3-*O*-Hydrosulphato-4-hydroxyphenethylamine (Dopamine 3-*O*-Sulphate), a Metabolite Involved in the Sclerotization of Insect Cuticle

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Dopamine 3-*O*-sulphate (3-*O*-hydrosulphato-4-hydroxyphenethylamine) was isolated from newly ecdysed cockroaches, *Periplaneta americana* (L.), and its structure established by chemical and physical techniques and by synthesis. Relatively high concentrations (about 1 $\mu\text{mol/g}$ wet wt.) of dopamine 3-*O*-sulphate exist in the newly ecdysed insect, and these concentrations decrease sharply as sclerotization of the cuticle proceeds. At least 40% of the radioactivity of ^{14}C -labelled dopamine 3-*O*-sulphate injected into newly ecdysed nymphs was recovered in the sclerotized cuticle 7–12 days after the injection. However, less than 1% of the radioactivity of injected dopamine 3-*O*-[^{35}S]sulphate was recovered, and this value was not appreciably different from that for the incorporation of $\text{Na}_2^{35}\text{SO}_4$. Apparently, little or none of the sulphate moiety of dopamine 3-*O*-sulphate is incorporated directly into the cuticle as the intact sulphate ester. These observations are discussed in relation to current concepts of cuticular sclerotization in insects.

Sulphate conjugates of phenols (Bauman, 1876*a,b*; 1878–79) and phenolic amines (review by Goodall, 1959) have been long known in vertebrates. Generally speaking, they are considered to be excretory metabolites, although recently Jenner & Rose (1973) suggest with justification that ‘the *O*-sulpho-conjugates of dopamine may represent convenient forms in which the compounds are transported in the mammalian system as well as representing metabolic intermediates’; indeed the greater proportion of urinary phenolic amines in man are represented mainly by sulphate esters (Goodall, 1959; La Brosse & Mann, 1960; Rutledge & Hoehn, 1973). In insects, sulphate conjugates have been detected *in vivo* in many species after treatment with various phenolic compounds, and again, the excretory nature of these conjugates is almost certain (Smith, 1955, 1964, 1968; Binning *et al.*, 1967; Yang & Wilkinson, 1971, 1972).

The present paper describes dopamine 3-*O*-sulphate (3-*O*-hydrosulphato-4-hydroxyphenethylamine), a naturally occurring sulphate ester of dopamine that exists in large amounts in the newly ecdysed cockroach, *Periplaneta americana* (L.). Far from being an excretory metabolite of dopamine, dopamine 3-*O*-sulphate appears to play an important role in the sclerotization of this insect’s cuticle.

Experimental

Insects

Cockroaches [*Periplaneta americana* (L.)] were reared on dry chicken mash in an insectary main-

tained at 28°C and approx. 60% relative humidity, with 12 h of illumination/day.

Extraction of metabolites from cockroaches

About 250 g of newly ecdysed nymphs and adults was homogenized in 800 ml of ethanol in a Waring-type blender. After centrifuging at 38 000*g* for 20 min at 0–4°C, the precipitate was washed with a further 800 ml of 80% (v/v) ethanol and the combined supernatants were concentrated to about 100 ml on a rotary evaporator at temperatures not exceeding 30°C. After centrifuging at 38 000*g* for 1.5 h at 0–4°C, the supernatant was shaken with an equal volume of chloroform, and centrifuged as described above for 10 min. The clear upper aqueous phase was used for the isolation of dopamine 3-*O*-sulphate.

Isolation of dopamine 3-*O*-sulphate

The extract from above was pumped on to a column (50 cm $\times$ 1 cm) of Dowex 50 (X4; 200–400 mesh; H^+ form) resin. The effluent from this column was passed directly on to a second column (50 cm $\times$ 1 cm) of Dowex 1 (X4; 200–400 mesh; acetate form) resin and the system was washed with 300 ml of water. The Dowex 50 column was then disconnected and discarded, and the Dowex 1 column was eluted with a succession of 150 ml of each of 0.05*M*-, 0.10*M*-, 0.15*M*- and 0.20*M*-acetic acid. The flow rate was 1 ml/min, and fractions (5 ml) were collected. The effluent was monitored by spotting

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10 μ l of each fraction on Whatman no. 1 chromatography paper and spraying this with the diazotized sulphanilic acid reagent ('diazotized' reagent) of Pauly (1904).

Dopamine 3-*O*-sulphate was displaced from the column by the 0.15M-acetic acid (fractions 65–76) and was the only phenolic compound that produced a red colour with the 'diazotized' reagent. 'Diazotized'-positive fractions were pooled, dried on a rotary evaporator (20°C) and the residue was dissolved in about 2ml of water. Samples (0.5ml) were loaded on to Whatman 3MM chromatography papers (25cm $\times$ 57cm) for descending chromatography in solvent 1. After development for 18h, the chromatograms were allowed to dry at room temperature for 24h. Dopamine 3-*O*-sulphate (R_F 0.2) was located by guide strips cut from each side of the chromatogram, and eluted from the paper with water. Finally, the pooled dopamine 3-*O*-sulphate solutions were concentrated to about 1ml and chromatographed on a column (50cm $\times$ 1cm) of Sephadex G-10 with water as the eluent. Fractions (5ml) were collected and the effluent from the column was monitored as above. 'Diazotized'-positive fractions were pooled and dried on a rotary evaporator (20°C), to give a yield of 13mg of white powder.

*Preparation of ^{14}C -labelled dopamine 3-*O*-sulphate and dopamine 3-*O*-[^{35}S]sulphate*

This was done *in vivo*. Five tanned nymphs (4th and 5th instars) were injected with 5 μ Ci of 3,4-dihydroxyphenyl[1- ^{14}C]ethylamine hydrochloride (30–50mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). After 1h at 24°C, the nymphs were extracted as described and ^{14}C -labelled dopamine 3-*O*-sulphate was isolated by a scaled-down version of the preceding method. For dopamine 3-*O*-[^{35}S]sulphate, ten tanned nymphs were each injected with 50 μ Ci of carrier-free $\text{Na}_2^{35}\text{SO}_4$ (The Radiochemical Centre); they were extracted 3 days after the injection and the extract was worked up as above. Radioactivity during the isolation was monitored by removing samples (5–50 μ l) of each fraction and counting these for radioactivity in 10ml of scintillant mixture consisting of 2,5-diphenyloxazole (6g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.1g)/litre of toluene–Triton X-100 (2:1, v/v) in a Beckman LS-230 scintillation counter. Radioactivity on paper and thin-layer chromatograms was located by using a Dunnschicht-Scanner II (Berthold, Wildblad/Schwarzwald, Germany). The final products had a radiochemical purity of at least 99%. ^{14}C -labelled dopamine 3-*O*-sulphate had 1.24 $\times 10^6$ c.p.m./ml at the time when it was used for experimental purposes (see below). Specific radioactivity was not determined because of the small weight of material present.

Synthesis of dopamine O-sulphates

A mixture of dopamine 3-*O*-sulphate, dopamine 4-*O*-sulphate and dopamine 3,4-*O*-disulphate was prepared by a method similar to that of Tallan *et al.* (1955) for the synthesis of tyrosine *O*-sulphate. Dopamine hydrochloride (2g) was added slowly to 20ml of conc. H_2SO_4 and kept at -12°C with constant stirring. After 1h, the reaction mixture was poured into a slurry of 65g of finely divided $\text{Ba}(\text{OH})_2$ in ice-water (400ml) and stirred for 20min. BaSO_4 was removed by centrifugation at -5°C , and solid CO_2 (4g) was then added to the supernatant. The resulting precipitate was removed by centrifugation and the volume of the supernatant was concentrated to about 50ml on a rotary evaporator (20°C) for use in ion-exchange chromatography.

*Isolation of synthetic dopamine 3-*O*-sulphate and dopamine 4-*O*-sulphate*

The supernatant from the previous stage was pumped on to a column (50cm $\times$ 1cm) of Dowex 50 (X4; 200–400 mesh; H^+ form) resin to remove unchanged dopamine. The effluent was passed directly on to a second column (50cm $\times$ 1cm) of Dowex 1 (X4; 200–400 mesh; acetate form), and the system was washed with water and acetic acid solutions exactly as described under 'Isolation of dopamine 3-*O*-sulphate'. Samples (10–30 μ l) of each 'diazotized'-positive fraction from the Dowex 1 column were chromatographed on Whatman no. 1 paper in solvent 1. Dopamine 3-*O*-sulphate (red 'diazotized'-positive spots, R_F 0.2) was displaced from the column by 0.15M-acetic acid before dopamine 4-*O*-sulphate. Most of the dopamine 4-*O*-sulphate was found in the 0.2M-acetic acid fractions. Some overlap occurred, and fractions containing both compounds were pooled and rechromatographed on Dowex 1. Dopamine 3,4-*O*-disulphate, which is considerably more acidic than the monosulphate esters, remained bound to the Dowex 1 resin and was not investigated.

Fractions were pooled, dried, and chromatographed on Sephadex G-10 as described above. Yields were: dopamine 3-*O*-sulphate, 22mg of white flakes; dopamine 4-*O*-sulphate, 59mg of white powder.

Analytical methods

Methylation. Portions (5 μ mol) of the natural and synthetic compounds were dissolved in 1ml of 2.5M-NaOH at 0°C and 10 μ mol of dimethyl sulphate was added. After warming to 24°C, the suspension was stirred at intervals during 30min. NaOH was removed on a short column 15cm $\times$ 1cm of Dowex 50 resin (H^+ form) after which the methylated com-

pound was heated at 100°C in 1M-HCl for 1 h. HCl was removed *in vacuo* and a solution of the compound was passed through a short column 15 cm × 1 cm of Dowex 1 resin (acetate form). The effluent was concentrated to about 0.5 ml for chromatography and for further degradation.

Alkaline-nitrobenzene oxidation. Portions (0.4 ml) of the methylated compounds prepared as described above were oxidized to the corresponding aldehydes (vanillin or isovanillin) by the alkaline-nitrobenzene-oxidation method of Stone & Blundell (1951). Sodium was removed from the reaction mixture on a short column 15 cm × 1 cm of Dowex 50 resin (H⁺ form) and the effluent from the column, which had a strong odour of vanilla, was concentrated to about 0.5 ml. Samples were electrophoresed on Whatman no. 1 chromatography paper in aq. 1% (w/v) (NH₄)₂CO₃, pH 8.9, on a flat-bed apparatus, which clearly separated vanillin from isovanillin.

Spectrophotometry. The i.r. spectra of the natural and synthetic sulphate esters of dopamine were measured on a Unicam SP. 200G grating i.r. recording spectrophotometer on pressed discs of KBr containing about 1 mg of the compound. The u.v. spectra were determined in 0.1 M-HCl and 0.1 M-NaOH with a Unicam SP. 800 recording spectrophotometer.

Determination of dopamine and sulphate. Dopamine was determined spectrophotometrically by measuring its absorption at 279 nm in 0.1 M-HCl, or less commonly by the cadmium-ninhydrin method of Atfield & Morris (1961). Sulphate was determined by the barium chloroanilate method of Lloyd (1959). The presence of dopamine did not interfere with the accuracy of the sulphate determinations.

Chromatography. Solvent 1, butan-1-ol-acetic acid-water (4:1:1, by vol.); solvent 2, propan-1-ol-

NH₃ (sp.gr. 0.910)-ethyl acetate (5:2:3, by vol.); solvent 3, propan-2-ol-NH₃ (sp.gr. 0.910)-ethyl acetate-water (45:17:30:8, by vol.); solvent 4, butan-1-ol-NH₃ (sp.gr. 0.880)-ethanol (1:1:1, by vol.).

T.l.c. was carried out on pre-coated plastic sheets of silica gel N-HR (Macherey, Nagel & Co., Düren, Germany).

Results

Chemical properties

Hydrolysis products. Portions (2.16 mg) of the natural and synthetic sulphate esters were heated in 1.0 M-HCl at 100°C for 1 h in sealed tubes. HCl was removed *in vacuo* and the sample was dissolved in water for dopamine and sulphate determinations. High-voltage electrophoresis, at pH 6.5, and paper chromatography in solvent 1 indicated that dopamine was the only 'diazo'-positive ninhydrin-positive product of hydrolysis in each of the samples. Quantitative measurements established that dopamine and sulphate were present on hydrolysis in equimolar ratio, and that all of the weight of the natural and synthetic compounds was accounted for by dopamine and sulphate. Subsequent studies revealed that the sulphate esters of dopamine were hydrolysed by very mild conditions, i.e. heating in 0.1 M-HCl for 10 min at 100°C.

Methylation products. A summary of the methylation products derived from the natural and synthetic sulphate esters of dopamine is given in Table 1. It is evident that the natural compound had a free 4-hydroxyl group on the benzene ring and hence is

Table 1. *Methylated products of the natural sulphate ester of dopamine isolated from P. americana, and of the synthetic sulphate esters of dopamine*

The sulphate group was removed from the compounds after methylation by acid hydrolysis. Alkaline-nitrobenzene oxidation of the methylated products was carried out as described in the text. Authentic 3-methoxy-4-hydroxyphenethylamine (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) had *R_F* 0.75 and 0.70 in solvents 2 and 3 respectively; authentic 4-methoxy-3-hydroxyphenethylamine (Regis Chemical Co., Chicago, Ill., U.S.A.) had *R_F* 0.65 and 0.63 in solvents 2 and 3 respectively.

Compound	<i>R_F</i> of methylated compound after removal of sulphate group (thin-layer silica gel N-HR)		Identity of methylated compound	Product after alkaline- nitrobenzene oxidation of methylated compound
	Solvent 2	Solvent 3		
Natural compound	0.65	0.63	4-Methoxy-3-hydroxy- phenethylamine	Isovanillin
Synthetic dopamine 3- <i>O</i> -sulphate	0.65	0.63	4-Methoxy-3-hydroxy- phenethylamine	Isovanillin
Synthetic dopamine 4- <i>O</i> -sulphate	0.75	0.70	3-Methoxy-4-hydroxy- phenethylamine	Vanillin

Table 2. *Physical properties of dopamine 3-O-sulphate isolated from P. americana, synthetic dopamine 3-O-sulphate and synthetic dopamine 4-O-sulphate*

Compound	Melting point (°C)	Light-absorption maxima (nm) and extinction coefficient, ϵ (litre $\cdot$ mol ⁻¹ $\cdot$ cm ⁻¹)		Solvent ...	R_f value (thin-layer silica gel N-HR)		
		In 0.1 M-HCl	In 0.1 M-NaOH		2	3	4
Natural dopamine 3-O-sulphate	254–256 (decomp.)	276nm $\epsilon = 2088$	296nm $\epsilon = 3740$		0.21	0.41	0.62
Synthetic dopamine 3-O-sulphate	256–257 (decomp.)	276nm $\epsilon = 2085$	296nm $\epsilon = 3744$		0.21	0.42	0.62
Synthetic dopamine 4-O-sulphate	264–265 (decomp.)	274nm $\epsilon = 2372$	293nm $\epsilon = 4316$		0.13	0.27	0.52

dopamine 3-O-sulphate. Alkaline-nitrobenzene oxidation of the methylated compound yielded the expected aldehyde, isovanillin. The identity of the synthetic esters was also established by these experiments.

Physical properties

Natural and synthetic dopamine 3-O-sulphate had virtually identical melting points, light-absorption maxima, extinction coefficients and R_f values, and these properties are distinctly different from those of dopamine 4-O-sulphate (Table 2). The i.r. spectra (Fig. 1) indicate that natural and synthetic dopamine 3-O-sulphate are identical in the wavelength of all absorption bands. Distinguishable differences exist between wavelengths of absorption bands in dopamine 3-O-sulphate and dopamine 4-O-sulphate.

Amounts of dopamine 3-O-sulphate in *P. americana* at various stages in the life cycle

Nymphs (4th and 5th instars) or adults of known post-ecdysis age (8–15 individuals) were extracted and dopamine 3-O-sulphate was partially purified by a scaled-down version of the Dowex 50/Dowex 1 ion-exchange system described above, except that dopamine 3-O-sulphate was displaced from the Dowex 1 column with 0.5 M-acetic acid without prior washing of the column with any other acetic acid solutions. Samples of the partially purified extract were chromatographed on paper in solvent 1 together with known amounts of authentic dopamine 3-O-sulphate. The cadmium–ninhydrin method of Atfield & Morris (1961) was used to determine dopamine 3-O-sulphate in the chromatographed extract, reference being made to the authentic material.

Highest amounts of dopamine 3-O-sulphate were found in newly ecdysed nymphs (0.82 μ mol/g wet wt.)

and newly ecdysed adults (0.87 μ mol/g). Within only 4–5 h after ecdysis, concentrations of dopamine 3-O-sulphate had fallen by about 70% (nymphs, 0.26 μ mol/g; adults, 0.31 μ mol/g). At 16 h after ecdysis, only traces of dopamine 3-O-sulphate remained, and 2 days after ecdysis this compound could not be detected by the present method.

Incorporation of radioactivity from radioactively labelled dopamine 3-O-sulphate into the cuticle of *P. americana*

Newly ecdysed nymphs (4th and 5th instars, 10–45 min after shedding the old cuticle) were injected with a known amount (c.p.m.) of ¹⁴C-labelled dopamine 3-O-sulphate, dopamine 3-O-[³⁵S]sulphate or Na₂³⁵SO₄ by using a finely drawn glass needle and micrometer syringe. After 7–12 days the nymphs were frozen in liquid N₂ and their cuticles were peeled from the underlying epidermis as they thawed. Each cuticle fragment was rubbed thoroughly against filter paper to remove any adhering tissue. The cuticle fragments were washed vigorously with three changes each of water, ethanol and ether, and then air dried. The dried fragments were placed in 2 ml of a solution containing equal volumes of acetic acid and H₂O₂ (30%, w/v) and kept at 60°C for 18 h in a tightly capped scintillation vial. After cooling, the extract was divided into four portions and each portion was counted in 15 ml of scintillant [5g of 2,5-diphenyl-oxazole/litre of toluene–2-ethoxyethanol (5:3, v/v)].

The data in Table 3 show that a large proportion (at least 40%) of the radioactivity of ¹⁴C-labelled dopamine 3-O-sulphate injected into the body cavity of newly ecdysed nymphs was incorporated into the cuticle during sclerotization. However, less than 1% of the radioactivity of dopamine 3-O-[³⁵S]sulphate was recovered in the sclerotized cuticle, and this value is not appreciably different from that for the incorporation of Na₂³⁵SO₄.

Fig. 1. Infrared spectra of (a) dopamine 3-O-sulphate isolated from *P. americana*, (b) synthetic dopamine 3-O-sulphate and (c) synthetic dopamine 4-O-sulphate

The spectra were measured on pressed KBr discs containing about 1 mg of the compound.

Table 3. Radioactivity incorporated into the cuticle of *P. americana* from labelled dopamine 3-O-sulphate
The labelled metabolites were injected into the body cavity of newly ecdysed 4th- and 5th-instar nymphs. After 7–12 days, their sclerotized cuticles were isolated, washed, solubilized and counted for radioactivity as described in the text. Values marked * are corrected for radioisotope decay.

Radioactive metabolite	Injected (c.p.m./insect)	Recovered (c.p.m./cuticle)	Recovery (%)
¹⁴ C-Labelled dopamine 3-O-sulphate	124070	52230	42.1
Dopamine 3-O-[³⁵ S]sulphate	181250	1580*	0.9
Na ₂ ³⁵ SO ₄	166620	2330*	1.4

Discussion

There can be little reasonable doubt that the compound isolated from newly ecdysed nymphs and adults of the cockroach, *P. americana*, is dopamine 3-*O*-sulphate (3-*O*-hydrosulphato-4-hydroxyphenethylamine). Chemical analysis demonstrated that the compound was composed only of dopamine and sulphate in equimolar ratio. Methylation and alkaline-nitrobenzene-oxidation experiments confirmed the presence of a free 4-hydroxyl group and established unambiguously that the compound was a 3-*O*-ester of dopamine. Critical comparison of the physical properties of the isolated compound with those of synthetic dopamine 3-*O*-sulphate revealed virtually identical melting points, extinction coefficients, i.r. spectra, and the R_F values on paper chromatography and silica-gel t.l.c. were also similar. The radioactive labelling of dopamine 3-*O*-sulphate *in vivo* after injection of insects with 3,4-dihydroxyphenyl[1- 14 C]ethylamine or $\text{Na}_2^{35}\text{SO}_4$ proved that dopamine 3-*O*-sulphate is a biosynthetic product and is not derived directly from the insect's food.

As mentioned in the introduction, sulphate conjugates of phenols and phenolic amines in vertebrates and insects have usually been considered to be excretory metabolites, but see Jenner & Rose (1973). This does not appear to be the case for dopamine 3-*O*-sulphate in the cockroach. On the contrary, the data indicate that dopamine 3-*O*-sulphate is an important metabolite involved in the sclerotization of this insect's cuticle.

Sclerotization of insect cuticle is thought to involve a reaction between the oxidation products of *o*-dihydroxybenzene compounds and proteins (Pryor, 1940). Karlson's school has firmly established the existence of *N*-acetyldopamine in the larva of the fly, *Calliphora* (Karlson *et al.*, 1962; Sekeris & Karlson, 1962), and their evidence that this is the cuticular tanning agent is sound if not completely convincing. The presence of *N*-acetyldopamine has been reported in *Drosophila*, *Tenebrio*, *Schistocerca* and *Periplaneta* (Karlson & Herrlich, 1965; Sekeris & Herrlich, 1966; Murdock *et al.*, 1970), but on more tenuous evidence. However, the presence of relatively large amounts of dopamine 3-*O*-sulphate in newly ecdysed cockroaches, as reported in the present paper, suggests that the situation with respect to cuticular tanning may be more complex than originally envisaged by Karlson *et al.* (1962).

The radioactivity of ^{14}C -labelled dopamine 3-*O*-sulphate injected into newly ecdysed cockroaches is incorporated into the cuticle during sclerotization, and the extent of incorporation is large (at least 40%). However, less than 1% of the radioactivity of injected dopamine 3-*O*-[^{35}S]sulphate was recovered in the sclerotized cuticle, and this value is not appreciably different from that for the incorporation of radio-

activity from $\text{Na}_2^{35}\text{SO}_4$. On this basis it seems reasonable to conclude that little or none of the sulphate moiety of dopamine 3-*O*-sulphate is incorporated directly into the cuticle as the intact sulphate ester.

That the dopamine moiety of dopamine 3-*O*-sulphate, but not the sulphate moiety, becomes incorporated into cuticle structure during sclerotization leaves open the question of the biochemical function of this sulphate ester in the cockroach. It can be suggested that dopamine 3-*O*-sulphate is a sequestered form of dopamine, protected from indole formation by the presence of the 3-*O*-sulphate group, that might serve as a precursor (after hydrolysis) of the presumed sclerotizing agent, *N*-acetyldopamine. A somewhat analogous situation appears to exist in *Drosophila*. Here, tyrosine *O*-phosphate is accumulated by growing larvae and represents a sequestered form of tyrosine, which is utilized for sclerotization of the puparium. During sclerotization, tyrosine *O*-phosphate is hydrolysed and the released tyrosine is converted into dihydroxyphenylalanine, dopamine and *N*-acetyldopamine (Mitchell & Lunan, 1964; Lunan & Mitchell, 1969). By contrast, however, recent studies in this laboratory (R. P. Bodnaryk & P. C. J. Brunet, unpublished work) have revealed that dopamine 3-*O*-sulphate is not hydrolysed before further metabolism in *P. americana* and that in fact *N*-acetylation first occurs.

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