Comparative Detoxication

11. CONJUGATIONS OF 1-NAPHTHOL AND SOME OTHER PHENOLS IN HOUSEFLIES AND LOCUSTS*

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(*Received* 23 *October* 1963)

Insects from a variety of Orders have been shown to detoxify phenols by conjugation with glucose and this mechanism has been suggested as a systematic biochemical characteristic of insects (Smith, 1955*a*). The phenols may be foreign compounds or may be those formed in the normal course of metabolism (Smith, 1955*b*, 1964; Kent & Brunet, 1959; Stay & Roth, 1962; Butenandt, Biekert, Kübler & Linzen, 1960).

On the other hand, a glucosiduronate of 3-hydroxykynurenine is said to be present in silkworm pupae (Inagami, 1955), and Terriere, Boose & Roubal (1961) detected 1-naphthyl glucosiduronic acid and glucosiduronic acid derivatives of 1,2-dihydro-1,2-dihydroxynaphthalene and 1,2-dihydronaphthol in the excreta of flies dosed with 1-naphthol. No glucosides were reported.

Possible explanations of this apparent difference between flies and other insects were that the extent of conjugation of naphthol differed from that of other phenols or that there were qualitative species differences in the glycosidic detoxications.

We have therefore examined the rates of conjugation of a range of phenols, including 1-naphthol, in locusts in which the glucosidic detoxication is well established, and have also studied the conjugation of 1-naphthol and some other phenols in houseflies.

EXPERIMENTAL

Materials

Reference compounds. Glucosides and glucosiduronic acids of *m*-aminophenol, 4-methylumbelliferone and 8-quinolinol were samples prepared previously in this Laboratory (Mead, Smith & Williams, 1955; Smith, 1953, 1955*a*; Robinson, 1956). *p*-Hydroxyphenyl glucoside (arbutin, i.e. the glucoside of quinol) and *o*-formylphenyl glucoside (helicin, i.e. the glucoside of salicylaldehyde) were commercial samples. 1-Naphthyl β-D-glucoside tetra-acetate, m.p. 173°, $[\alpha]_D^{20}$ −72° (*c* 1 in $CHCl_3$), was prepared by the Glazer & Wulwek (1924) procedure and deacetylated with sodium in methanol to give 1-naphthyl β-D-glucoside, m.p. 170°, $[\alpha]_D^{20}$ −76° (*c* 1 in water). 1-Naphthyl glucosiduronic acid, m.p. 195°, $[\alpha]_D^{20}$ −94° (*c* 0·3 in ethanol), was isolated from rabbit urine after dosing with 1-naphthol (cf. Berenbom & Young, 1951). Potassium 1-naphthyl sulphate and sulphates of the other phenols were prepared by the general method of Burkhardt & Lapworth (1926).

1-Naphthol was a commercial sample resublimed before use. Disodium 1-naphthyl orthophosphate was a commercial sample (from British Drug Houses Ltd.).

Methods

Insects and dosing. Houseflies were obtained from the Rothamsted Experimental Station as pupae. After emergence the flies were left for 2 days and then transferred in batches of 100 to 20 cm. crystallizing dishes covered with gauze. They were fed on sucrose and milk. Locusts were obtained from the Anti-Locust Research Centre as fifth-instar hoppers. Halved carcasses ('fillets') were prepared as described by Cohen & Smith (1964).

Incubations. Two locust 'fillets' (from one locust) were covered with 2 ml. of grasshopper saline, pH 6·5 (Carlson, 1946; but with the glucose omitted), containing 6 μmoles of the phenol being studied and incubated at 37° in air.

Small volumes, usually 0·1–0·2 ml., were withdrawn after 0·5 hr. and the glucoside was estimated. Formation of glucoside by locust 'fillets' from 4-methylumbelliferone was linear with time for 3–4 hr.

Paper chromatography. This was carried out and compounds were detected by methods described previously (Mead *et al.* 1955; Myers & Smith, 1954; Smith, 1955*a*; Cohen & Smith, 1964) (see Table 1). In addition, salicylaldehyde and its glucoside were detected as brown spots by spraying with saturated 2,4-dinitrophenylhydrazine in 2N-HCl followed by 2N-NaOH. Quinol conjugates gave brown–red colours when sprayed with a stabilized diazotized *p*-nitroaniline (aq. 0·1% Bretamine Fast Red GG).

1-Naphthol conjugates gave a dark-purple fluorescence under ultraviolet irradiation (with a Hanovia Chromatolite lamp). After hydrolysis, the liberated 1-naphthol gave a light-blue fluorescence in ultraviolet light and gave a blue colour when sprayed with ethanolic 0·01% dichloroquinone-chloroimide and saturated aq. $NaHCO_3$. A 1:5 dilution of *Helix pomatia* gastric juice hydrolysed 1-naphthol conjugates of glucuronic acid, sulphuric acid or glucose. Emulsin (British Drug Houses Ltd.; 1% in water) hydrolysed only the glucoside, and a spray of N-HCl, allowed to dry at room temperature, hydrolysed the ethereal sulphate.

Except in experiments with 8-quinolinol, quantitative determinations of glucosides were made after paper chromatography. Small volumes (usually 0·1 ml.) of the saline incubation media were banded on 4 cm. strips of Whatman no. 1 paper and separated in butan-2-one–water

* Part 10: Cohen, Smith & Turbert (1964).

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Table 1. *R_F values of some phenolic conjugates*

The solvent systems used were: A, propan-1-ol–aq. ammonia (sp.gr. 0·88) (7:3, v/v); B, butan-1-ol–ethanol–water (17:3:20, by vol.); C, butan-1-ol–aq. ammonia (sp.gr. 0·88)–water (4:1:5, by vol.); D, butan-1-ol–acetic acid–water (4:1:5, by vol.); E, butan-2-one–water (2:1, v/v); F, dimethylformamide–butan-1-ol–water (4:25:1, by vol.); G, butan-2-one–2N-NH_3 (2:1, v/v); H, pyridine–'amyl alcohol'–water (7:7:6, by vol.). The 'amyl alcohol' used in solvent H was a commercial product obtained from British Drug Houses Ltd.

	R_F values							
Solvent	A	B	C	D	E	F	G	H
4-Methylumbelliferonyl sulphate	—	—	0·6	0·6	0·7	0·4	0·9	0·7
4-Methylumbelliferonyl glucoside	—	—	0·7	0·7	0·4	0·4	0·6	0·7
4-Methylumbelliferonyl glucosiduronic acid	—	—	0·1	0·6	0·1	0·2	0	0·3
8-Quinolyl sulphate	0·6	—	0·4	0·5	0·3	0·4	—	0·6
8-Quinolyl glucoside	0·8	—	0·8	0·5	0·4	0·4	—	0·7
8-Quinolyl glucosiduronic acid	0·4	—	0·2	0·3	0·1	0·2	—	0·3
1-Naphthyl sulphate	0·9	0·6	0·6	0·7	0·7	—	0·7	0·9
1-Naphthyl glucoside	0·9	0·8	0·8	0·8	0·9	—	0·8	0·9
1-Naphthyl glucosiduronic acid	0·7	0·4	0·3	0·8	0·4	—	0·1	0·4
4-Aminophenyl sulphate	—	—	0·2	0·5	0·3	0·4	—	—
4-Aminophenyl glucoside	—	—	0·3	0·4	0·3	0·4	—	—
4-Aminophenyl glucosiduronic acid	—	—	0·1	0·2	0·1	0·2	—	—

(solvent E) in which all the phenols ran at the solvent front. Glucosides were located by their fluorescence or by comparison with reference material in the region R_F 0·3–0·8 and the zones cut out and eluted by standing in the required volume of the colour reagents described below.

Colorimetric determinations

m-*Aminophenyl glucoside.* *m*-Aminophenyl glucoside in 2 ml. of water was mixed with 0·5 ml. of ethanolic 1% (w/v) *p*-dimethylaminobenzaldehyde and 0·5 ml. of acetic acid. After 10 min. the colour was measured against a reagent blank at 440 mμ in a Unicam SP. 500 spectrophotometer.

m-*Nitrophenyl glucoside.* *m*-Nitrophenyl glucoside in 2 ml. of water was mixed with 1 ml. of 1% (w/v) $TiCl_3$ in 0·1N-HCl and left for 0·5 hr. at room temperature. The amino compound was then measured against a reagent blank as described above.

p-*Hydroxyphenyl glucoside.* *p*-Hydroxyphenyl glucoside (arbutin) in 2 ml. of water was mixed with 0·2 ml. of saturated aq. $NaHCO_3$, and 0·5 ml. of aq. 0·2% Brentamine Fast Red GG was added. The colour was measured against a reagent blank at 540 mμ.

o-*Formylphenyl glucoside.* *o*-Formylphenyl glucoside (helicin) in 4 ml. of water was treated with 0·5 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2N-HCl, and after 10 min. 0·5 ml. of 2·5N-NaOH was added. After a further 10 min. the colour was measured against a reagent blank at 460 mμ.

Calculations from these procedures were made by reference to calibration curves constructed by using the stated volume of solutions of known concentrations. These were linear over the range 10–100 μg. of glucoside/tube, and the recoveries of known quantities, after chromatography, were in the range 85–95%.

Fluorimetric determinations

These were carried out with the Aminco–Bowman spectrophotofluorimeter and solutions were diluted if necessary to bring them to a linear part of the concentration–fluorescence curve.

4-*Methylumbelliferonyl glucoside.* 4-Methylumbelliferonyl glucoside was estimated as described by Smith & Turbert (1961).

8-*Quinolyl glucoside.* 8-Quinolyl glucoside in 0·1N-acetic acid was activated by light at 360 mμ and fluoresced at 480 mμ. A filter with cut-off at 405 mμ was used in front of the photomultiplier. Fluorescence of the glucoside was depressed by concentrations of the free phenol greater than 10 μg./ml. Portions (0·1 ml.) of the saline incubation medium were therefore diluted to 10 ml. with 0·1N-acetic acid, which decreased the 8-quinolinol concentration below this value. Concentrations of glucoside were calculated by comparison with the response of quinine sulphate solution (0·1 μg./ml.) in 0·1N-H_2SO_4 which was calibrated in terms of 8-quinolyl glucoside. Solutions to be estimated normally contained 0·1–1 μg. of the glucoside/ml., and at this concentration recoveries of glucoside were within ±5%.

1-*Naphthyl glucoside.* 1-Naphthyl glucoside in 0·1N-HCl was activated by light at 300 mμ and fluoresced at 345 mμ. It was estimated, after chromatography in butan-2-one–water (solvent E) by soaking the cut-out sections in 5 ml. of 0·1N-HCl and measuring the fluorescence after 2 hr. Solutions from paper strips containing 0·01 μg./ml. could be measured without interference from scattered light, and solutions in most experiments were in the range 0·1–1 μg./ml., from which recoveries were within ±5%.

The glucosiduronic acid and ethereal sulphate of 1-naphthol had the same activation and fluorescent wavelengths as the glucoside but differed in fluorescence intensity.

RESULTS

Rate of conjugation of phenols in locust 'fillets'. Solutions of phenols were incubated in saline with locust 'fillets' as described above and the glucoside present after 0·5 hr. was assayed colorimetrically or fluorimetrically. In an attempt to control variations in different batches of locusts, the rate of conjugation of 4-methylumbelliferone was mea-

Table 2. *Glucosidation of phenols by locust 'fillets'*

The rates of conjugation are expressed as μmoles of glucoside formed/hr./locust under the conditions described in the text.

No. of experiments		Rates of conjugation: With phenols	Rates of conjugation: With 4-methylumbelliferone controls
6	*m*-Nitrophenol	0·24 (0·13–0·35)	0·24 (0·15–0·41)
6	*m*-Aminophenol	0·22 (0·17–0·41)	
9	Quinol	0·88 (0·37–2·57)	0·61 (0·08–1·24)
9	Salicylaldehyde	0·24 (0·07–0·40)	
14	8-Quinolinol	0·77 (0·39–1·37)	0·37 (0·09–1·08)
10	1-Naphthol	0·22 (0·16–0·30)	—
	4-Methylumbelliferone	0·41 (0·08–1·24)	—

sured on locusts from each group at the same time as each other phenol. Considerable variation was found in the experimental rates in individual locusts, these ranging from 27 to 420 μg. of 4-methylumbelliferone/hr./locust. The results are summarized in Table 2.

Estimations of the conjugation rate of 8-quinolinol at intervals during the fifth instar and for 10 days after moulting into adults showed no clear relation to the moult cycle. All the values obtained from individual locusts were scattered within the range 0·59–1·80 μmoles/hr./locust.

Qualitative paper-chromatographic examination of the saline media, with larger volumes than were used for the quantitative assay, provided no evidence for metabolites other than the monoglucosides of each phenol.

Conjugation of phenols in houseflies. Houseflies were allowed to feed on sucrose and milk to which 10 mg. of *m*-aminophenol, 4-methylumbelliferone, 8-quinolinol or 1-naphthol had been added/ml. These doses were non-toxic. Batches of 100–300 flies were ground with aq. 80 % (v/v) ethanol after 2–7 days and the ethanol extracts evaporated to small volume *in vacuo*. Fat was removed by shaking with 20 ml. of ether, and the aqueous layer was examined on paper chromatograms.

Excreta left on the side of the glass dishes housing the flies were also collected in aq. 80 % (v/v) ethanol and examined chromatographically in the solvents given in Table 1.

Glucoside conjugates of all the phenols were readily detected on the chromatograms and smaller amounts of ethereal sulphates were present in excreta from flies dosed with 1-naphthol, *m*-aminophenol and 4-methylumbelliferone. Glucosiduronic acid derivatives could not be detected in these experiments.

Excreta from 300 flies that had fed on 1-naphthol for 7 days were collected in aq. 80 % (v/v) ethanol and separated on a 15 cm.-wide sheet of Whatman no. 1 paper in solvent system A. Reference compounds were also run as a mixed chromatogram with another sample of the excreta. The zones corresponding to the reference 1-naphthyl glucosiduronic acid and the combined 'glucoside–sulphate' zone were cut out and eluted in 0·1 N-ammonia.

A sample of the combined 'glucoside–sulphate' zone (R_F 0·8–0·9) was subjected to paper electrophoresis at 10 v/cm. in 0·02 N-sodium hydroxide for 1 hr. with reference conjugates by using the technique described by Smith (1958). Strong reactions were found, coincident with the 1-naphthyl hydrogen sulphate (+8 cm.) and 1-naphthyl glucoside (−2 cm.).

The bulk of the 'glucoside–sulphate' fraction was evaporated to a syrup and treated with 0·5 ml. of acetic anhydride and 0·5 ml. of pyridine overnight; 1·0 ml. of water was then added and, on standing, needles (10 mg.) of 1-naphthyl β-D-glucoside tetra-acetate were obtained. These had m.p. and mixed m.p. 172–173°, and $[\alpha]_D^{20}$ −68° (*c* 0·5 in chloroform) (Found: C, 60·1; H, 5·5. Calc. for $C_{24}H_{26}O_{10}$: C, 60·7; H, 5·5 %).

The 'glucosiduronic acid' eluate (R_F 0·6–0·8) was concentrated and transferred to a 5 cm.-wide strip of Whatman no. 1 paper for electrophoresis in 0·02 N-sodium hydroxide. After 1 hr. at 10 v/cm. the zone corresponding to reference 1-naphthyl glucosiduronate was eluted with water and chromatographed in solvents A, B, C and D. A weak spot of a conjugate of a phenol that could be hydrolysed by *Helix pomatia* enzyme was present in each system, but this did not coincide with that of the reference 1-naphthyl glucosiduronic acid in any solvent.

In other experiments with 100–300 flies, excreta were separated in solvents A, B and C, and zones corresponding to the 1-naphthyl glucosiduronic acid were eluted and examined in the fluorimeter. These eluates had the fluorescence characteristic of 1-naphthyl conjugates (i.e. activation at 300 mμ and fluorescence at 345 mμ) but were 50–100-fold less fluorescent than the zones containing 1-naphthyl glucoside. Moreover, they did not differ

in fluorescence properties from control eluates of paper or of eluates of zones not corresponding to a known naphthol conjugate.

In addition to the glucoside and sulphate conjugates of the four phenols studied, houseflies formed a third conjugate, which had low R_F values in the alkaline solvent systems used. These could be hydrolysed on paper to the corresponding phenols by *Helix pomatia* enzyme at pH 4 and, more readily, by a phosphatase preparation (desiccated ileum) at pH 11. The conjugate from excreta of flies dosed with 1-naphthol also had the same ionophoretic properties as synthetic 1-naphthyl phosphate at pH 12.

Rate of conjugation of 1-naphthol in flies. Houseflies were dosed topically with 15 μg. of 1-naphthol in 1 μl. of olive oil, and at hourly intervals up to 5 hr. three flies were homogenized in 2 ml. of ethanol and centrifuged. Portions of the supernatant were chromatographed in solvent system C, and the zones corresponding to 1-naphthyl glucosiduronic acid and 1-naphthyl glucoside were cut out and measured fluorimetrically. The glucosiduronic acid fluorescence was not significantly different from the paper controls and showed no increase with time. The amount of 1-naphthyl glucoside found corresponded to a rate of synthesis of 50 μg./hr./fly. In two other experiments in which houseflies were injected with the same dose and later crushed and transferred in water to the paper, rates of 40 and 50 μg./hr./fly were found.

DISCUSSION

The rates of conjugation of different phenols with glucose vary considerably in individual locusts, but the average values do not suggest that structure has any marked effect on the reaction rate. Houseflies also conjugate phenols with glucose, and taking into account the relative weights of a locust (about 1 g.) and the housefly (about 20 mg.) the flies appear to be somewhat more effective in this conjugation.

With the procedures used it has not been possible to identify with certainty any glucuronic acid conjugate in the houseflies. In similar experiments blowflies have also yielded mainly glucosidic conjugates together with smaller amounts of ethereal sulphates. If present, the glucuronic acid conjugates only occur in amounts some 50 times smaller than the main glucosidic metabolite. In the work of Terriere *et al.* (1961) ^{14}C-labelled 1-naphthol was used and some unidentified metabolites were detected in a range of R_F values where 1-naphthyl glucoside might have been expected. No quantitative results were reported and it is not possible to deduce the relative quantities of this and the 1-naphthyl glucosiduronic acid.

The UDP-glucuronic acid necessary for glucosiduronic acid conjugations in mammals has not been found in insects, and in Orthoptera the UDP-glucuronate glucuronyltransferase is absent (Smith & Turbert, 1961; Dutton, 1962). More recently G. J. Dutton (personal communication) found no enzymic evidence for the formation of glucosiduronic acids in flies, though the formation of glucosides was detected.

Small amounts of glucuronic acid conjugates could possibly be produced by a transglycosidation reaction (Dedonder, 1961) catalysed by glucuronidase (Fishman & Green, 1957), though the physiological significance of this reaction in detoxication is doubtful. Insect gut and excreta contain a variety of glycosidases (Robinson, 1956, 1957, 1964; Powning & Irzykiewicz, 1962), and it is conceivable that the major glucoside metabolite could give rise to a glucosiduronic acid derivative by transglycosidation if a source of glucuronic acid was present, possibly as a polysaccharide in food or media.

A third conjugate, possibly a phosphate ester, was also present in the excreta of houseflies dosed with the phenols. The nature of these conjugates, which have R_F values in some solvent systems not very different from those of the glucosiduronic acids, is being further investigated. Phosphate esters have not been identified as detoxication products of phenols in insects, but 2-amino-1-naphthol is conjugated with phosphate in dogs (Boyland, Kinder & Manson, 1961).

The establishment of a glucoside conjugation as the major glycosidic detoxication mechanism in houseflies strengthens the belief that this reaction is a characteristic biochemical feature of insects. Consideration of the branches of the phylogenetic tree where the two sugar detoxications are found shows that the glucuronic acid conjugation is confined to vertebrates. It is found in mammals, reptiles, birds, amphibia (Smith, 1964) and fish (Huang & Collins, 1962). In invertebrates where a glycoside detoxication occurs, glucose is used. This may have appeared several times in evolution, since glucoside formation occurs in a snail (Dutton, 1962), in crustaceans (Smith, 1964) and in myriapods (Eisner, Eisner & Hurst, 1963) as well as in insects. In arthropods the mechanism may have developed later than in the vertebrate phylum, since the arachnids (spiders, scorpions, ticks, harvestmen) so far examined have no glycosidic detoxication (Hitchcock & Smith, 1963; and unpublished work).

SUMMARY

1. The rate of glucosidation, in locust tissues, of *m*-aminophenol, *m*-nitrophenol, quinol, salicylaldehyde, 8-quinolinol, 1-naphthol and 4-methylumbelliferone have been measured.

2. No marked dependence on structure was observed.

3. Glucosides and ethereal sulphates of *m*-aminophenol, 1-naphthol and 4-methylumbelliferone were detected in excreta of houseflies dosed with these phenols.

4. Glucuronic acid conjugates were not detected by the colorimetric and fluorimetric procedures used.

We are grateful to the Insecticide Department, Rothamsted Experimental Station, for supplies of houseflies, and to the Anti-Locust Research Centre for financial support and supplies of locusts.

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The Effect of Concentration on Glucose Phosphorylation and Incorporation into Glycogen in the Livers of Foetal and Adult Rats and Sheep

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(*Received* 28 *October* 1963)

Adult rat and guinea-pig livers have been shown to contain two types of hexokinase, one specific for glucose and mannose, glucokinase (EC 2.7.1.2), and a second non-specific enzyme, hexokinase (EC 2.7.1.1), both of which phosphorylate glucose in the 6-position (Walker, 1962; Viñuela, Salas & Sols, 1963; Ballard & Oliver, 1964). The glucokinase present in adult rat liver, but absent in foetal rat liver, has a Michaelis constant for glucose of 10–40 mM (DiPietro, Sharma & Weinhouse, 1962; Walker, 1962; Ballard & Oliver, 1964). This property would permit enzymic control over the rate of glucose phosphorylation in response to changes in the concentration of blood glucose over the normal range 3–10 mM (Cole & Harned, 1938). This form of glucokinase would thus enable the rat liver to regulate the uptake of glucose from the blood and thus the amount of glycogen stored in the liver.

In ruminants, e.g. sheep, large quantities of volatile fatty acids, principally acetic acid, propionic acid and butyric acid, are produced by fermentation of carbohydrate in the rumen and provide much of the energy for the animal (Elsden & Phillipson, 1948). Glucose is not absorbed even if present in the diet, because it is rapidly metabolized by the rumen micro-organisms (Weller & Gray, 1954). Glucose required for the metabolism of