Studies on the *meta* and *para* *O*-sulphation of the catechol compound 3,4-dihydroxybenzoic acid by rat liver sulphotransferase *in vitro*

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The enzymic *meta* and *para* *O*-sulphation of 3,4-dihydroxybenzoic acid was investigated *in vitro* with a dialysed high-speed supernatant from rat liver. The *O*-sulphated products were identified by comparison with the reference compounds. The chemical synthesis and identification of the reference *O*-sulphate esters is described in detail. The sulphotransferase activity of the dialysed supernatant from rat liver towards 3,4-dihydroxybenzoic acid was 580 pmol of 3-*O*-sulphate and 120 pmol of 4-*O*-sulphate formed/min per mg of protein at the optimal pH of 7.4. The *meta/para* ratio of *O*-sulphation was independent of pH, time of incubation, concentration of enzyme and presence of dithiothreitol. The *O*-sulphate esters of 3,4-dihydroxybenzoic acid were found to be good substrates for the arylsulphatase reaction at pH 5.6. The arylsulphatase activity of a dialysed preparation from rat liver was 4.0 nmol of 3-*O*- and 5.7 nmol of 4-*O*-sulphate ester hydrolysed/min per mg of protein, respectively. Arylsulphatase from *Helix pomatia* had an activity of 620 pmol of 3-*O*-sulphate and of 16.6 nmol of 4-*O*-sulphate ester hydrolysed/min per unit (μ mol/h) of sulphatase.

Conjugation with sulphate is an important route for the metabolism of catecholamines and related compounds in liver and brain tissue of various mammals (Sugden & Eccleston, 1971; Meek & Neff, 1973; Wong, 1978, 1979). From results of early studies (Dodgson & Williams, 1949; Dodgson *et al.*, 1955) it may be inferred that *O*-sulphation of catechol compounds occurs on the 4-hydroxyl group. This is in agreement with the view that the 3-hydroxyl group is subject to *O*-methylation (Axelrod *et al.*, 1958; Daly *et al.*, 1960). However, several recent studies have shown that dopamine (3,4-dihydroxyphenethylamine) is preferentially sulphated at the 3-hydroxyl group *in vitro* by rat liver and brain (Jenner & Rose, 1973) and *in vivo* in man (Jenner & Rose, 1974; Bronaugh *et al.*, 1975; Arakawa *et al.*, 1979). Apart from these results, nothing is known about the relative importance of 3-*O*-sulphation and 4-*O*-sulphation in catecholamine metabolism.

Sulphoconjugates are often considered as excretory products of catecholamine metabolism. However, the presence of arylsulphatases (EC 3.1.6.1) in liver and brain tissue points to additional roles for these metabolites, such as local and/or temporary inactivation, or participation in enzymic *O*-methylation, as suggested by Miyazaki *et al.* (1969) and Brooks & Horn (1971).

In view of the postulated influence of *O*-sulphation on the *O*-methylation of catechols, we

decided to investigate the 3-*O*- and 4-*O*-sulphation of 3,4-dihydroxybenzoic acid by a high-speed supernatant from rat liver. Our choice of this sulphate acceptor is justified by its suitability as a substrate for the catechol *O*-methyltransferase (EC 2.1.1.6) reaction, which enables us to study these enzyme reactions simultaneously. We also present in detail the chemical synthesis of the two *O*-sulphate esters of 3,4-dihydroxybenzoic acid. These compounds have been identified and quantified by reverse-phase paired-ion high-pressure liquid chromatography. Furthermore, the activity of arylsulphatase from a high-speed rat liver supernatant towards the *meta* and *para* *O*-sulphate esters of 3,4-dihydroxybenzoic acid is described.

Materials and methods

Chemicals

3,4-Dihydroxybenzoic acid from Aldrich, Beerse, Belgium, was recrystallized from water before use. Plates for preparative t.l.c. (catalogue no. 5717; silica gel 60 F₂₅₄; 2 mm thickness), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid and 2,6-dichloroquinone-4-chloroimide (Gibbs reagent) were purchased from Merck, Darmstadt, West Germany. DEAE-Sephadex A-25 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Arylsulphatase (S-9626, type H-1 from *Helix pomatia*, containing 19800 units/g; 1 unit will hydrolyse

1.0 μ mol of *p*-nitrocatechol sulphate/h at pH 5.0 at 37°C) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Adenosine 3'-phosphate 5'-phosphosulphate, more than 99% pure, was prepared enzymically and analysed as described earlier (Pennings *et al.*, 1978; Pennings & Van Kempen, 1979). All other chemicals were of analytical grade.

High-pressure liquid chromatography

All analyses were carried out on a Waters Associates (Milford, MA, U.S.A.) liquid chromatograph consisting of a M-6000A pump, a model U6K injector and a model 440 absorbance detector, capable of monitoring at 254 nm. Solvent system A, for chromatography of the sulphate esters, consisted of 3 mM-tetrabutylammonium perchlorate as the pairing agent in 0.05 M-sodium acetate in methanol/water (1:4, v/v), adjusted to pH 6.5 with 1 M-acetic acid. Solvent system B, for chromatography of the hydrolysis products, consisted of 0.05 M-acetic acid in methanol/water (1:4, by vol.), final pH 3.2. All separations were carried out at room temperature on a LiChrosorb 10 RP 18 stainless-steel column (0.46 cm $\times$ 25 cm; Chrompack, Middelburg, The Netherlands) at a flow rate of 2 ml/min. Peak areas were measured with an analogue integrator.

The amounts of products formed by the various reactions were always calculated from relative calibration curves (Brown, 1973).

Preparation of enzymes

Adult Wistar rats (200–300 g) of either sex were used.

A high-speed supernatant in iso-osmotic KCl was made from rat liver by the method of Brunngraber (1958) and then dialysed at 4°C for 16 h against 200 vol. of 5 mM-Tris/HCl buffer, pH 7.4, containing 0.5 mM-dithiothreitol. Precipitated material was removed by centrifugation. The resulting enzyme preparation was used for the sulphotransferase reaction.

For the preparation of arylsulphatase enzymes a rat liver high-speed supernatant in water was prepared in the same way. The supernatant was dialysed at 4°C for 30 h against 100 vol. of 5 mM-sodium acetate buffer, pH 5.6. The dialysis residue obtained after removal of precipitated material by centrifugation was used for the arylsulphatase reaction.

Assay of sulphotransferase activity

The standard incubation mixture (1 ml; final pH 7.4) consisted of: 0.1 M-Tris/HCl buffer, pH 7.4; 0.5 mM-adenosine 3'-phosphate 5'-phosphosulphate; 1 mM-dihydroxybenzoic acid; 3 mM-dithiothreitol; 1 mM-MgCl₂; 37 mM-KH₂PO₄ adjusted with KOH to

pH 7.4 and 0.25 ml of the crude enzyme preparation, containing 3–4 mg of protein.

The reaction, carried out at 37°C for 30 min, was started by addition of enzyme and terminated by placing the tubes in boiling water for 2 min. After cooling and centrifugation, the total supernatant was applied to a column (0.4 cm $\times$ 8 cm) of DEAE-Sephadex A-25 (Cl⁻ form). The column was washed with 3 ml of water, 3.5 ml of 0.075 M-HCl and 2 ml of 1 M-HCl. The *O*-sulphated products were eluted with 4 ml of 1 M-HCl. After addition of 4-hydroxybenzoic acid as the internal standard, the 3-*O*- and 4-*O*-sulphated products were identified and quantified by high-pressure liquid chromatography in solvent system A. In the blanks, prepared by adding either the sulphate donor or the sulphate acceptor after termination of the reaction, no *O*-sulphated products were formed. Recoveries were determined by adding known amounts of *O*-sulphated products to blanks before and after the incubation.

Assay of arylsulphatase activity

Total arylsulphatase activity in rat liver supernatant was determined according to the instructions of Worwood *et al.* (1973) with the exception that we used a different type and concentration of substrate. The standard assay mixture (1 ml, final pH 7.4) contained: 0.25 M-sodium acetate buffer, pH 5.6; 2 mM of the 3-*O*- or 4-*O*-sulphate ester of 3,4-dihydroxybenzoic acid and 100 μ l of the dialysed rat liver supernatant, containing 0.8 mg of protein. For blanks, enzyme was added after incubation. Recoveries were determined by adding known amounts of 3,4-dihydroxybenzoic acid to blanks. Incubation was for 10 min at 37°C. The reaction was stopped by placing the tubes in boiling water for 2 min. The supernatant obtained after cooling and centrifugation was applied to a column (0.4 cm $\times$ 8 cm) of DEAE-Sephadex A-25 (Cl⁻ form). The column was washed with 3 ml of water and then with 1.0 ml of 0.075 M-HCl. The sulphohydrolysed product was eluted with 3 ml of 0.075 M-HCl and, after addition of 4-hydroxybenzoic acid as the internal standard, was measured by high-pressure liquid chromatography in solvent system B.

Arylsulphatase activities towards the 3-*O*- and 4-*O*-sulphate esters were also tested in an enzyme preparation from *Helix pomatia*. Incubation was with 250 μ g (4.95 units of enzyme). Other conditions were unchanged.

Gibbs test

The products obtained by the persulphate oxidation method, as described below, were chromatographed on silica gel in butan-1-ol/acetic acid/water (12:3:5, by vol.). After drying, the chromatograms were sprayed with 1% (w/v) Gibbs reagent in

ethanol and then exposed to NH_3 vapour. In this way, a free phenolic hydroxyl group situated *para* to an unsubstituted position can be detected by the appearance of an intense blue colour (Jenner & Rose, 1973).

Determination of protein

This was done by the method of Lowry *et al.* (1951), with horse serum albumin (Rijks Instituut voor de Volksgezondheid, The Netherlands) as a standard.

Experimental and results

Chemical synthesis of the 3-O- and 4-O-sulphate esters of 3,4-dihydroxybenzoic acid

Preliminary studies indicated that treatment of 3,4-dihydroxybenzoic acid with pyridine/ SO_3 (Roy & Trudinger, 1970) yielded a mixture of two sulphate esters. These could not be separated preparatively from each other by various chromatographic procedures, including anion-exchange chromatography and t.l.c. Therefore, a different approach to their synthesis was used. The persulphate oxidation of phenols (Elbs, 1893; Smith, 1951) is known to give O-sulphate esters as intermediates. Although the yields were low (2–5%), this method was used in the present work as it offered a means of preparing the desired O-sulphate esters of 3,4-dihydroxybenzoic acid.

3-Hydroxy-4-sulphonyloxybenzoic acid. 3-Hydroxybenzoic acid (17g) was set aside at room temperature in 500ml of water containing KOH (35g) and $\text{K}_2\text{S}_2\text{O}_8$ (35g). After 48h, the dark-coloured solution was acidified to pH 4 with 2M- H_2SO_4 and the free phenol was extracted with diethyl ether. The aqueous solution was neutralized and evaporated *in vacuo*. The residue was extracted four times with 50ml of methanol. The resulting methanolic solution was concentrated to 10ml *in vacuo* and partially purified by preparative t.l.c. on silica gel (in methanol/chloroform, 8:1 v/v). The material in the zone with R_F 0.7–0.9 was eluted with water. From the resulting solution the required sulphate ester could be purified on a column (4cm $\times$ 22cm) of DEAE-Sephadex A-25. The column was eluted with 0.3M-triethylammonium hydrogen carbonate buffer, pH 7.5, at a flow rate of approx. 20ml/h. The desired product was eluted after approx. 3 litres of buffer had passed through the column. After evaporation of solvent, the product was stored at 4°C.

4-Hydroxy-3-sulphonyloxybenzoic acid. This product was synthesized in the same way from 4-hydroxybenzoic acid. After preparative t.l.c., the product (R_F 0.7–0.9) was eluted with methanol. It appeared to be homogeneous by high-pressure liquid chromatography in solvent system A. The solution

was evaporated to a few ml *in vacuo* and, after addition of 200ml of ethyl acetate, the white precipitate was collected, dried *in vacuo* over P_2O_5 , and stored at 4°C.

Both sulphate esters were highly hygroscopic and readily turned brown on exposure to air by absorption of water. For analytical purposes, concentrations were determined by measuring the amount of 3,4-dihydroxybenzoic acid liberated after acid hydrolysis for 45 min in 1M-HCl at 80°C, as described below.

Identification of the synthesized 3-O- and 4-O-sulphate esters of 3,4-dihydroxybenzoic acid

High-pressure liquid chromatography. The products obtained by the persulphate oxidation method were found to be homogeneous by high-pressure liquid chromatography in solvent system A. Furthermore, it was verified that these products co-chromatographed with both the products obtained by treatment of 3,4-dihydroxybenzoic acid with pyridine/ SO_3 . When the pairing agent, tetrabutylammonium perchlorate, was excluded from the solvent system, both products were eluted with the void volume, indicating their polar nature. Fig. 1 shows the optimal separation of the sulphate esters.

Acid hydrolysis. A small amount of either product was dissolved in 1ml of 1M-HCl and placed in a waterbath at 80°C. The rate of hydrolysis was followed qualitatively by chromatography of 5 μ l samples of the reaction mixture taken at 15 min intervals. In both cases there appeared only one product, at the expense of starting material, which co-chromatographed with authentic 3,4-dihydroxybenzoic acid. Hydrolysis of the sulphate esters was complete within 45 min.

Liberated 3,4-dihydroxybenzoic acid was quantified in solvent system B. An internal standard (4-hydroxybenzoic acid) was added before hydrolysis.

Enzymic hydrolysis. After incubation with arylsulphatase from rat liver or from *Helix pomatia*, both the sulphate esters yielded one and the same product, co-chromatographing with authentic 3,4-dihydroxybenzoic acid. Recovery of this product from the incubation mixture was 96%. A typical chromatogram is shown in Fig. 2.

The activity of arylsulphatase from rat liver was 4.0nmol of 3-O-sulphate ester and 5.7nmol of 4-O-sulphate ester hydrolysed/min per mg of protein. Neither sulphate ester appeared to be a specific substrate for arylsulphatase A, as determined by the method of Worwood *et al.* (1973). The specificity of the sulphate esters as substrates for arylsulphatase B cannot be studied with crude rat-liver enzyme preparations.

The activity of arylsulphatase from *Helix pomatia* was 620pmol and 16.6nmol of ester hydrolysed/min

Fig. 1. High-pressure liquid chromatography of the chemically synthesized *O*-sulphate esters of 3,4-dihydroxybenzoic acid

The esters were chromatographed in solvent system A on a LiChrosorb 10 RP 18 column. Chromatographic details are given in the Materials and methods section. Retention times for the internal standard 4-hydroxybenzoic acid (*a*), 3-hydroxy-4-sulphonyloxybenzoic acid (*b*) and 4-hydroxy-3-sulphonyloxybenzoic acid (*c*) were 3.0, 8.4 and 11.7 min, respectively.

per enzyme unit for the 3-*O*- and the 4-*O*-sulphate, respectively. With the latter substrate, the reaction went to completion within 25 min under standard assay conditions.

The enzymic hydrolysis was also followed qualitatively by direct injection into the high-pressure liquid chromatograph of 5 μ l samples of the assay mixture taken at various time intervals. In this way, the disappearance of sulphate ester and the concomitant appearance of 3,4-dihydroxybenzoic acid was verified.

Gibbs test. The product prepared from 3-hydroxybenzoic acid gave an intense blue colour with this reagent, whereas the isomeric product from 4-hydroxybenzoic acid gave no colour with this

Fig. 2. Determination of arylsulphatase activity by high-pressure liquid chromatography

Incubation was carried out with enzyme from rat liver and with 3-hydroxy-4-sulphonyloxybenzoic acid as the substrate. 3,4-Dihydroxybenzoic acid was determined in the DEAE-Sephadex eluate after a 7.5 μ l injection. The analysis was performed in solvent system B on a LiChrosorb 10 RP 18 column. Experimental details are given in the Materials and methods section. Retention times for 3,4-dihydroxybenzoic acid (*a*) and the terminal standard 4-hydroxybenzoic acid (*b*) were 3.6 and 6.6 min, respectively.

reagent. This indicated that the former product still contained an intact 3-hydroxy group.

U.v. absorption spectra. The u.v.-absorption maxima were determined in solvent system A (3-*O*-sulphate, ϵ_{246} 13800 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$; 4-*O*-sulphate, ϵ_{237} 7600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$). At 254 nm the molar absorption of the 3-*O*-sulphate ester was 4.3 times that of the 4-*O*-sulphate ester.

Sulphotransferase reaction

Recoveries of 4-hydroxy-3-sulphonyloxybenzoic acid and 3-hydroxy-4-sulphonyloxybenzoic acid from the incubation mixture were 93% and 90%, respectively. Loss of sulphate esters during the incubation amounted to 2–4%, which may be due to residual arylsulphatase activity.

The enzymically formed products co-chromato-

graphed with the corresponding chemically synthesized *O*-sulphate esters of 3,4-dihydroxybenzoic acid. In addition, these products yielded also 3,4-dihydroxybenzoic acid after chemical hydrolysis in 1M-HCl or enzymic hydrolysis with arylsulphatase from *Helix pomatia* or from rat liver. The activity of the rat liver sulphotransferase preparation was 580pmol of 3-*O*-sulphate and 120pmol of 4-*O*-sulphate formed/min per mg of protein. The *meta/para* ratio of *O*-sulphation was 4.8.

Effects of pH, time of incubation and enzyme concentration

The pH optimum of the sulphotransferase reaction was 7.4 in 0.1M-Tris/HCl buffer, the range studied being 6.7–8.5. Recoveries of products were constant throughout this range. The rate of the reaction was linear with time for 40min. During the sulphotransferase experiments we used saturating concentrations of the sulphate donor. This was done to avoid deviations from linearity that may result from degradation of the sulphate donor by other enzymes present in the rat liver supernatant. Dialysis of this enzyme preparation was necessary to obtain a linear relationship between the rate of the reaction and the concentration of enzyme up to the highest amount of enzyme studied, i.e. 8mg/ml. The activity was not decreased by this procedure, provided that dithiothreitol was included in the dialysis buffer. Omission of this thiol-specific reagent led to an approx. 50% loss of sulphotransferase activity without affecting, however, the *meta/para* ratio of *O*-sulphation. This ratio did not depend on pH, time of incubation nor enzyme concentration.

Discussion

It is often assumed that sulphoconjugation of dihydroxycatecholamine metabolites occurs preferentially at the 4-hydroxyl group. However, the present results and those of Jenner & Rose (1973) argue against this view. Although the *meta/para* ratio of *O*-sulphation may differ, both studies indicate that 3-*O*-sulphation predominates at physiological pH, apparently irrespective of the charge of the substituent side chain. It is therefore interesting to identify the site of sulphoconjugation of such metabolites as 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylene glycol and 3,4-dihydroxyphenylethanol in mammalian tissues.

The preferential 3-*O*-sulphation may have a significant effect on the action of catechol *O*-methyltransferase, which *O*-methylates catecholamine metabolites, likewise, primarily at the 3-hydroxyl group (Axelrod & Tomchick, 1958; Creveling *et al.*, 1972). Thus *O*-sulphation and *O*-methylation may be competing reactions in the mammalian system.

The experiments of Miyazaki *et al.* (1969)

suggested that *O*-sulphation might have a directing effect on *O*-methylation of catecholamine metabolites. Considering the present results and those of Jenner & Rose (1973), a preferential formation of 4-*O*-methylated metabolites would be expected. However, this interpretation of the role of *O*-sulphation is less likely, as 4-*O*-methylation is considered to give rise to toxic, or at least unwanted, metabolites and has been implicated in the etiology of mental disease (Marzullo & Friedhoff, 1979).

Apart from its traditional role as detoxification mechanism, the possibility remains that *O*-sulphation may give rise to transport forms of catecholamine metabolites in the mammalian system. The presence of arylsulphatases in many tissues supports such a view. The distribution of sulphotransferase activity among various rat organs also suggests that its role is not restricted to detoxification (Foldes & Meek, 1974). Interestingly, both sulphate esters of 3,4-dihydroxybenzoic acid were found to be good substrates for the arylsulphatase reaction.

Dialysis of the crude enzyme preparation was necessary to obtain a linear relationship between the rate of the sulphotransferase reaction and the concentration of enzyme. Apparently, low-molecular-weight inhibitors of sulphotransferase or activators of adenosine 3'-phosphate 5'-phosphosulphate breakdown are present in rat liver. We did not observe the presence of high-molecular-weight inhibitors. This contrasts with the findings of others (Davis, 1975; Anderson & Weinshilbourn, 1979). However, these authors used non-saturating concentrations of a less pure preparation of the sulphate donor adenosine 3'-phosphate 5'-phosphosulphate (approx. 0.04 and 0.4 μ M, respectively). When the concentration of crude enzyme is raised, this must invariably lead to an apparent inhibition, due to the presence of other enzymes that bind and/or destroy the sulphate donor. It is therefore stressed that saturating concentrations of the sulphate donor must be used for the assay of sulphotransferase activity in crude enzyme preparations to avoid these inhibitory effects.

The present study also describes a convenient method to measure the sulphoconjugates of 3,4-dihydroxybenzoic acid. The introduction of tetrabutylammonium perchlorate as a pairing agent in a reverse-phase high-pressure liquid-chromatographic solvent system increases the retention of such polar compounds as sulphate esters. The method can therefore be of general importance, and could be applicable to the assay of a number of endogenous sulphate esters in biological fluids.

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