronates, often contain a variety of other hydrolases, including phosphatase.

Inagami's (1955) identification of a chromatographic spot as (3-hydroxykynurenine glucosid)uronate from silkworms appears to depend on its low R_f value, difficulty of hydrolysis and behaviour as an acid on electro-osmosis. These are the properties of the (3-hydroxykynurenine glucosid)uronate identified by Dalglish (1952) as a tryptophan metabolite in vertebrates, but it is likely that a phosphate conjugate could have very similar properties.

The nature of the metabolite of Diptera (dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) and its methyl ether in *Prodenia* larvae that Zayed *et al.* (1965) and Hassan *et al.* (1965) have

identified as a glucosiduronate is not clear. The product liberated by alkaline hydrolysis was extracted into ether and identified as glucuronic acid by paper chromatography with ammoniacal silver nitrate and methyl orange as detection reagents. Their ether extract contained material giving a Molisch reaction but the low solubility of glucuronic acid in ether makes it unlikely that they were dealing with this compound.

In studies of the metabolism of insecticides in insects the water-soluble metabolites are often less easily characterized than those extractable with organic solvents. It has usually been assumed that these are conjugates with either sulphuric acid or glucose, but it is now necessary to consider the possibility of phosphate formation if a phenol is an intermediate in the metabolic degradation of an insecticide. Unidentified polar metabolites have been reported as metabolites of the insecticide Sevin (1-naphthyl *N*-methylcarbamate) (Edelfrawi & Hoskins, 1961; Dorrough & Casida, 1964), and it is probable that in some insects these include 1-naphthyl phosphate.

The occurrence of aryl phosphates in Nature is not common. Tyrosine *O*-phosphate has been identified as a normal metabolite in the larvae of the fruitfly *Drosophila* (Mitchell & Lunan, 1964) and an aryl phosphate may be an intermediate in oxidative phosphorylation (Hatefi, 1963). Menadione is excreted in urine as the phosphate ester of 1,4-dihydroxy-2-methylnaphthalene (Jaques, Millar & Spinks, 1954) and the phenolic hallucinogen psilocin (2-dimethylaminoethyl-4-hydroxyindole) occurs in some fungi as its phosphate, psilocybin (Downing, 1962). Apart from these examples the only case of the formation of an aryl phosphate as a detoxication product is in the metabolism of 2-naphthylamine, which in dogs and man is excreted as di-2-amino-1-naphthyl hydrogen phosphate and 2-amino-1-naphthyl dihydrogen phosphate (Boyland, Kinder & Manson, 1961; Troll, Tessler & Nelson 1963). The first of these compounds was very easily hydrolysed to the corresponding monoaryl phosphate, but this was probably a consequence of the presence in this metabolite of an *o*-amino group since in general the diaryl phosphates are stable to acid (Brown 1963).

We were unable to detect any diaryl phosphates in the present study, though reference sodium di-1-naphthyl phosphate added to homogenates of insects could readily be reisolated and detected on chromatograms by our procedures.

Though there is no doubt of the presence of monoaryl phosphates in excreta and extracts of flies and grass grubs treated with phenols, it is not yet certain that these arise physiologically. Insect tissue and gut often contain high phosphatase activity that could conceivably convert excreted phenols into their phosphates by a phosphotrans-

ferase action from a suitable donor or during the drying of the excreta. We have not been able to demonstrate the formation of phosphate conjugates as artifacts from phenols or from phenyl sulphates in freshly voided fly excreta or vomit, but attempts to demonstrate the biochemical formation of these conjugates in homogenates of insect tissues have, as yet, also been without success.

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REFERENCES

- Berenbom, M. & Young, L. (1951). *Biochem. J.* **49**, 165.
 Boyland, E., Kinder, C. H. & Manson, D. (1961). *Biochem. J.* **78**, 175.
 Brown, D. M. (1963). *Advanc. org. Chem.* **3**, 75.
 Dalgleish, C. E. (1952). *Biochem. J.* **52**, 3.
 Dorrough, H. W. & Casida, J. E. (1964). *J. agric. Fd. Chem.* **12**, 294.
 Downing, D. F. (1962). *Quart. Rev. chem. Soc., Lond.*, **16**, 133.
 Dutton, G. J. & Ko, V. (1964). *Comp. Biochem. Physiol.* **11**, 269.
 Edelfrawi, M. E. & Hoskins, W. M. (1961). *J. econ. Ent.* **54**, 401.
 Friedman, O. M. & Seligman, A. M. (1950). *J. Amer. chem. Soc.* **72**, 624.
 Gessner, T. & Smith, J. N. (1960a). *Biochem. J.* **75**, 165.
 Gessner, T. & Smith, J. N. (1960b). *Biochem. J.* **75**, 172.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hassan, A., Zayed, S. M. A. D. & Abdel-Hamid, F. M. (1965). *Biochem. Pharmacol.* **14**, 1577.
 Hatefi, Y. (1963). *Advanc. Enzymol.* **25**, 275.
 Hitchcock, M. (1965). Ph.D. Thesis: University of London.
 Inagami, K. (1955). *Nippon Nôgei-Kagaku Kaishi*, **29**, 918.
 Jaques, L. B., Millar, G. J. & Spinks, J. W. T. (1954). *Schweiz. med. Wschr.* **84**, 792.
 Jordan, T. W. (1966). M.Sc. Thesis: Victoria University of Wellington.
 Marsh, C. A. & Levvy, G. A. (1958). *Biochem. J.* **68**, 617.
 Mitchell, H. K. & Lunan, K. D. (1964). *Arch. Biochem. Biophys.* **106**, 219.
 Myers, C. M. & Smith, J. N. (1954). *Biochem. J.* **56**, 498.
 Smith, J. N. (1953). *Biochem. J.* **55**, 156.
 Smith, J. N. (1955). *Biochem. J.* **60**, 436.
 Smith, J. N. (1958). *Biochem. J.* **69**, 509.
 Smith, J. N. (1964). In *Comparative Biochemistry*, vol. 6, p. 403. Ed. by Florkin, M. & Mason, H. S. New York: Academic Press Inc.
 Smith, J. N. & Turbert, H. B. (1964). *Biochem. J.* **92**, 127.
 Terriere, L. C., Boose, R. B. & Roubal, W. T. (1961). *Biochem. J.* **79**, 620.
 Troll, W., Tessler, A. N. & Nelson, N. (1963). *J. Urol.* **89**, 626.
 Whitehead, J. E. M., Morrison, A. R. & Young, L. (1952). *Biochem. J.* **51**, 585.
 Zayed, S. M. A. D., Hassan, A. & Hussein, T. M. (1965). *Z. Naturf.* **20**, 587.