Species Differences in the Conjugation of 4-Hydroxy-3-methoxyphenylethanol with Glucuronic Acid and Sulphuric Acid

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(Received 24 September 1975)

The biosynthesis of the glucuronide and sulphate conjugates of 4-hydroxy-3-methoxyphenylethanol was demonstrated *in vitro* by using the high-speed supernatant and microsomal fractions of liver respectively. These two conjugates were also produced simultaneously by using the post-mitochondrial fraction of rat, rabbit or guinea-pig liver. In contrast only the glucuronide was synthesized by human liver and only the sulphate by mouse and cat livers. Neither of these conjugates was formed by the kidney or the small or large intestine of the rat. A high sulphate-conjugating activity was observed in mouse kidney; the rate of sulphation of 4-hydroxy-3-methoxyphenylethanol with kidney homogenate and high-speed supernatant preparations was 1.8 times greater than with liver preparations. The sulpho-conjugates of 4-hydroxy-3-methoxyphenylethanol and 4-hydroxy-3-methoxyphenylglycol were also formed by enzyme preparations of rabbit adrenal and rat brain; the glycol was the better substrate in the latter system. Mouse brain did not possess any sulphotransferase activity. For the conjugation of 4-hydroxy-3-methoxyphenylethanol by rabbit liver, the K_m for UDP-glucuronic acid was 0.22 mM and that for Na_2SO_4 was 3.45 mM. The sulphotransferase has a greater affinity for 4-hydroxy-3-methoxyphenylethanol than has glucuronyltransferase, as indicated by their respective K_m values of 0.036 and 1.3 mM. It was concluded that sulphate conjugation of 4-hydroxy-3-methoxyphenylethanol predominates in most species of animals.

4-Hydroxy-3-methoxyphenylethanol is a metabolite of dopa (3,4-dihydroxyphenylalanine) and dopamine (3,4-dihydroxyphenethylamine) (Goldstein *et al.*, 1960). After administration of dopa or dopamine, 4-hydroxy-3-methoxyphenylethanol was found in the blood and central nervous system (Goldstein & Gerber, 1963), where inactivation was thought to occur by conjugation (Goldstein, 1964). It is present in normal human urine (Goldstein *et al.*, 1961) and may be quantitatively determined by g.l.c. (Karoum *et al.*, 1971; Braestrup, 1972). From these determinations, which include hydrolysis, it was inferred that the alcohol occurs in brain and urine as a conjugate, the nature of which is unknown. Studies with sulphotase and β -glucuronidase have shown that a sulphate conjugate of the alcohol predominates in human urine, whereas the rat excreted this compound mainly as a glucuronide (Karoum *et al.*, 1973). The sulphate conjugate was identified in rat brain after administration of $\text{Na}_2^{35}\text{SO}_4$ (Eccleston & Ritchie, 1973). The present study was undertaken to investigate the conjugation of 4-hydroxy-3-methoxyphenylethanol *in vitro* in different tissues and in different animal species.

Materials

4-Hydroxy-3-methoxyphenylethanol (homovanillyl alcohol, 99% pure) and 4-hydroxy-3-

methoxyphenylglycol (piperazine salt, 99% pure) were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. UDP-glucuronic acid (triammonium salt, 99% pure), ATP (sodium salt, 99% pure), D-glucuronic acid, D-glucuronolactone, β -glucuronidase (type B-1, prepared from bovine liver, containing 500 000 Fishman units per g of solid) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. UDP-[U- ^{14}C]glucuronic acid (specific radioactivity 290 or 302 mCi/mmol) and $\text{Na}_2^{35}\text{SO}_4$ (specific radioactivity 24 or 67 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks, U.K.

Methods

Preparation of enzymes

Adult male animals were used. The rats were of the albino Wistar strain, and the other animals were local hybrids. Their body weights were as follows: rat, 150–200 g; rabbit, 1–1.2 kg; mouse, 25–30 g; guinea pig, 200–300 g. A liver biopsy (150 mg) was obtained from a male patient (T.C.S.) aged 62, suffering from moderately differentiated adenocarcinoma of the descending colon. The biopsy was normal in appearance.

(a) *Glucuronyltransferase*. This enzyme was prepared by the procedure of Wong & Sourkes (1967). The microsomal pellet obtained after centrifugation

of the post-mitochondrial fraction at 105000g for 1 h was suspended in ice-cold 0.15M-KCl so that 1 ml of this suspension corresponded to 1 g fresh weight of tissue. This was subjected to overnight dialysis at 4°C against 10mM-EDTA (disodium).

(b) *Sulphate-activating and sulpho-transferring enzymes.* The supernatant obtained above is referred to as the high-speed supernatant; it contains these enzymes.

(c) *Post-mitochondrial fraction.* A 10% (w/v) homogenate of liver, kidney, adrenal or brain was prepared in cold 0.15M-KCl. Centrifugation of this homogenate at 15000g for 30min removed the mitochondria leaving the supernatant as the post-mitochondrial fraction.

Reaction conditions

(a) *Glucuronidation.* The incubation medium was the same as described previously (Wong, 1971), except for the aglycone, which was 4-hydroxy-3-methoxyphenylethanol in this case. The medium contained, in a final volume of 200 μ l, the following (final concentrations in parentheses): 4-hydroxy-3-methoxyphenylethanol (2mM); MgCl₂ (5mM); UDP-[U-¹⁴C]glucuronic acid (3.32 or 6.64 μ M) and/or unlabelled UDP-glucuronic acid (1mM) and 0.5M-Tris/HCl buffer, pH7.8. The reaction was started with 50 μ l of the rabbit liver microsomal preparation, containing 3.7mg of protein/ml. The protein content was determined by the procedure of Lowry *et al.* (1951).

(b) *Sulphation.* The procedure described for the sulphation of 4-hydroxy-3-methoxyphenylglycol (Wong, 1975) was followed, but 4-hydroxy-3-methoxyphenylethanol [³⁵S]sulphate was measured.

(c) *Simultaneous glucuronidation and sulphation.* For this, 50 μ l of the post-mitochondrial fraction of liver, kidney or brain was used. The incubation medium contained 4-hydroxy-3-methoxyphenylethanol (2mM), MgCl₂ (8mM) dissolved in dithiothreitol (3mM), 0.5M-Tris/HCl buffer (pH8.0), UDP-[U-¹⁴C]glucuronic acid (3.32 or 4.32 μ M), ATP (8mM) and Na₂³⁵SO₄ (0.65 or 3.1mM).

For all the three systems above, incubation was carried out at 37°C in a metabolic shaker, the times of incubation being 10 or 15min for glucuronidation, 30min for sulphation and 15 or 30min for simultaneous glucuronide and sulphate conjugations. The reactions were stopped by adding 50 μ l each of ZnSO₄ (10%, w/v) and Ba(OH)₂ (0.3M). The precipitate was removed by centrifugation, and 25 or 50 μ l of the supernatant was chromatographed on Whatman no. 1 paper (56cm $\times$ 1.2cm) by the descending technique in solvent A [propan-2-ol/NH₃/water (8:1:1, by vol.)] for preparations (a) and (c) and in solvent B [butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase)] for preparation (b). After the chromatogram had been developed, 2cm fractions

were counted for radioactivity in 5ml of scintillator containing 0.4% 2,5-diphenyloxazole and 0.025% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. The radioactivity of 4-hydroxy-3-methoxyphenylethanol [¹⁴C]glucuronide or 4-hydroxy-3-methoxyphenylethanol [³⁵S]sulphate or both were measured. Standards of Na₂³⁵SO₄ and UDP-[U-¹⁴C]glucuronic acid were chromatographed and similarly counted for radioactivity.

Hydrolysis of biosynthetic 4-hydroxy-3-methoxyphenylethanol glucuronide

The material contained in the radioactive peak thought to be 4-hydroxy-3-methoxyphenylethanol glucuronide was eluted from ten paper strips, each with radioactivity greater than 10000 c.p.m. The *R_F* value of this peak was not determined, as the solvent front had moved off the chromatogram. However, this conjugate was characterized as a peak at 19.5cm from the origin on the chromatogram developed by the descending technique at room temperature (29°C) in solvent A for 26h. One portion of the above eluate was incubated overnight at pH5.0 with bovine liver β -glucuronidase (5000 Fishman units). Another was boiled at 100°C for 1 h with 6M-HCl and the third was untreated. Then 50 μ l of each was chromatographed on paper and developed in solvent B. Standards of authentic 4-hydroxy-3-methoxyphenylethanol, D-glucuronic acid and D-glucuronolactone were chromatographed simultaneously. The free alcohol was located with Folin-Ciocalteu reagent (Waldi, 1965) or diazotized sulphanilic acid (Smith, 1960), and glucuronic acid and its lactone were detected with naphtharesorcinol reagent (Smith, 1960). To identify the alcohol released from biosynthetic 4-hydroxy-3-methoxyphenylethanol glucuronide, a large amount of the unlabelled conjugate was first produced and subjected to acid and enzymic hydrolysis. The hydrolysates were chromatographed on cellulose-coated t.l.c. plates in solvent B.

Results

Formation and hydrolysis of 4-hydroxy-3-methoxyphenylethanol glucuronide

4-Hydroxy-3-methoxyphenylethanol [¹⁴C]glucuronide was synthesized *in vitro* by the transfer of [¹⁴C]glucuronic acid from UDP-[U-¹⁴C]glucuronic acid to the alcohol. Under the same experimental conditions, 4-hydroxy-3-methoxyphenylglycol was also glucuronidated. The chromatographic procedure used for the separation of the glucuronide was used but the time of development was extended to 40h. The distance traversed by this glucuronide and UDP-glucuronic acid were 16.5 and 3.5cm respectively from the origin.

Treatment of unlabelled 4-hydroxy-3-methoxyphenylethanol glucuronide with β -glucuronidase

liberated 4-hydroxy-3-methoxyphenylethanol, which gave a purple coloration with the Folin reagent and orange coloration with diazotized sulphanilic acid. This aglycone has R_F 0.89 on a cellulose-coated t.l.c. plate developed in solvent B. Labelled glucuronic acid released from 4-hydroxy-3-methoxyphenylethanol [^{14}C]glucuronide after acid and β -glucuronidase hydrolysis was located as a radioactive peak with R_F 0.18 on a paper chromatogram developed in solvent B. This peak was coincident with that of authentic glucuronic acid. In the acid hydrolysate, another radioactive peak with R_F 0.41 was observed. This presumably contained glucuronolactone, which has the same mobility in this solvent system. Conceivably, lactonization had occurred as a result of boiling with HCl.

Kinetic data on the transglucuronidation of 4-hydroxy-3-methoxyphenylethanol by rabbit liver microsomal fractions

Effect of pH. The pH optimum for the transglucuronidation of 4-hydroxy-3-methoxyphenylethanol was 8.2, when 0.5M-Tris/HCl buffer was used.

Effect of time of incubation. The formation of 4-hydroxy-3-methoxyphenylethanol [^{14}C]glucuronide increased with time of incubation. Linear velocity was obtained for reaction up to 15 min.

Effect of enzyme concentration. There was increased formation of 4-hydroxy-3-methoxyphenylethanol [^{14}C]glucuronide with increasing concentration of enzyme.

Measurement of K_m of 4-hydroxy-3-methoxyphenylethanol. The rate of reaction over the concentration range 0.02–2 mM was determined. In one experiment, only radioactive UDP-glucuronic acid at a final concentration of 6.64 μM was used and in the other 1 mM unlabelled nucleotide was also added. The K_m values for 4-hydroxy-3-methoxyphenylethanol obtained from Lineweaver–Burk (1934) plots of the above sets of data were 1.33 and 1.25 mM respectively.

Measurement of K_m of UDP-glucuronic acid. At very low nucleotide concentration (0.42–16.6 μM), a linear relationship was established between the rate of formation of 4-hydroxy-3-methoxyphenylethanol glucuronide and the nucleotide concentration up to 3.3 μM . The addition of unlabelled UDP-glucuronic acid (0.01–1 mM) at a fixed concentration (3.32 μM) of radioactive UDP-glucuronic acid increased the transglucuronidation reaction progressively. A Lineweaver–Burk plot of the second set of data gave a K_m of 0.22 mM for UDP-glucuronic acid.

Formation and hydrolysis of 4-hydroxy-3-methoxyphenylethanol sulphate

The sulphate conjugate has R_F 0.48 on a paper chromatogram developed in solvent B. When the

hydrolysis studies carried out on 4-hydroxy-3-methoxyphenylglycol sulphate (Wong, 1975) were performed on labelled and unlabelled 4-hydroxy-3-methoxyphenylethanol sulphate, $\text{Na}_2^{35}\text{SO}_4$ and 4-hydroxy-3-methoxyphenylethanol were shown to be products of acid hydrolysis, indicating that the conjugate formed was 4-hydroxy-3-methoxyphenylethanol sulphate.

Kinetic data on the sulphation of 4-hydroxy-3-methoxyphenylethanol by rabbit liver

Measurement of K_m of 4-hydroxy-3-methoxyphenylethanol for the sulphotransferase reaction. The 'active' sulphate adenosine 3'-phosphate 5'-sulphatophosphate was first generated from $\text{Na}_2^{35}\text{SO}_4$ by using the high-speed supernatant of rabbit liver (Wong, 1975). The reaction was stopped by boiling. Before the second incubation in which $^{35}\text{SO}_4$ from adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate was transferred to 4-hydroxy-3-methoxyphenylethanol, another portion of the same enzyme preparation was added with introduction of EDTA to inhibit the sulphate-activating system (Brünngraber, 1958). The K_m for 4-hydroxy-3-methoxyphenylethanol obtained from the double-reciprocal plot was 0.036 mM.

Measurement of K_m of Na_2SO_4 . Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate formed from $\text{Na}_2^{35}\text{SO}_4$ by the high-speed supernatant preparation of rabbit liver was measured by the transfer of its sulphate to 4-hydroxy-3-methoxyphenylethanol and harmol (Wong, 1974). With both substrates, the K_m value for Na_2SO_4 was 3.45 mM.

Sulpho-conjugation of 4-hydroxy-3-methoxyphenylethanol by kidney, brain and adrenal

This was measured in the high-speed supernatant and 10% homogenate preparations; the rates of sulphation by mouse kidney were 1.8 and 1.84 times greater than the rates with liver preparations. From this, it was inferred that the sulpho-conjugating activity measured in the 10% homogenate reflects closely its activity in the high-speed supernatant preparation. Neither the rat nor rabbit kidney formed 4-hydroxy-3-methoxyphenylethanol sulphate. Of the other tissues examined, rat, but not mouse brain, and rabbit adrenal were able to conjugate 4-hydroxy-3-methoxyphenylethanol and 4-hydroxy-3-methoxyphenylglycol with sulphate, but the small and large intestines of rat did not show any activity. The amount of glycol sulphate formed by rat brain was 4.73 times more than 4-hydroxy-3-methoxyphenylethanol sulphate.

Simultaneous formation of 4-hydroxy-3-methoxyphenylethanol glucuronide and sulphate

The sulphation and glucuronidation was further

confirmed in a single reaction with crude 10% homogenate or post-mitochondrial fraction of liver of rat, rabbit or guinea pig. Of all the tissues tested, rat liver was the most suitable for this simultaneous reaction as it contained relatively high activity of both transferases. The chromatographic profile of Fig. 1 showed the complete separation of the glucuronide and sulphate peaks from their labelled precursors. It must be emphasized that the reaction conditions used here are not optimum for both reactions. Thus the conjugates formed simultaneously were not determined quantitatively. This system is, however, extremely suitable for studying the overall pattern of conjugation of 4-hydroxy-3-methoxyphenylethanol. Mouse and cat liver did not form any glucuronide, but the sulphate conjugate was produced in appreciable amounts. In contrast, a 10% homogenate of human liver showed formation of 4-hydroxy-3-methoxyphenylethanol glucuronide, but the sulphate conjugate was not produced. Thus the modes of conjugation of 4-hydroxy-3-methoxyphenylethanol by hepatic tissues differ in various animals.

Fig. 1. Radiochromatogram showing the biosyntheses of 4-hydroxy-3-methoxyphenylethanol [^{14}C]glucuronide (peak B) and 4-hydroxy-3-methoxyphenylethanol [^{35}S]sulphate (peak C) by rat liver

These peaks are separated from the labelled precursors UDP-[U- ^{14}C]glucuronic acid and $\text{Na}_2^{35}\text{SO}_4$ (peak A), both of which have the same mobility in solvent system A. The ordinates show the radioactivity (c.p.m.) per fraction of the chromatogram: left ordinate (—) and right ordinate (....). The abscissa shows the distance traversed in 26 h by radioactive compounds during descending chromatography.

Discussion

There is a distinct pattern of conjugation of 4-hydroxy-3-methoxyphenylethanol in different animals. The liver was the only organ capable of forming the glucuronide, but, in addition to liver, kidney, brain and adrenal of various animals could synthesize the sulphate conjugate. The high affinity of sulphotransferase for the glycol accounted for its presence exclusively as the sulphate conjugate in rat brain (Schanberg *et al.*, 1968). Mouse brain on the contrary is devoid of this activity, which explained the absence of the glycol sulphate from its cerebral tissue (Sharman, 1973). Whether the high renal activity in mouse facilitates the clearance of the glycol and alcohol remains to be studied.

In general, most animals seem to form the sulphate conjugate preferentially as far as the catecholamines and their metabolites are concerned. Measurements of the urinary concentrations of these compounds and studies *in vivo* tend to substantiate this generalization, e.g. the sulpo-conjugates of the following have been reported: adrenaline (Richter, 1940), dopamine (Jenner & Rose, 1973), 4-hydroxy-3-methoxyphenylglycol (Schanberg *et al.*, 1968), 4-hydroxy-3-methoxyphenylethanol (Goldstein *et al.*, 1960; Eccleston & Ritchie, 1973), dihydroxyphenylglycol (Eccleston & Ritchie, 1973) and 3-O-methyl-adrenaline and -nor-adrenaline (Sharman, 1973). An analysis of the sulphate and glucuronide values in these references showed that in most cases the former predominates. In this study, rabbit hepatic sulphotransferase has 36 times greater affinity for 4-hydroxy-3-methoxyphenylethanol than does glucuronyltransferase. Thus when the concentration of this substrate is limiting, sulphation would undoubtedly be the preferential route of conjugation of this alcohol. With harmol, an exogenous substrate, the sulphate conjugate was also formed more readily *in vivo* (Mulder & Hagedoorn, 1974) and *in vitro* (Mulder, 1975).

The significance of the sulpo-conjugates of 4-hydroxy-3-methoxyphenylethanol and 4-hydroxy-3-methoxyphenylglycol in brain metabolism deserves further investigation. The metabolic sequence proposed for these neutral metabolites of the catecholamines, i.e. their passage out of the brain with subsequent peripheral conjugation and final return to the brain (Taylor & Laverty, 1969), may occur in the mouse, but it is probably of secondary importance in the rat whose cerebral tissue possesses the entire sulpo-conjugatory machinery (Wong, 1975). This alcohol is believed to be of low significance in brain metabolism (Braestrup, 1973) and it is present in very low concentration in brain and cerebral-spinal fluid (Karoum *et al.* 1971; Braestrup, 1972). As peripheral tissues could conjugate this alcohol with glucuronic acid and/or sulphuric acid, it would be interesting to find out if these conjugates participate as active

metabolic intermediates, both centrally and peripherally. In particular, the fates of the sulphates of 4-hydroxy-3-methoxyphenylethanol and 4-hydroxy-3-methoxyphenylglycol in brain metabolism should be examined since the latter was found in rat brain exclusively in this form.

I thank Miss Theresa Yeo Huay Cheng for her skilful and diligent technical assistance and the Wellcome Trust for financial support. I also thank Mr. Ho Soon Teck of the Department of Surgery, University of Singapore, for the specimen of human liver.

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