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Studies on the Sulphation of 3,4-Dihydroxyphenylethylamine (Dopamine) and Related Compounds by Rat Tissues

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The formation of sulpho-conjugates of 3,4-dihydroxyphenylethylamine (dopamine) and related compounds was examined in preparations of rat tissues. Liver high-speed-supernatant preparations readily transferred sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to dopamine under standard conditions. The main product was identified as the 3-*O*-sulphate. The preparation also sulphated the 3- and 4-methoxy derivatives but to a lesser extent (44% and 95% respectively) relative to dopamine. Brain preparations possessed only half the activity of liver but formed both the 3- and 4-*O*-sulphates in the molar ratio of 1.7:1. L-3,4-Dihydroxyphenylalanine (L-dopa) in both tissue preparations did not yield any significant amount of sulpho-conjugate when the dopa decarboxylase present was inhibited. The sulphotransferase activity of preparations was doubled in the presence of dithiothreitol and it was concluded that L-tyrosine methyl ester sulphotransferase was the enzyme involved. A method for the preparation of authentic dopamine 3-*O*-sulphate and 4-*O*-sulphate was developed.

Studies on the metabolism of ^{14}C-labelled L-3,4-dihydroxyphenylalanine (L-dopa) in the rat (Gey & Pletscher, 1964) indicated that an appreciable proportion of the amine fraction extractable from the tissues and representing mainly dopamine, was present in a sulpho-conjugated form. More recent studies (Goodall & Alton, 1968, 1972; Imai *et al.*, 1972) have shown that metabolism of ^{14}C-labelled 3,4-dihydroxyphenylethylamine (dopamine) and L-dopa in human subjects is followed by the excretion of considerable amounts of sulpho-conjugated metabolites, including a sulpho-conjugate of dopamine. These observations clearly demonstrate that this group of catecholamines readily become sulpho-conjugated in the mammalian system *in vivo*, but their ability to accept sulphate in the biological sulphating system of the cytosol *in vitro* has never been fully investigated. Moreover, the precise identity and metabolic role of the sulpho-conjugates detected in tissues and urine remains obscure.

In view of the influence of the metabolic pathways of L-dopa and dopamine on the effectiveness of these compounds in the treatment of Parkinson's disease (see Calne & Sandler, 1970), a detailed investigation of the formation of sulpho-conjugates of these compounds in the mammalian system was undertaken. This study has demonstrated that sulphation of dopamine and various derivatives readily occurs in rat liver and probably represents a significant metabolic pathway for these catecholamines.

Materials and Methods

Materials

Chemicals. L-3,4-Dihydroxyphenylalanine (L-dopa), 3,4-dihydroxyphenylethylamine (dopamine) hydrochloride and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Koch–Light Ltd., Colnbrook, Bucks., U.K. 4-Hydroxy-3-methoxybenzoic acid (vanillic acid), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), 3-methoxydopamine hydrochloride, pyridoxal 5′-phosphate and bovine serum albumin were obtained from Sigma (London) Chemical Co. Ltd., Kingston, Surrey, U.K. Dithiothreitol (Cleland's reagent) and 2,6-dichloroquinone-*p*-benzoquinone-4-chloroimine (Gibbs's reagent) were from BDH Chemicals Ltd., Poole, Dorset, U.K. 2,5-Diphenyloxazole was supplied by Beckman Instruments Inc., Palo Alto, Calif., U.S.A. The inhibitor N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine (RO 4-4602 or STH) and 4-methoxydopamine hydrochloride were gifts from Roche Products Ltd., Welwyn Garden City, Herts., U.K. *p*-Nitrophenol, obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., was recrystallized twice from water before use. Adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate was prepared by the method described by Mattock & Jones (1970). All other chemicals were AnalaR grade.

Animals. Young male and female Medical Research Council hooded rats (200–250g body wt.) were used throughout.

Methods

Sulphotransferase preparations from liver and brain. Rats were killed by a blow on the head and the tissues were quickly excised and chilled. After passage through a fine wire mesh to remove connective tissue, the pulp was weighed and homogenized as a suspension (liver 20%, w/v; brain 30%, w/v) in 0.25M-sucrose with the aid of a Tri-R homogenizer (Tri-R

International Co. Ltd., Rockville Centre, N.Y., U.S.A.; clearance between tube and Teflon pestle, 0.19mm) rotating at 4000rev./min, the suspension being forced past the pestle six times during 2min. The homogenate was then centrifuged at 100000$g_{av.}$ for 1h in the Spinco model L preparative ultracentrifuge in the no. 40 rotor. The clear supernatant was carefully removed from beneath the surface lipid layer and was either used on the same day or stored in 1ml portions at −20°C until required.

Paper chromatography and paper electrophoresis. Whatman no. 1 filter paper was used throughout. Development (descending) of chromatograms was with one of the following systems: A, butan-1-ol–acetic acid–water (50:12:25, by vol.); B, isobutyric acid–aq. 0.3M-NH_3 (5:3, v/v); C, 0.1M-Na_2CO_3–ethanol–aq. NH_3 (8:1:1, by vol.); D, propan-2-ol–0.1M-Na_2CO_3–aq. NH_3 (5:4:1, by vol.). Development was at 15°C for 16h (A, B and D) or 5h (C). Horizontal electrophoresis was performed at room temperature in 0.1M-$NaHCO_3$–Na_2CO_3 buffer, pH10, for 3h at a potential gradient of 10V/cm. Compounds were located on the paper by viewing under u.v. light and by spraying with 0.25% ninhydrin in acetone, followed by heating at 105°C for 5min. The 3- and 4-*O*-sulphate isomers gave red and pink spots respectively. Dopamine 4-*O*-sulphate could also be readily detected as a bright-blue spot by spraying with 1% (w/v) Gibbs's reagent in ethanol and then exposing the dried chromatogram to NH_3 vapour. Dopamine 3-*O*-sulphate gave no colour with this reagent. Radioactively labelled materials were also located by scanning or by radioautography as described.

Determination of sulphate ester formation. The composition of the reaction mixture was based on that used by Barford & Jones (1971*a*) and consisted of 10μl of enzyme preparation, 50μl of 0.3mM-adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate, 100μl of 0.1M-Tris–HCl buffer, pH7.5, containing the acceptor substrate and in some cases 0.48μmol of dithiothreitol. All reaction mixtures were incubated for 30min at 37°C and the reaction was terminated by placing the tubes in boiling water for 1min. Control determinations were made by adding the acceptor solution immediately before stopping the reaction. Chromatography or electrophoresis was carried out on 15μl portions of the supernatant fluid separable from the coagulated protein by centrifuging for 10min at 2000*g*. In experiments on the inhibition of dopa decarboxylase, the inhibitor was added to 10μl of enzyme in 50μl of the buffer containing 0.24μmol of dithiothreitol. After a 95min preincubation at 37°C the adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate solution and the acceptor (in 50μl of buffer containing 0.24μmol of dithiothreitol) were added and incubation was then continued as described above.

Detection and measurement of radioactivity. Radioactive components were located and measured quantitatively on dried chromatograms by the method of Jones & Dodgson (1965) with a Packard model 7200 radiochromatogram scanner. Radioautography was performed by exposing dried chromatograms to X-ray film (Ilford Industrial B) for up to 10 days. The radioactivity of solutions was determined by adding 100μl samples to 10ml of a scintillation mixture consisting of 2,5-diphenyloxazole (7g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.7g) dissolved in toluene–methyl oxitol (4:1, v/v; 1 litre) and counting in the Packard Tri-Carb model 3375 liquid-scintillation spectrometer.

Protein determination. This was done by the biuret method with crystalline freeze-dried bovine serum albumin as the standard (Gornall *et al.*, 1949).

Gibbs's test. A sensitive qualitative test for the detection of a free phenolic hydroxyl group situated *para* to an unsubstituted position in the aromatic nucleus was performed by mixing small quantities of the compound with 2,6-dichloroquinone-*p*-benzoquinone-4-chloroimine in alkaline solution according to the instructions of Gibbs (1927). Such compounds give an intense blue in this test.

Experimental and Results

Preparations

Dopamine O-monosulphates. The sulpho-conjugate of dopamine, which was detected in urine by Goodall & Alton (1968) after administration of the amine, was claimed to be the *O*-monosulphate. However, these workers did not attempt to identify the metabolite absolutely. Preliminary studies on the sulphation of dopamine *in vitro* in these laboratories also indicated that *O*-monosulphates were formed. It was therefore necessary to obtain synthetic authentic samples of these compounds to establish the identity of these products. Although dopamine 3-*O*-sulphate has been prepared by definitive synthesis (Hagedüs, 1963) the method is tedious and the yield is poor. A method for the preparation of dopamine 3- and 4-*O*-sulphate was therefore developed.

Dopamine 3- and 4-O-sulphate. Dopamine hydrochloride (0.4g, 2.1mmol) that had been kept *in vacuo* over P_2O_5 for 24h was added with stirring over 20min to 1.1ml (20.6mmol) of H_2SO_4 (sp.gr. 1.86) maintained at 0°C throughout. The reaction mixture was then quickly poured with vigorous stirring into 10ml of water–crushed ice mixture and the resulting solution was applied immediately to a column (20cm × 1.5cm) of Dowex 50 (X8, 200–400 mesh, H^+ form; Dow Chemical Co., Mich., U.S.A.) and eluted with water. The first portion of the eluate (approx. 37ml), which was strongly acidic (below pH3) and contained inorganic sulphate, was rejected. The next portion (approx. 50ml) was collected and the volume decreased to 10ml *in vacuo* at 30°C. Storage of the

resulting solution in a frozen state for 24h and subsequent thawing yielded a crop of white crystals of dopamine 4-*O*-sulphate, which were filtered off at the pump, washed quickly with a little ice-cold water and recrystallized twice from water. The final product was washed with ethanol and ether and dried *in vacuo* over P_2O_5. Yield: 60mg (Found: ester SO_4^{2-}, 41.7; C, 41.3; H, 4.7; N, 6.2; $C_8H_{11}O_5NS$ requires ester SO_4^{2-}, 41.2; C, 41.2; H, 4.8; N, 6.0%).

The mother liquor from the first crop of dopamine 4-*O*-sulphate crystals was then worked up for the 3-*O*-sulphate isomer by first concentrating to 5ml *in vacuo* at 30°C and then applying to a column (30cm×1cm) of Dowex 1 anion-exchange resin (X8; 200–400 mesh; acetate form). The column was eluted with 0.2M-acetic acid at a flow rate of 0.5ml/min, the eluate being monitored at 275nm. The fraction containing the dopamine 3-*O*-sulphate (560–780ml) was taken to dryness *in vacuo* at 30°C, final traces of acetic acid being removed by adding 50ml of ethanol to the residue and evaporating to dryness as before. The residue was then recrystallized twice from hot ethanol–water mixture (4:1, v/v), the crystals being washed finally with ethanol and ether and dried *in vacuo* over P_2O_5. Yield: 50mg (Found: ester SO_4^{2-}, 43.4; C, 41.1; H, 4.5; N, 5.7; $C_8H_{11}O_5NS$ requires ester SO_4^{2-}, 41.2; C, 41.2; H, 4.8; N, 6.0%).

Ultraviolet and infrared spectra

The u.v.-absorption spectra of the compounds were determined in 0.1M-HCl (3-*O*-sulphate, $\epsilon_{276} = 1900$; 4-*O*-sulphate, $\epsilon_{274} = 2100$ litre·mol^{-1}·cm^{-1}) and in 0.1M-NaOH (3-*O*-sulphate, $\epsilon_{296} = 3600$; 4-*O*-sulphate, $\epsilon_{292} = 4000$ litre·mol^{-1}·cm^{-1}) by using a Pye-Unicam SP. 800 recording spectrophotometer. The spectra were consistent with the existence of one free hydroxyl group. I.r. spectra were determined by using Nujol mulls of the compounds and KBr discs on the Perkin–Elmer Infracord recording spectrometer and were consistent with the existence of a single sulphated phenolic hydroxyl group in each case.

Determination of the absolute structure of dopamine 3- and 4-O-sulphate

The esters were identified by subjecting them to exhaustive methylation, Hofmann degradation, permanganate oxidation and acid hydrolysis sequentially. This treatment converted the 4-*O*-sulphate and the 3-*O*-sulphate isomers respectively into vanillic acid and isovanillic acid, which were identified chromatographically.

To 1.5ml of methanol was added 25mg of the ester sulphate and 100mg of Ag_2O. Methyl iodide (100μl) was then added dropwise with shaking and the mixture was refluxed for 3h. After the solvent was evaporated off, 5ml of 2M-NaOH was added and the mixture was refluxed for a further 20min. Finely powdered $KMnO_4$ (17mg) was then added and the flask was warmed and shaken until the $KMnO_4$ colour had disappeared. The brown precipitate that formed was filtered off at the pump, washed well with water and the combined pale-yellow filtrates were then brought to pH1 with HCl and refluxed for 1h to hydrolyse the ester sulphate. The solution was saturated with NaCl and the vanillic acid derivative extracted by shaking with three separate 10ml portions of ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4, concentrated to 1ml and chromatographed in solvent system A. Samples of authentic vanillic acid and isovanillic acid were also chromatographed. Compounds were located on the paper by spraying dried chromatograms lightly on both faces with diazotized *p*-nitroaniline reagent (Mathieu & Revol, 1967). The single purple azo-derivative obtained from the 4-*O*-sulphate and 3-*O*-sulphate ester corresponded to vanillic acid (R_F 0.86) and isovanillic acid (R_F 0.82) respectively. To establish the identity of these derivatives still further, the areas of the chromatograms corresponding to the coloured azo-derivatives were then eluted from the paper with methanol. After concentration *in vacuo* the products were chromatographed together with azo-derivatives of authentic vanillic acid and isovanillic acid in solvent systems C and D. The mobility of the azo-derivative obtained from the 4-*O*-sulphate and 3-*O*-sulphate esters corresponded exactly with the azo-derivatives of vanillic acid (R_F: system C, 0.17; system D, 0.84) and isovanillic acid (R_F: system C, 0.43; system D, 0.57).

L-*Dopa and dopamine as sulphate acceptors*

Preliminary experiments in which various concentrations of L-dopa (0.50–6.25mM) were incubated with adenosine 3′-phosphate 5′-[^{35}S]sulphatophosphate and rat liver preparations resulted in the formation of only one major ^{35}S-labelled component, having a chromatographic mobility identical with that of dopamine 3-*O*-sulphate in the two solvent systems used (R_F: system A, 0.39; system B, 0.45). This component did not appear when the enzyme preparation was preincubated in the presence of N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine, a specific inhibitor of L-dopa decarboxylase. Further, addition of 10μM-pyridoxal 5′-phosphate, a cofactor of dopa decarboxylase, to the enzyme preparation, resulted in a twofold increase in the amount of sulphated product formed. These results suggested that dopamine produced by decarboxylation of dopa was the true acceptor in the sulphation experiments (Table 1).

In support of this conclusion, on incubating various concentrations of dopamine with the rat liver enzyme, the same single major component, chromatographic-

Table 1. *Sulphation of* L-*dopa and dopamine by rat liver supernatant in the presence and absence of* L-*dopa decarboxylase cofactor and inhibitor*

Experimental details are given in the text. Dithiothreitol was included in the systems used for all determinations. STH is N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine hydrochloride.

Acceptor (1.6mM)	Cofactor or inhibitor (final concn. 10μM)	Preincubation period (min)	Sulphate transferred (nmol of ^{35}S/h per mg of protein)	Sulphation relative to dopamine 3-*O*-[^{35}S]-sulphate formation (%)
Dopamine	—	0	75.1	100
L-Dopa	—	0	15.9	21
Dopamine	Pyridoxal 5'-phosphate	0	65.8	88
L-Dopa	Pyridoxal 5'-phosphate	0	27.4	37
Dopamine	—	95	32.9	44
L-Dopa	—	95	1.5	2
Dopamine	Inhibitor (STH)	95	35.3	47
L-Dopa	Inhibitor (STH)	95	0	0

ally identical with the 3-*O*-sulphate, was formed. Preincubation of the enzyme with the inhibitor of dopa decarboxylase and addition of pyridoxal 5'-phosphate as before, had no effect (Table 1).

Several minor radioactive components, one of them having a mobility coincident with that of the authentic 4-*O*-sulphate isomer, were detectable on chromatograms but these were present in small amounts only relative to the major product (less than 1%). A comparison of the activity of the enzyme preparation towards dopamine and *p*-nitrophenol, a recognized substrate for phenolsulphotransferase (Mattock & Jones, 1970; Barford & Jones, 1971*a*), showed that the former is the better sulphate acceptor. The presence of 3mM-dithiothreitol in the incubation mixture increased the amount of product formed from dopamine by 134%, whereas the effect on the formation of *p*-nitrophenyl sulphate when *p*-nitrophenol was the acceptor was very much less (35%). It also simultaneously decreased the tendency of the catecholamine to oxidize.

The results indicated that the enzyme responsible for the sulphation of dopamine might be the rat liver L-tyrosine methyl ester sulphotransferase described by Mattock & Jones (1970). A sample of this enzyme (supplied by J. G. Jones of this department) was indeed found to sulphate dopamine to give exactly the same product as formed with the high-speed supernatant.

Identification of the product of dopamine sulphation as dopamine 3-*O-sulphate*

Isotope-dilution experiment. An incubation mixture prepared as described above but with tenfold quantities of reactants and in the presence of 3mM-dithiothreitol was incubated at 37°C for 1h. After the reaction was stopped and the protein precipitated by immersing in boiling water for 1min, 1ml of the supernatant was applied as 15μl spots to chromatography paper, followed by development with solvent system A. To determine the precise localization of the product on the paper, the dried chromatograms were subjected to radioautography for 2 days. The areas corresponding to the major product were cut from the chromatogram and eluted with water. The eluate was filtered and evaporated to 1ml *in vacuo* at 30°C. After the purity of the product had been checked by re-chromatographing in solvent systems A and B, 50mg of pure authentic unlabelled dopamine 3-*O*-sulphate was added. The solution was evaporated to dryness *in vacuo* at 30°C, and the crystals were then added to 1ml of hot ethanol and hot water was added dropwise with stirring until solution was complete. After cooling, the crystals were separated by centrifuging and dried *in vacuo* over P_2O_5. A sample (2mg) was retained and the remainder was recrystallized a further five times, a 2mg sample being retained after each recrystallization. The specific radioactivity of the samples was determined by liquid-scintillation counting of 100μl portions of a solution prepared by dissolving 2mg of the material in 500μl of 0.1M-NaOH. The specific radioactivity of the samples was constant within experimental error, indicating that the ester was indeed the 3-*O*-sulphate isomer.

Chromatography on Dowex 1. Confirmatory evidence that this conclusion was correct was obtained by chromatography of the product on Dowex 1 resin. The product remaining after the final recrystallization in the isotope-dilution experiment (18mg) was dissolved in 5ml of water and to this solution was added 10mg each of the authentic dopamine 3- and 4-*O*-sulphate. The material was then passed through the Dowex 1 ion-exchange column exactly as described for the preparation of the authentic esters. The eluate was monitored for absorption at 275nm and for

radioactivity by scintillation counting of 100μl samples of the 10ml fractions. The elution pattern of the radioactivity coincided exactly with that of the authentic dopamine 3-*O*-sulphate and was well separated from the peak that gave a positive reaction with Gibbs's reagent corresponding to the 4-*O*-sulphate isomer, which emerged later. The identity of the component as the 3-*O*-sulphate was also confirmed by electrophoresis.

3-Methoxydopamine and 4-methoxydopamine as sulphate acceptors

The 3- and 4-methoxydopamine hydrochlorides were compared with dopamine hydrochloride as sulphate acceptors by using the liver sulphotransferase preparation. A single ^{35}S-labelled product was formed with both of the methoxy derivatives but whereas the sulphation of the 4-methoxy compound occurred to almost the same extent as dopamine itself, the 3-methoxydopamine was sulphated to less than half this value (Table 2). Sulphation in the 4-position by liver preparations can therefore occur but this is not appreciable unless the 3-position is blocked.

Dopamine sulphation in rat brain preparations

The ability of dopamine to undergo sulphation was determined by using whole homogenate and high-speed-supernatant preparations of rat brain, under the conditions described above, in the presence of dithiothreitol. Only the supernatant fraction possessed any appreciable sulphotransferase activity, but this was approximately half that of liver on a protein basis. Moreover, significant amounts of two products were formed in these experiments. The major one (R_F 0.39, solvent A), corresponded exactly to dopamine 3-*O*-sulphate, whereas the minor one (R_F 0.35, solvent A) had the same mobility as the dopamine 4-*O*-sulphate (Table 3). The identity of the products as the 3- and 4-*O*-sulphate esters of dopamine was confirmed by subjecting the reaction mixture to chromatography on Dowex 1 anion-exchange resin in the presence of the unlabelled authentic esters as described above. In this experiment the radioactivity emerged from the column coincidently with the 3- and 4-*O*-sulphate peaks and in approximately the same proportions, as determined directly by scanning paper chromatograms of the reaction mixture (3-*O*-sulphate, 73%; 4-*O*-sulphate, 27%).

The sulphation of dopamine was considerably decreased if dithiothreitol was omitted from the reaction mixture, even when freshly prepared supernatant was used. Storage of the supernatant under the conditions described above for liver led to a rapid loss of activity, which could not be restored by adding dithiothreitol as in the case of liver. Brain preparations were only feebly active towards *p*-nitrophenol and this activity was largely unaffected by the presence of dithiothreitol. When L-dopa was used as the sulphate acceptor, no radioactive products were formed.

Discussion

The results of this study strongly indicate that conjugation with sulphate may represent a significant aspect of the metabolic fate of L-dopa and its derivatives in the mammalian body, and the claims that the major circulating form of dopamine may be a sulphoconjugate (Imai & Tamura, 1970) seem to receive

Table 2. *Sulphation of 3- and 4-methoxydopamine by rat liver supernatant*

Experimental details are given in the text. Dithiothreitol was included in the systems used for all determinations.

Acceptor (6.2mM)	Sulphate transferred (nmol of ^{35}S/h per mg of protein)	R_F of product (solvent A)	Sulphation relative to dopamine 3-*O*[^{35}S]sulphate formation (%)
Dopamine	61.4	0.39	100
4-Methoxydopamine	58.6	0.41	95
3-Methoxydopamine	27.1	0.36	44

Table 3. *Sulphation of dopamine and p-nitrophenol by rat brain preparations*

Experimental details are given in the text. Dithiothreitol was included in the systems used for all determinations.

Acceptor	Concn. of acceptor (mM)	Sulphate transferred (nmol of ^{35}S/h per mg of protein)	Percentage of isomers formed	
			3-*O*-Sulphate	4-*O*-Sulphate
Dopamine	6.2	29.7	62.5	37.5
p-Nitrophenol	2.0	1.7	—	—

support. The finding that L-dopa itself does not apparently undergo any sulpho-conjugation, whereas dopamine readily does so, is not altogether surprising since a similar situation obtains with tyrosine and tyramine in the sulphating system of the liver cytosol (Segal & Mologne, 1959; Barford & Jones, 1971*a*). These observations add further support to the conclusions that the enzyme responsible for all these sulpho-conjugations is the L-tyrosine methyl ester sulphotransferase, first described by Mattock & Jones (1970). Barford & Jones (1971*b*) have in fact proposed that the physiological function of this enzyme is the sulphation of biogenic amines and the affinity of the enzyme for these substrates appears to be similar (W. N. Jenner, unpublished results).

The finding that the 3-hydroxyl rather than the 4-hydroxyl group is conjugated preferentially in rat liver at first sight appears to be the reverse of what might be expected from observations on the metabolism of dihydric phenols possessing a third substituent other than —OH (Dodgson & Williams, 1949; Dodgson *et al.*, 1950). Most of these conclusions related to conjugation with glucuronic acid, although Dodgson *et al.* (1955) confirmed that the rabbit metabolized 4-chlorocatechol to yield 2-hydroxy-4-chlorophenyl sulphate. It seems reasonable to expect that the nature of the substituent group might direct the position of conjugation in substituted dihydroxyphenols, but clarification of this problem must await further study of the sulphotransferase mechanism. Various workers have, however, assumed (without experimental evidence) that the catecholamine conjugates they have detected in the mammalian system and urine after the administration of L-dopa and dopamine were the 4-*O*-sulphates (Goodall & Alton, 1972). In view of our present results it will be interesting to identify these metabolites.

The formation of the 3-*O*-sulpho-conjugate of dopamine in the mammalian system may have a significant effect on the action of catechol *O*-methyltransferase (EC 2.1.1.a), which readily produces the 3-methoxy derivative of dopamine *in vivo*. Miyazaki *et al.* (1969) have observed that sulphation has a directing effect on the methylation of 2-hydroxyoestrone and have proposed that a similar situation may apply for the catecholamines. The formation of both the 3-*O*-sulphate and 4-*O*-sulphate in brain preparations may have some special significance since *O*-methylation of these amines is thought to represent a fundamental aspect of brain biochemistry.

It is noteworthy that both the 3-*O*-sulphate and 4-*O*-sulphate form internal salts carrying little electrical charge at physiological pH, thus resembling the *O*-sulphate ester of 5-hydroxytryptamine. This property might be expected to confer a greater facility for penetrating biological membranes when compared with the parent compound, but for 5-hydroxytryptamine this does not appear to be the case (Rose & Bleszynski, 1971).

The possibility remains 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, the function of which might be regulated through the action of the arylsulphate sulphohydrolase enzymes (EC 3.1.6.1).

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