Water-Soluble Metabolites of *p*-Nitrophenol and 1-Naphthyl *N*-Methylcarbamate in Flies and Grass Grubs

FORMATION OF GLUCOSE PHOSPHATE AND PHOSPHATE CONJUGATES

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Metabolites isolated from houseflies dosed with 1-naphthol or *p*-nitrophenol were identified as the phosphate and glucose phosphate conjugates of these phenols by titrations, hydrolysis, ionophoresis, i.r. spectra and mixed melting point. [³H]Carbaryl (1-naphthyl *N*-methylcarbamate) was metabolized by houseflies, blowflies and grass grubs to water-soluble metabolites which had chromatographic and ionophoretic behaviour similar to those of the conjugates of 1-naphthol with glucose, sulphate, phosphate and glucose 6-phosphate.

The insecticide carbaryl (Sevin, 1-naphthyl *N*-methylcarbamate) is extensively metabolized in both insects (Eldefrawi & Hoskins, 1961; Andrawes & Dorough, 1967) and mammals (Leeling & Casida, 1966; Knaak *et al.*, 1965; Dorough & Casida, 1964) and a complex mixture of hydroxylation products may be formed. In intact insects or mammals, the initial, mainly hydroxylated, metabolites are subsequently conjugated and a major part of the detoxified insecticide may be present in extracts or excreta as water-soluble metabolites. In insects, these as yet have not been well characterized.

1-Naphthol, which could be formed from carbaryl by hydrolysis, has been shown to conjugate with glucose, sulphate and phosphate in flies and grass grubs and to give rise to a further water-soluble metabolite (Binning *et al.*, 1967) which was tentatively identified as a glucoside 6-phosphate conjugate (Heenan & Smith, 1967). We have now examined the behaviour of carbaryl itself in these insects to see if the same types of conjugates are present. We also present further evidence, from isolations, to confirm the formation of phosphate esters and phenyl glucoside phosphates in the metabolism of phenols by houseflies.

Materials and Methods

Reference compounds

Conjugates of 1-naphthol and *p*-nitrophenol with sulphate, glucose and phosphate were samples used previously (Binning *et al.*, 1967).

Attempts to prepare the glucoside 6-phosphate conjugates of 1-naphthol and *p*-nitrophenol by the method described by Fischer (1914) were unsuccessful and reaction of POCl₃ with the glucoside at –25°C

gave mainly a cyclic phosphate. *p*-Nitrophenyl β-glucoside (1 g) was dissolved in 3.25 ml of pyridine, cooled to –25°C and treated with 0.3 ml of POCl₃ in 1.1 ml of pyridine, also at –25°C. After 1 h water (1.1 ml) was added and the solution allowed to warm to room temperature (20°C). A further 11 ml of water was added and the mixture evaporated *in vacuo* to remove the pyridine. Traces of pyridine were removed by filtration through a cation-exchange column in the H⁺ form (Amberlite IR-105) and the eluate was evaporated to dryness. The residue was dissolved in 2 ml of water and a little cyclohexylamine added to bring the pH to 8.5. Addition of 2 vol. of acetone gave a white crystalline precipitate which was recrystallized from ethanol–ether. This appeared to be the cyclohexylamine salt of *p*-nitrophenyl β-glucoside 4',6'-phosphate, m.p. 224°C, [α]_D²⁰ –76 ± 5° (c 1.0 in water) Found: C, 47.3; H, 6.4; N, 5.8; C₁₈H₂₇N₂O₁₀P requires C, 46.8; H, 5.9; N, 6.1 %.

On paper ionophoresis (Smith, 1958) this compound behaved as a monobasic strong acid and lacked the i.r. band at 970 cm^{–1} characteristic of ionized phosphomonoesters (Shimanouchi *et al.*, 1964).

A similar preparation using 1-naphthyl glucoside gave a product which was not crystallized but which also behaved as a strong monobasic acid on paper electrophoresis.

Only traces of conjugates were obtained by Fischer's (1914) second method using Ba(OH)₂ as base, though the 1-naphthol and *p*-nitrophenol conjugates so obtained were different by chromatography and ionophoresis from the cyclic phosphate prepared above. The yields of conjugates were too low for isolation as cyclohexylamine salts, though the solutions contained material identical in behaviour with the metabolites described below.

Synthesis of the glucoside 6-phosphates was attempted by a route via the glucoside triacetates. *p*-Nitrophenyl glucoside (8.41 g) and trityl chloride (7.86 g) were dissolved in dry pyridine and sealed; after 20 h acetic anhydride (28 ml) was added with an additional 28 ml of pyridine. After a further 20 h the mixture was poured into 2 litres of water and the granular tritylated acetylated derivative collected. *p*-Nitrophenyl 2,3,4-triacetyl-6-trityl- β -D-glucopyranoside crystallized from ethanol, m.p. 110°C, $[\alpha]_D^{20}$ $7 \pm 5.0^\circ$ (*c* 1.0 in ethanol), (Found: C, 66.5; H, 5.4; N, 1.8; $C_{37}H_{35}NO_{11}$ requires C, 66.4; H, 5.3; N, 2.1%). 1-Naphthyl 2',3',4'-triacetyl-6'-trityl- β -D-glucoside was prepared similarly. It had m.p. 169°C, $[\alpha]_D^{20}$ $-14 \pm 5^\circ$ (*c* 1.0 in chloroform). (Found: C, 72.8; H, 5.9; $C_{41}H_{38}O_9$ requires C, 73.0; H, 5.7%).

The naphthyl triacetyl trityl glucoside (15 g) was suspended in 50 ml of acetic acid and 1 equivalent of 38% (w/v) HBr in acetic acid added. After 5 min the trityl bromide was removed and the filtrate poured into 1 litre of water. The 1-naphthyl 2',3',4'-triacetyl- β -D-glucopyranoside was filtered and recrystallized from methanol or toluene. It had m.p. 164°C and $[\alpha]_D^{20}$ $-117 \pm 5^\circ$ (*c* 1.0 in chloroform) (Found: C, 61.1; H, 5.5; $C_{22}H_{24}O_9$ requires C, 61.1; H, 5.6%).

p-Nitrophenyl 2,3,4-triacetyl- β -D-glucopyranoside was prepared similarly and crystallized from toluene with solvent of crystallization. It had m.p. 124°C and $[\alpha]_D^{20}$ $-21 \pm 5^\circ$ (*c* 1.0 in ethanol) Found: C, 54.2; H, 5.3; N, 2.9; loss at 120°C, 9.5; $C_{18}H_{21}NO_{11} \cdot 0.5C_7H_8$ requires C, 54.5; H, 5.3; N, 3.0; loss at 120°C 9.7%.

p-Nitrophenyl glucoside triacetate (640 mg) in 5 ml of dry pyridine was frozen at -40°C and dibenzyl phosphorochloridate, prepared from 1.31 g of dibenzyl phosphite and 0.66 g of *N*-chlorosuccinimide (Kenner *et al.*, 1952), was added. The flask was shaken till the mixture became homogeneous and the mixture was frozen again at -20°C overnight. Water (5 ml) was added and after the mixture reached room temperature the pyridine was removed *in vacuo*. The residual oil was taken up in ether, washed with 1M-HCl, 5% (w/v) NaHCO_3 and water, dried over Na_2SO_4 and evaporated. The residue was crystallized from toluene and the *p*-nitrophenyl 2,3,4-triacetyl- β -D-glucopyranoside 6-(dibenzyl phosphate) had m.p. 115°C, $[\alpha]_D^{20}$ $-21 \pm 5^\circ$ (*c* 1.0 in ethanol) (Found: C, 56.3; H, 5.2; N, 2.0; P, 4.7; $C_{32}H_{34}NO_{14}P$ requires C, 55.9; H, 5.0; N, 2.1; P, 4.5%). This compound had i.r. bands at 1280cm^{-1} and 1005cm^{-1} corresponding to P=O and P-O-alkyl absorption, but the band at 3570cm^{-1} attributable to a free hydroxyl group was absent. Attempts to remove the benzyl group hydrolytically were not successful.

A similar preparation carried out with 1-naphthyl glucoside triacetate yielded an oil which was not crystallized and which did not take up H_2 when treated with H_2 and palladium black at room temperature and pressure.

Attempts to phosphorylate the triacetylglucosides were also carried out by the method of Tener (1961). A mixture of 1 g of *p*-nitrophenyl glucoside triacetate, 1.25 g of 2-cyanoethyl dihydrogen phosphate and 3.4 g of dicyclohexylcarbodi-imide was left for 2 days in a stoppered flask in 20 ml of dry pyridine at 20°C and then 10 ml of water was added. After 1 h the *NN'*-dicyclohexylurea was removed and the filtrate evaporated *in vacuo* to an oily residue.

This was dissolved in 50 ml of aq. 20% (w/v) NH_3 and heated in a stoppered flask for 2 h at 55°C . A small precipitate was removed and the filtrate evaporated to small bulk *in vacuo*. The residue was chromatographed on three sheets of Whatman no. 3MM paper and the zone with R_F 0.2, located by the colour reactions for nitro compounds, was cut out and eluted with 0.1M- NH_3 . This eluate was passed through a short column (10 cm $\times$ 1 cm) of ion-exchange resin (Amberlite IR120; H^+ form; Rohm and Haas Co., Philadelphia, Pa., U.S.A.) to remove bases, adjusted to pH 8 with cyclohexylamine and concentrated to 1 ml *in vacuo*. Addition of acetone to this solution precipitated the cyclohexylamine salt. After crystallization from acetone the compound believed to be *p*-nitrophenyl β -D-glucoside 6-(dicyclohexylammonium phosphate) had m.p. 174°C , $[\alpha]_D^{20}$ -94 ± 5 (*c* 1 in H_2O) (Found: C, 47.3; H, 7.6; N, 7.0. $C_{24}H_{24}N_3O_{11}P \cdot 2\text{H}_2\text{O}$ requires C, 46.8; H, 7.5; N, 6.8%).

The analogous 1-naphthyl derivative was prepared similarly and crystallized from acetone-water. The 1-naphthyl β -D-glucoside 6'-(dicyclohexylammonium phosphate) had m.p. 196°C (Found: C, 54.2; H, 7.6; $C_{28}H_{45}N_2O_9P \cdot 2\text{H}_2\text{O}$ requires C, 54.2; H, 8.0%).

[^3H]Carbaryl was prepared from 1-naphthol, tritiated by the procedure of Yavorsky & Gorin (1962) and Hilton & O'Brien (1964). The 1-[^3H]naphthol was purified by t.l.c. on silica gel G (E. Merck A.G., Darmstadt, Germany) in di-isopropyl ether-iso-octane (1:5, v/v). The 1-naphthol zone (R_F 0.5) was scraped into a tube and treated with methyl isocyanate in ether containing a trace of triethylamine. After 24 h in the sealed tube the ether was evaporated and the residue chromatographed on silica gel G with di-isopropyl ether-iso-octane (1:5, v/v) to separate [^3H]carbaryl (R_F 0.25) from the trace of residual 1-naphthol. The carbaryl was eluted with ethanol and assayed spectrophotometrically. The eluate containing 3 mg of carbaryl (41 mCi/mmol) was evaporated and redissolved in acetone to give a solution (0.5 $\mu\text{g}/\mu\text{l}$) for dosing.

1-[^{14}C]Naphthol (1 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Insects and dosing

Houseflies (*Musca domestica*), blowflies (*Lucilia sericata*) and grass grubs (*Costelytra zealandica*)

were strains used earlier (Binning *et al.*, 1967) and were maintained and dosed as then described.

Radioactivity measurements

These were made in a Packard 4312 scintillation spectrometer as previously described (Binning *et al.*, 1967).

Paper chromatography and ionophoresis

Methods described earlier were used (Binning *et al.*, 1967). In addition, phosphate esters were detected on paper chromatograms by the sulphosalicylic acid- FeCl_3 reagent of Wade & Morgan (1953) and reducing sugars with the *m*-phenylenediamine reagent (Chargaff *et al.*, 1948).

Results

Separation and isolation of the 1-naphthol metabolites

Pupae of 2000–10000 houseflies were allowed to hatch into adults in large glass tanks, through which a current of air was drawn, and allowed to feed on a 1% (w/v) solution of 1-naphthol in milk together with dry sucrose for a week or until all had died. Dead flies and pupae cases were discarded and the excreta washed off the sides of the glass tank by rubbing with aq. 80% (v/v) ethanol. The washings were centrifuged to remove insoluble material and concentrated to small volume *in vacuo* for paper chromatography.

The extract was chromatographed on sheets of Whatman no. 3MM paper in butan-1-ol-acetic acid-water (4:1:5, by vol.) and the zones of metabolites were located either by observing the blue fluorescence under u.v. light or by counting sections cut from the edge of the paper in runs to which the extract of excreta from flies dosed with 1- ^{14}C -naphthol had been added. Three zones were located at R_F 0.5, 0.75 and 0.85 as well as a 1-naphthol zone at R_F 0.95. Zones with R_F 0.75 and 0.85 contained 1-naphthyl sulphate and glucoside respectively (Binning *et al.*, 1967). The zone of R_F 0.5 was eluted in 0.1M- NH_3 and purified by rechromatography in butan-1-ol- NH_3 (sp.gr. 0.88)-water (4:1:5, by vol.), the solvent being allowed to drip from the front for 16h. Two zones were now visible, the slower one corresponding to reference 1-naphthyl phosphate and the faster one to the 'metabolite X' of Binning *et al.* (1967). The latter zone was eluted in 0.1M- NH_3 and purified by rechromatography in 2-methylpropan-2-ol- NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.). The metabolite chromatographed and co-chromatographed identically with the reference 1-naphthyl glucoside 6-phosphate, prepared by Fischer's (1914) second method, in each of the solvents used by Binning *et al.* (1967).

The u.v.-absorption spectrum (measured in Beckman DB) of the eluate between 250 and 350nm

was similar to that of other naphthol conjugates, with λ_{max} at 280 and 319nm in both 0.1M-HCl and 0.1M-NaOH.

The ionophoretic behaviour of the metabolite was characteristic of a dibasic acid having pK_a values near 1.5 and 5.5 (Fig. 1), similar to 4-naphthyl phosphate, though with lower mobility. Samples of the synthetic glucoside 6-phosphate conjugate prepared above behaved identically with the metabolite.

A portion of the eluate containing metabolite equivalent to about 1mg of 1-naphthol by spectrophotometric assay was passed through a short column (2cm $\times$ 5cm) of Amberlite IR-105 cation-exchanger to remove metal ions and evaporated to dryness to remove volatile acids. The residue was dissolved in 1.5ml of water and titrated with 0.1M-NaOH in a Radiometer automatic titrator. The titration curve had two steps, one indicating a strong acid ionization and the second corresponding to pK_a 6.0.

From the alkali required to titrate the second equivalent, the molar concentration of the solution was calculated and hence, from the u.v. spectrum, the molar extinction coefficient of the metabolite. This ($\log \epsilon_{\text{max}}$ 3.6) was of the same order as for other naphthol conjugates and suggested that the molecule contained two ionizable hydrogen atoms to each naphthol fragment.

Bases were removed from the purified extract from 20000 flies by passage through a column (50mm $\times$ 7mm) of cation-exchange resin (Amberlite IR-105). The pH of the eluate was adjusted to 8.5 by addition

Fig. 1. Paper ionophoresis of conjugates of 1-naphthol and *p*-nitrophenol

○, Phosphate, and □, synthetic and biosynthetic glucoside 6-phosphate conjugates of (a) *p*-nitrophenol and (b) 1-naphthol. Separations were made at 40V/cm on Whatman 3MM paper for 1.5h.

of cyclohexylamine, the solution evaporated to 0.1 ml and then diluted with 5 ml of acetone. The precipitate (5mg) after recrystallization from ethanol-ether appeared to be the monocyclohexylamine salt of a 1-naphthyl glucoside phosphate, m.p. 156°C, $[\alpha]_D^{20} -49 \pm 3^\circ$ (c 1.0 in water) (Found: C, 52.5; H, 6.9; N, 3.4. Calc. for $C_{22}H_{32}NO_9P \cdot H_2O$: C, 52.5, H, 6.8; N, 2.8%). The i.r. spectrum of this material measured in Nujol mulls had a strong band at 970cm^{-1} , characteristic of fully ionized phosphomonoesters and which may be used to distinguish them from phosphodi-esters, which have a strong band at 1080cm^{-1} (Shimanouchi *et al.*, 1964). Similar strong bands were present in glucose 6-phosphate barium salt, disodium AMP, disodium GMP, *p*-nitrophenyl disodium phosphate, *m*-nitrophenyl dicyclohexylamine phosphate, disodium α -glycerophosphate and disodium β -glycerophosphate. This band was not present in di-(*p*-nitrophenyl) sodium phosphate.

Further recrystallization from ethanol decreased the melting point of this product and ionophoretic experiments then showed the presence of a contaminant having only one acidic function.

The zone on the large-scale chromatogram identified as 1-naphthyl phosphate was also eluted with 0.1M-NH₃ and treated with ion-exchange resin and cyclohexylamine as described for the glucoside 6-phosphate metabolite. The dicyclohexylamine salt of 1-naphthyl dihydrogen phosphate (30mg) was isolated, with m.p. and mixed m.p. 215°C (Found: C, 58.9; H, 8.7; N, 6.2. Calc. for $C_{22}H_{35}N_2O_4P \cdot 1.5H_2O$: C, 58.8; H, 8.5; N, 6.2%).

Separation and isolation of the *p*-nitrophenol metabolites

Groups of 2000–10000 houseflies were fed with *p*-nitrophenol in milk as described above for 1-naphthol and the extract of the excreta was chromatographed on Whatman no. 3MM paper in butan-1-ol-acetic acid-water (4:1:5, by vol.). Metabolites were located

by the quenching of u.v. fluorescence or by the colour reactions described by Binning *et al.* (1967). Four zones were present at R_F 0.45, 0.55, 0.65, 0.75, as well as unchanged *p*-nitrophenol at R_F 0.95. The zones with R_F 0.55, 0.65 and 0.75 were identified as the conjugates of *p*-nitrophenol with phosphate, sulphate and glucose respectively (Binning *et al.*, 1967). The zone with R_F 0.45, corresponding to 'metabolite X' of Binning *et al.* (1967), was eluted with 0.1M-NH₃ and purified by rechromatography in butan-1-ol-NH₃ (sp.gr. 0.88)-water (4:1:5, by vol.) and in butan-1-ol-acetic acid-water (4:1:5, by vol.).

The purified metabolite was chromatographically homogeneous and in behaviour identical with synthetic *p*-nitrophenol glucoside 6-phosphate prepared by Fischer's (1914) second method in each of the paper-chromatographic systems used by Binning *et al.* (1967). It differed chromatographically (R_F 0.36) from the cyclic phosphate prepared above, which had R_F 0.66 in propan-1-ol-NH₃ (sp.gr. 0.88) (7:3, v/v).

The u.v. spectrum of the eluate was similar to those of other *p*-nitrophenol conjugates, with λ_{max} at 298nm both in 0.1M-HCl and in 0.1M-NaOH. It behaved on ionophoresis and titration like the naphthol metabolite, as a dibasic acid with one pK_a value of 6.0 and also a strong acid function with a pK_a near 1.5, though with a lower mobility than *p*-nitrophenyl dihydrogen phosphate (Fig. 1). As with the 1-naphthol metabolite, the $\log \epsilon_{\text{max}}$ (3.84) calculated from the second step of the titration curve was similar to that of other *p*-nitrophenol conjugates, indicating the presence of one *p*-nitrophenyl group for two titration equivalents.

Spectrophotometric assays were made of the *p*-nitrophenol and phosphate ion (Fiske & SubbaRow, 1925) released by hydrolysis with 1M-HCl at 100°C and by 0.1M-NaOH at 80°C. *p*-Nitrophenol was released faster than phosphate in both systems, but this was more marked in acid solution and after 2h treatment with 1M-HCl, 80% of the nitrophenol

Table 1. Paper chromatography of partial hydrolysis products of a *p*-nitrophenol metabolite

Solvents were upper layers of: A, butan-1-ol-acetic acid-water (4:1:5, by vol.); B, 2-methylpropan-2-ol-NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.).

	R_F		Colour reactions with:			
	A	B	1M-NaOH	<i>m</i> -Phenylenediamine	FeCl ₃ -sulphosalicylic acid	U.v. fluorescence
Glucose	0.2	0.6		Yellow		—
Glucose 6-phosphate	0.1	0.2		Yellow	White	—
<i>p</i> -Nitrophenol	0.9	0.9	Yellow			Quench
Metabolite	0.4	0.7	—	—	White	Quench
	0.4	0.7	—	—	White	Quench
	0.9	0.9	Yellow	—	—	Quench
Hydrolysis products	0.2	0.6	—	Yellow	—	—
	0.1	0.2	—	Yellow	White	—

but only 30% of the total phosphate had been liberated. Portions of the solution taken at this time and examined by paper chromatography showed the presence of some unchanged metabolite together with *p*-nitrophenol, glucose 6-phosphate and a little glucose (Table 1).

The *p*-nitrophenol conjugate in extracts from 20000 houseflies was converted into its cyclohexylamine salt as described for the 1-naphthol conjugate. This (10mg) after crystallization from water containing a little cyclohexylamine, appeared to be the *dicyclohexylamine salt of a p-nitrophenol glucoside phosphate*, m.p. 159°C, $[\alpha]_D^{20} -68 \pm 5^\circ$ (c 1.0 water) (Found: C, 47.0; H, 7.7; $C_{24}H_{42}N_3O_{11}P \cdot 2H_2O$ requires C, 46.8; H, 7.5%).

The i.r. spectrum of the salt had a strong band at 970cm^{-1} characteristic of phosphomonoester salts, similar to that in the naphthol metabolite and other ionized monoesters of phosphoric acid. It also had a weak absorption band at 900cm^{-1} which was also present in *p*-nitrophenyl β -D-glucoside. Absorption at this wavelength has been thought characteristic of pyranoside β -D-glucosides and is absent from α -D-pyranosides (Barker *et al.*, 1956).

The zone with R_F 0.55 on the large-scale chromatogram identified as *p*-nitrophenyl phosphate was eluted as described above and converted into its dicyclohexylamine salt, m.p. 185°C (yield 20mg) (Found: C, 51.6; H, 7.8; N, 10.1. Calc. for $C_{18}H_{32}N_3O_6P$: C, 51.8; H, 7.7; N, 10.1%).

Separation of the carbaryl metabolites

Twenty houseflies, ten blowflies or ten grass grubs were dosed topically with $0.5\mu\text{g}$ of $[^3\text{H}]$ carbaryl in $1\mu\text{l}$ of acetone and kept for 24 h at room temperature in conical flasks. They were ground up in 8 ml of acetone in all-glass Potter-Elvehjem homogenizers, and 2 ml of water was added before refluxing at 60°C for 4 h along with 1 mg of the non-radioactive phosphate, glucose and sulphate conjugates. Carbaryl (0.5 g) and 1-naphthol (0.5 g) were also added.

The centrifuged extracts were concentrated to small bulk, then made up to 5 ml with water and extracted with $4 \times 5\text{ml}$ of benzene. Counts made of these extracts in three separate experiments with each insect showed that the benzene-soluble radioactivity recovered was 30–34% of the dose in houseflies, 35–38% in blowflies and 45–51% in grass grubs.

Portions of the aqueous layer were chromatographed on Whatman no. 4 paper in butan-1-ol-ethanol-water (17:3:20, by vol.; Binning *et al.*, 1967) and 1 cm sections counted for radioactivity in scintillation vials after positions of the reference conjugates had been marked.

In each case (Fig. 2), peaks of radioactivity were located at the R_F values of the three reference con-

jugates used, though additional bands were present, including a major radioactive zone near the origin. Vials re-counted after removal of the paper strips contained no radioactivity.

The sections of paper were rinsed with toluene to remove scintillation chemicals, dried and then treated with 1 drop of a 1% (w/v) solution of ileum alkaline phosphatase (BDH Ltd., Poole, Dorset, U.K.) or β -glucosidase (Koch-Light, Colnbrook, Bucks., U.K.). When these had dried they were returned to the vials and the extractable and non-extractable radioactivity was measured as described above.

Toluene-soluble radioactivity was now found under the naphthyl glucoside and naphthyl phosphate zones as well as from the peak near the origin in papers treated with either of the enzymes. No radioactivity was rendered toluene-soluble from the 1-naphthyl sulphate peak.

In similar experiments with houseflies, blowflies and grass grubs the radioactive zones which corresponded to the reference glucoside, sulphate and

Fig. 2. Radiochromatograms of water-soluble metabolites of $[^3\text{H}]$ carbaryl in insects

Extracts were made as described in the text from (a) houseflies, (b) blowflies or (c) grass grubs, and chromatograms were run in butan-1-ol-ethanol-water (17:3:20, by vol.). Bars indicate the position of co-chromatographed conjugates of 1-naphthol with: 1, phosphate; 2, sulphate; 3, glucose.

Fig. 3. Ionophoresis of eluates of paper-chromatographed zones from grass grubs dosed with [^3H]carbaryl

Zones containing sulphate, phosphate and glucose conjugates from grass-grub extracts chromatographed as in Fig. 2 were eluted and separated ionophoretically at 10 V/cm on Whatman no. 1 paper for 2 h. (a) 'Phosphate' zone; (b) 'sulphate' zone; (c) 'glucoside' zone of Fig. 2(c). Bars indicate positions taken up by reference conjugates of 1-naphthol with glucose (1), sulphate (2) and phosphate (3).

phosphate peaks were eluted with 0.1M-NH₃ and subjected to ionophoresis at pH 2.5, 9.0 and 12.5. This showed that these eluates were not homogeneous (e.g. Fig. 3), but in each appropriate eluate a major zone with ionophoretic properties corresponding to the reference sulphate, glucoside or phosphate was present.

In addition to the reference aryl phosphate the 'phosphate' eluate from experiments with all three insects contained a major peak of radioactivity with a mobility of about half that of reference 1-naphthyl phosphate, similar in behaviour to the glucoside phosphate conjugate (cf. Fig. 1).

Discussion

The metabolite isolated from excreta supports our earlier conclusions (Heenan & Smith, 1967) that the conjugates of 1-naphthol and *p*-nitrophenol in insects may include both phosphate esters of the phenols and conjugates with a phosphorylated glucose. It is probable that the compounds present in extracts of excreta are glucoside 6-phosphate glycosides, since on hydrolysis of the *p*-nitrophenol

metabolite glucose 6-phosphate was detected after chromatography.

We were, however, unable to confirm this by comparison of synthetic and biosynthetic crystalline material. Both materials were dibasic acids with pK_a values characteristic of phosphate monoesters but had different values for melting point and optical activity. Synthesis via the triacetyl glucosides would have been expected to yield the 6-phosphates, but it is possible that migration of phosphate from the 6 to the 4 position could have occurred in either synthetic or biosynthetic samples on recrystallization.

The glucoside phosphate conjugate was present in excreta of both houseflies and blowflies, but not to any significant extent in extracts of the grass grub after dosing with 1-naphthol. On the other hand, when [^3H]carbaryl was administered, substantial amounts of material behaving like a glucoside phosphate were formed by grass grubs (Figs. 2 and 3), and it is possible that this is a conjugation reaction which can occur generally in insects.

Extracts from all three insects which had been dosed with the insecticide gave rise to chromatographic zones behaving like the sulphate and glucoside

conjugates of 1-naphthol, which after elution and subsequent ionophoresis had major constituents migrating similarly to these conjugates. The 'phosphate' zones of these chromatograms contained roughly equal amounts of material which behaved, on ionophoresis, like an aryl phosphate and material which migrated at about half this rate. This was similar in behaviour to the glucoside phosphate.

Previous experience with ionophoretic separation and identification of detoxication products at different pH values (Smith, 1958; Kikal & Smith, 1959; Cohen & Smith, 1964; Hitchcock & Smith, 1964, 1966; Binning *et al.*, 1967) suggests that this technique is valuable in distinguishing between the different types of ionized conjugation products, e.g. sulphates and glucuronides, but of little value in resolving mixtures of the same type of conjugate. Thus all phosphates, ethereal sulphates or glucuronides which are without other ionized functions have very similar mobilities. The ionophoretic experiments (e.g. Fig. 3) described above do not therefore identify the radioactive metabolites as 1-naphthol conjugates, though they suggest strongly that these metabolites are sulphate, glucoside, phosphate and probably glucoside phosphate conjugates of a phenol derived from 1-naphthol or carbaryl. Other work on the organic-soluble metabolites of carbaryl (Dorough & Casida, 1964; Andrawes & Dorough, 1967; Tsukamoto & Casida, 1967) has shown that a variety of phenolic and other hydroxylated metabolites may be formed from this insecticide, and these are potential precursors for the conjugates found in the aqueous fraction. The ionophoretic pattern at pH 12.5, however, suggests that mono-conjugates of a dihydroxynaphthalene do not form a significant proportion of the detoxication products, since the migrations of the main radioactive peaks did not suggest the presence of any additional phenolic hydroxyl groups.

The isolation of two representatives of this type of detoxication product of phenols makes the properties of the glucoside phosphates of some interest, since similar compounds could be encountered as metabolites of other insecticides or phenols in insects. Glucoside phosphate conjugates of phenols have also been reported in plants (Nanayakkara *et al.*, 1970).

The glucoside phosphates have characteristic ionophoretic properties which allow them to be distinguished from other ionized, water-soluble metabolites such as sulphates, phosphates and glucuronides. They show the increase in mobility between pH 6 and 7 which is also characteristic of the aryl phosphates (Fig. 1) but, possibly because of their carbohydrate content, their overall mobility is less than that of the aryl phosphates. In alkaline solution their mobility is distinct from and greater than that of the ethereal sulphates and they are also

distinguished from the sulphates by their much greater resistance to hydrolysis.

The new metabolites have the β -glycoside link, since their optical rotations are similar to those of the corresponding glucosides. 1-Naphthyl β -D-glucoside 6-phosphoric acid ($[\alpha]_D^{20} -75^\circ$) and *p*-nitrophenyl β -D-glucoside 6-phosphoric acid ($[\alpha]_D^{20} -103^\circ$) are similar to the corresponding glucoside and glucuronic acid conjugates (1-naphthyl β -D-glucoside $[\alpha]_D^{20} -76^\circ$; 1-naphthyl β -D-glucosiduronic acid $[\alpha]_D^{20} -94^\circ$; *p*-nitrophenyl β -D-glucoside $[\alpha]_D^{20} -105^\circ$; *p*-nitrophenyl β -D-glucosiduronic acid $[\alpha]_D^{20} -103^\circ$; see respectively Smith & Turbert, 1964; Binning *et al.*, 1967; Berenbom & Young, 1951; Bray, 1953). Some evidence for the β -configuration may also be drawn from the i.r. spectra, which had a band at 900cm^{-1} attributable to the pyranoside β -D-glucoside structure (Barker *et al.*, 1956).

The glycoside linkage in the conjugates was much more resistant to acid or alkaline hydrolysis than that in the corresponding β -glucosides or glucuronides. This is analogous to the situation with glucose 1,6-diphosphate, where the glycosidic link is more stable than in glucose 1-phosphate (Leloir & Cardini, 1963). The stabilizing effect of the bulky acidic substituent at C-5 is also shown in the glucuronides, which are more resistant to hydrolysis than the glucosides (Bemiller, 1967). In *p*-nitrophenyl glucoside 6-phosphate the half-time for the hydrolysis at pH 12 and 80°C was 75 min; under the same conditions the half-life of *p*-nitrophenyl glucoside is only 2 min (Piskiewicz & Bruice, 1967). The hydrolysis of the naphthyl metabolite was even slower and no 1-naphthol could be detected under the hydrolysis conditions.

The glucoside 6-phosphates were also resistant to enzymic hydrolysis, and treatment of chromatograms with β -glucosidase (emulsin; Koch-Light) or mixed hydrolases from snail gastric juice did not liberate phenols under conditions where glucosides and glucuronides were readily broken down.

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