Kinetics and Mechanism of the Rat Brain Phenol Sulphotransferase Reaction

By ED J. M. PENNINGS, REIN VRIELINK and GODFRIED M. J. VAN KEMPEN
Department of Biochemistry, Psychiatric Institute Endegeest, Oegstgeest, The Netherlands

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Some properties of rat brain phenol sulphotransferase were investigated *in vitro* at pH 7.4. The enzyme was purified 10-fold by chromatography on DEAE-Sephadex A-50. It can be assayed with 4-hydroxy-3-methoxyphenylethylene glycol or 4-methylumbelliferone as the sulphate acceptor. The partially purified enzyme is stable for at least 1 week when stored at 4°C. It is, however, additionally activated (10–20%) and stabilized by 1 mM-dithiothreitol. The activity of the enzyme does not depend on the addition of exogenous Mg^{2+} . The pH optima for the sulphation of 4-hydroxy-3-methoxyphenylethylene glycol and 4-methylumbelliferone are 7.8 and 7.4 respectively. Substrate inhibition by the sulphate acceptor is apparent at concentrations over 0.05 mM. Initial-velocity studies in the absence and presence of product and dead-end inhibitors suggested that the mechanism of the rat brain sulphotransferase reaction is sequential ordered Bi Bi with a dead-end complex of enzyme with adenosine 3',5'-bisphosphate and sulphate acceptor. The sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate is the first substrate that adds to the enzyme, and the sulphate acceptor is the second substrate. The dissociation constant for the complex of enzyme with sulphate donor is 21 μ M. The sulphated substrate is the first product and adenosine 3',5'-bisphosphate is the second product that leaves the enzyme.

In rat brain the sulphate conjugates of 3,4-dihydroxyphenylethylene glycol and 4-hydroxy-3-methoxyphenylethylene glycol are considered to be the major noradrenaline metabolites (Schanberg *et al.*, 1968a; Sugden & Eccleston, 1971; Stone, 1973; Braestrup *et al.*, 1974). The enzyme responsible for the sulphation of these catecholamine derivatives, phenol sulphotransferase (3'-phosphoadenylyl sulphate-phenol sulphotransferase, EC 2.8.2.1), has been studied by several investigators (Meek & Neff, 1973; Foldes & Meek, 1973; Pennings *et al.*, 1976a). The fact that the formation of one molecule of a sulphated compound occurs at the expense of two molecules of ATP, and the high concentrations of 4-hydroxy-3-methoxyphenylethylene glycol sulphate in the brains of various mammalian species (Schanberg *et al.*, 1968b; Foldes & Meek, 1974), indicate that sulphate conjugation might be an important metabolic route of catecholamines in the brain. In this respect it has been suggested that conjugation with sulphate in brain is a means of detoxification, as it is in liver, by enabling rapid transport out of the brain of the highly polar products formed (Eccleston & Ritchie, 1973; Foldes & Meek, 1973). The formation of sulphated metabolites, however, may also have a function in controlling the direction of the enzymic *O*-methylation, as suggested by the experiments of Miyazaki *et al.* (1969) and Brooks & Horn (1971). Moreover, the presence of arylsulphatases in brain tissue leaves the possibility that the formation of sulphated

metabolites has a function in temporary inactivation of catecholamines and in that way forms a regulatory pathway. The choice of brain phenol sulphotransferase for the present study is justified by the specific function of the sulphating system in the brain and by the fact that the system in the brain is more homogeneous than, and possibly differs from, that in liver, as suggested by the different effect of probenecid [4-(dipropylsulphamoyl)benzoic acid] on the enzymic sulphation in homogenates of liver and brain (Pennings *et al.*, 1976b). We were also led to this study by the observation that the formation of 4-hydroxy-3-methoxyphenylethylene glycol sulphate in brain was subject to substrate inhibition, which prompted us to carry out a more detailed study of the kinetics of the brain enzyme.

Materials and Methods

Chemicals

4-Hydroxy-3-methoxyphenylethylene glycol in the form of its piperazine salt (Ro 04-4358-001) and 4-hydroxy-3-methoxyphenylethylene glycol sulphate (Ro-04-6028-000) were gifts from Hoffman-La Roche and Co., Basle, Switzerland. 4-Methylumbelliferone, purified by recrystallization from ethanol before use, Dowex 50W (X4, 100–200 mesh, H^+ form) and triethylamine were obtained from Baker Chemicals, Deventer, The Netherlands.

QAE [diethyl-(2-hydroxypropyl)aminoethyl]-Sephadex A-25 and DEAE-Sephadex A-50 and A-25 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ethane-1,2-diamine, redistilled before use, and *p*-anisidine, recrystallized from water, were obtained from BDH Chemicals, Poole, Dorset, U.K. All other chemicals were analytical grade.

Animals

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

Preparation and purification of the sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate

This was prepared enzymically from freeze-dried rat liver (Van Kempen & Jansen, 1972). Incubation was carried out for 2 h at 37°C. After addition of 44 mM-glucose the incubation was continued for another 2 h to convert most ATP into AMP. The remainder of the ATP still present in this preparation did not interfere with the subsequent separation. The reaction was stopped by heating in boiling water. The clear solution obtained after cooling and filtration was further purified by the method of Tsang *et al.* (1976). A volume of 250 ml of the preparation was diluted with an equal volume of 0.1 M-triethylammonium hydrogen carbonate buffer, pH 7.5, before application to a DEAE-Sephadex A-25 column (1.6 cm × 15 cm). Fractions (5 ml) were collected at a flow rate of approx. 20 ml/h. The fractions containing adenosine 3'-phosphate 5'-sulphatophosphate, eluted just after ATP, were freeze-dried and each fraction was redissolved in 1 ml of 0.22 M-Tris/HCl, pH 7.4. It was shown by t.l.c. on silica gel [in propanol/conc. NH₃ (sp. gr. 0.91)/water, 6:3:1, by vol.] that the sulphate donor thus purified was free of any ATP, ADP, AMP, adenosine 3',5'-bisphosphate and adenosine 5'-sulphatophosphate. The concentration of the product was calculated by assuming a molar absorption coefficient of 14.5×10^3 litre · mol⁻¹ · cm⁻¹ at 260 nm (Cherniak & Davidson, 1964).

Isolation and partial purification of phenol sulphotransferase

The enzyme was isolated from rat brain and partially purified by a shortened procedure by the method of Foldes & Meek (1973). All purification steps were carried out at 4°C. The brains of 20–30 rats, killed by decapitation, were quickly excised and washed in ice-cold 0.01 M-Tris/HCl buffer, pH 7.4. A 20% (w/v) homogenate was made in the same buffer by using an MSE homogenizer with a cooling device. After addition of NaCl (final concn. 0.1 M), the crude homogenate was centrifuged for 1 h at 2°C

and 100000g (r_{av} , 5.9 cm). The clear supernatant was applied to a DEAE-Sephadex A-50 column (1.6 cm × 20 cm) previously equilibrated with 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl. Non-adsorbed proteins were eluted with the same buffer, and the adsorbed proteins were eluted with 0.5 M-NaCl in 0.01 M-Tris/HCl, pH 7.4. Fractions (5 ml) were collected at a flow rate of approx. 20 ml/h. The enzyme was eluted in a single peak and stored at -20°C. One enzyme unit is defined as the amount of protein catalysing the formation of 1 nmol of product/min under standard assay conditions as described below. All enzyme activities were measured at 37°C and at pH 7.4, unless otherwise stated.

Determination of protein

Protein concentrations were determined by the method of Lowry *et al.* (1951), with horse serum albumin as a standard.

Assay of phenol sulphotransferase

Phenol sulphotransferase from rat brain was assayed with either 4-hydroxy-3-methoxyphenylethylene glycol or 4-methylumbelliferone as the sulphate acceptor, by procedures described previously (Van Kempen & Jansen, 1972; Van Kempen *et al.*, 1975), except that Triton X-100 was omitted and that both the assays were carried out on half the scale. The standard incubation mixture consisted of: 0.22 M-Tris/HCl buffer, pH 7.4; 0.05 mM-adenosine 3'-phosphate 5'-sulphatophosphate; 0.05 mM-4-hydroxy-3-methoxyphenylethylene glycol or 4-methylumbelliferone; 38 mM-KH₂PO₄; 1 mM-dithiothreitol; 0.5 mM-MgCl₂; 0.5–2 enzyme units. The reaction was started by adding the enzyme and terminated after 30 min by placing the tubes in boiling water for 2 min. After cooling, the amount of sulphate ester formed was assayed in the total supernatant fluid. In the blanks, made by adding the sulphate acceptor or sulphate donor after termination of the reaction, no sulphated product was observed during an incubation period of 30 min. Internal standards were made by adding known amounts of the sulphate ester to such blanks before incubation. Hydrolysis of the sulphate ester did not occur during the incubation.

Effect of pH on enzyme activities

At 37°C enzyme activities were measured at pH values between 6.4 and 9.0 in 0.22 M-Tris/HCl buffers. Controls were incubated simultaneously, in which the pH was monitored at intervals. It was observed that pH value did not change during the incubation. Assay conditions were the same as described above, except that the concentration of the sulphate acceptor was either 0.02 or 0.2 mM.

Kinetic studies

At the highest and lowest concentrations of substrates and inhibitors the rate of the sulphotransferase reaction appeared to be linear for at least 30 min at 37°C. Therefore all incubations were carried out for 30 min and enzyme activities thus obtained were considered to represent initial velocities.

The experiments were designed such that the results could be analysed in terms of the method developed by Cleland (1963*a,b*, 1970). Initial velocities of the sulphotransferase reaction were measured in the presence or absence of product or dead-end inhibitors. The concentrations of the substrates were varied from 6.7 to 50 μ M. Substrate inhibition was studied by varying the sulphate acceptor concentration up to 2 mM. All results shown are those of the kinetic studies with 4-hydroxy-3-methoxyphenylethylene glycol as the sulphate acceptor. The results are similar to those obtained with 4-methylumbelliferone as the acceptor. Double-reciprocal plots and replots of slopes and intercepts were always linear unless otherwise stated.

Evaluation of enzyme kinetic constants

K_s , K_m and V values were determined by the method of the direct linear plot as outlined by Eisenthal & Cornish-Bowden (1974). This method is statistically preferable to the usual methods, as it is far less dependent on assumptions about the nature of the experimental error and is rather insensitive to some wrong observations. However, when displaying the results of several experiments, it is difficult to avoid crowding. For this reason results are presented as double-reciprocal plots, the slopes and intercepts of such plots being K_m/V and $1/V$ respectively. Replots of slopes and intercepts were used to determine the apparent inhibition constants K_{is} and K_{ii} respectively (Cleland, 1963*b*, 1970).

Heat inactivation of phenol sulphotransferase and protection by its substrates

The enzyme preparation after DEAE-Sephadex chromatography was preincubated at 46°C for 8 min in the absence of dithiothreitol, cooled to 4°C and

then tested for activity at 37°C. Controls were kept at 4°C. Protection of the enzyme by its substrates was studied by adding 0.375 mM-sulphate donor or 0.2–2 mM-sulphate acceptor before preincubation. The final concentrations during the assay were 0.05 and 0.05–0.5 mM-respectively. Dithiothreitol was omitted during the assay. Other conditions were unchanged.

Results

Enzyme purification

Table 1 shows the results of the purification procedure. Enzyme activities were determined with 4-hydroxy-3-methoxyphenylethylene glycol as the sulphate acceptor. The results obtained with 4-methylumbelliferone as the substrate were similar, specific activities for the homogenate, 100000g supernatant and column fraction being 0.037, 0.123 and 0.40 unit/mg of protein respectively. The purification is approx. 10-fold.

Stability of the enzyme and effect of dithiothreitol on enzyme activity

In the absence of dithiothreitol the partially purified enzyme was stable for at least 1 week when stored at 4°C. Even after preincubation at 37°C for 1 h or at 40°C for 20 min no decrease in activity was observed. However, the addition of 1 mM-dithiothreitol to the partially purified enzyme was necessary for full activity, the stimulation being 10–20%. Moreover, when the enzyme activity was decreased owing to prolonged storage, it could be fully restored by the addition of 3 mM-dithiothreitol. These results are in marked contrast with the instability of crude enzyme preparations. After a 10 min incubation period at 37°C, 80% of the activity of a 10% (w/v) rat brain homogenate was lost and could not be restored by the addition of 3 mM-dithiothreitol.

Apparent K_m values for adenosine 3'-phosphate 5'-sulphatophosphate and either sulphate acceptor were not affected by the addition of dithiothreitol.

Enzyme activities of crude and partially purified preparations were additionally stabilized by the presence of the sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate.

Table 1. *Partial purification of rat brain phenol sulphotransferase*

The enzyme activities were determined with 4-hydroxy-3-methoxyphenylethylene glycol as the sulphate acceptor under standard incubation conditions as described in the Materials and Methods section.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Yield (%)	Purification (-fold)
1. Homogenate (20%, w/v)	220	3817	145	0.038	100	1
2. 100000g supernatant	136	873	104	0.120	72	3
3. DEAE-Sephadex A-50 chromatography	20	42	15	0.37	11	10

Effect of Mg^{2+} on enzyme activity

Addition of Mg^{2+} to a concentration of 1 mM very slightly (2–5%) stimulated the activity of the partially purified enzyme. EDTA up to 60 mM had no effect on enzyme activities.

Effect of pH on enzyme activity

At pH 7.4 we observed substrate inhibition by either sulphate acceptor in concentrations over 0.5 mM. However, substrate inhibition did not occur at pH 6.2 or 9.0. Therefore the effect of pH on enzyme activity was studied at two different concentrations of the sulphate acceptor, 0.2 and 0.02 mM (Fig. 1). When determined at 0.02 mM, the pH optima for the sulphation of 4-hydroxy-3-methoxyphenylethylene glycol and 4-methylumbelliferone were 7.8 and 7.4 respectively. However, when the concentrations of the sulphate acceptors were raised to 0.2 mM, the forms of the curves change and the pH optima shift to lower values. The studies described here were performed at pH 7.4 to allow comparison of the results obtained with the two acceptors.

Kinetic studies

Irreversibility of the sulphotransferase reaction. Initial-velocity studies of the forward and reverse reactions are essential in deducing a kinetic mechanism. Attempts were undertaken to demonstrate the reverse reaction by incubating the partially purified

enzyme at 37°C with various concentrations of the products of the forward reaction, adenosine 3',5'-bisphosphate and 4-methylumbelliferone sulphate (0.2–1.8 mM). The remainder of the sulphate ester was assayed after a 1 h incubation period. Nucleotide-dependent hydrolysis of 4-methylumbelliferone sulphate could not be demonstrated. In another experiment the enzyme was incubated at 37°C for 2 h with 0.5 mM 4-hydroxy-3-methoxyphenylethylene glycol sulphate, 0.001 mM- or 0.1 mM-adenosine 3',5'-bisphosphate and 0.1 mM-4-methylumbelliferone. Transfer of sulphate to 4-methylumbelliferone could not be shown, again indicating the irreversibility of the reaction. In accordance with this was the observation that 4-hydroxy-3-methoxyphenylethylene glycol sulphate, when tested as an alternate product inhibitor of the forward reaction with 4-methylumbelliferone as the substrate, had no effect in concentrations up to 2.5 mM. At higher concentrations the sulphate ester interfered with the assay method.

Initial-velocity studies in the absence of product. The results of one experiment are illustrated in Figs. 2 and 3 as double-reciprocal plots. As substrate inhibition by the sulphate acceptor was apparent at concentrations exceeding 0.05 mM, only the points obtained at lower concentrations were considered. The two series of lines were constructed from the experiment's points by the method of direct

Fig. 1. Effect of pH on the activity of partially purified phenol sulphotransferase from rat brain

Enzyme activities were determined as described in the Materials and Methods section at two constant concentrations (mM) of either sulphate acceptor: ○, 0.02; △, 0.2 for 4-hydroxy-3-methoxyphenylethylene glycol; ●, 0.02; ▲, 0.2 for 4-methylumbelliferone.

Fig. 2. Double-reciprocal plots of initial velocity versus adenosine 3'-phosphate 5'-sulphatophosphate concentration at constant concentrations of 4-hydroxy-3-methoxyphenylethylene glycol

Plots of reciprocal initial velocities, expressed in arbitrary fluorimetric units, against reciprocal adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) concentrations for the following concentrations (mM) of 4-hydroxy-3-methoxyphenylethylene glycol: ○, 0.01; △, 0.02; □, 0.1; ●, 0.5. Lines were constructed by the method of the direct linear plot (Eisenthal & Cornish-Bowden, 1974).

Fig. 3. Double-reciprocal plots of initial velocity against 4-hydroxy-3-methoxyphenylethylene glycol concentration at constant adenosine 3'-phosphate 5'-sulphatophosphate concentrations

Plot of reciprocal initial velocities, expressed in arbitrary fluorimetric units, against reciprocal concentrations of 4-hydroxy-3-methoxyphenylethylene glycol for the following adenosine 3'-phosphate 5'-sulphatophosphate concentrations (μM): ○, 9.7; △, 19.3; □, 58. The linear parts of the curves were constructed by the method of the direct linear plot (Eisenthal & Cornish-Bowden, 1974). Only those points were used that were obtained at concentrations of 4-hydroxy-3-methoxyphenylethylene glycol below 0.05 mM.

Fig. 4. Replots of slopes and intercepts of double-reciprocal plots (of initial velocity against adenosine 3'-phosphate 5'-sulphatophosphate concentration) as a function of reciprocal concentration of 4-hydroxy-3-methoxyphenylethylene glycol

Slopes and intercepts of Fig. 2 were replotted against reciprocal concentrations of 4-hydroxy-3-methoxyphenylethylene glycol. Symbols: ○, slopes; △, intercepts. The slope replot and linear part of the intercept replot were constructed by the method of the direct linear plot for two-substrate reactions (Eisenthal & Cornish-Bowden, 1974).

linear plot (Eisenthal & Cornish-Bowden, 1974), as described in the Materials and Methods section. Within experimental error, the vertical co-ordinates of the cross-over points of Figs. 2 and 3 are equal. To evaluate the kinetic constants K_s^A , K_m^A and K_m^B it will be assumed now, and justified later in the Discussion section, that adenosine 3'-phosphate 5'-sulphatophosphate is the first substrate (A) and the sulphate acceptor the second substrate (B). The mean values $\pm$ S.E.M. of K_s^A , K_m^A and K_m^B were $19 \pm 2 \mu\text{M}$, $11 \pm 2 \mu\text{M}$ and $32 \pm 2 \mu\text{M}$ ($n = 4$) respectively when determined with 4-hydroxy-3-methoxyphenylethylene glycol as the sulphate acceptor. The corresponding values were $23 \pm 2 \mu\text{M}$, $10 \pm 2 \mu\text{M}$ and $11 \pm 2 \mu\text{M}$ ($n = 3$) respectively when determined with 4-methylumbelliferone as the acceptor. The K_s^A , which represents the dissociation constant for the enzyme-adenosine 3'-phosphate 5'-sulphatophosphate complex, is independent of the sulphate acceptor used. The maximal velocities of the sulphotransferase reactions were 3.5 nmol of 4-hydroxy-3-methoxyphenylethylene glycol sulphate/min per enzyme unit and 3.4 nmol of 4-methylumbelliferone sulphate/min per enzyme unit. Slopes and intercepts of double-reciprocal plots with B as the non-varied substrate (Fig. 2) were plotted against the reciprocal concentrations of B (Fig. 4). The hyperbolic intercept replot

again revealed the substrate inhibition by the sulphate acceptor.

Substrate inhibition. Fig. 4 shows that only the intercept replot is hyperbolic and that the slope replot is a straight line, as expected. This type of substrate inhibition is uncompetitive (Cleland, 1970). Moreover, it was verified that the substrate inhibition by the sulphate acceptor was linear with concentration up to the highest concentration of the acceptor studied (2 mM).

Product inhibition. To obtain further information on the reaction mechanism and on the order of addition of the substrates to the enzyme, product-inhibition studies were undertaken, but were limited in that only adenosine 3',5'-bisphosphate could be used. Initial-velocity measurements, based on the determination of the sulphate ester formed, became inaccurate when this product was included in the incubation mixtures. The results are illustrated in Figs. 5 and 6 and the apparent inhibition constants K_{is} and K_{ii} are summarized in Table 2.

When adenosine 3',5'-bisphosphate was varied with respect to either sulphate acceptor, non-competitive inhibition was observed. In addition, it was observed that these kinetic constants increased as the concentration of the sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate was raised. The non-competitive inhibition dictates that the sulphate

acceptor at infinite concentrations cannot prevent adenosine 3',5'-bisphosphate from binding to the enzyme.

When adenosine 3',5'-bisphosphate was varied with respect to adenosine 3'-phosphate 5'-sulphatophosphate competitive inhibition was observed, which indicates that adenosine 3',5'-bisphosphate binds to the same enzyme form as does the sulphate donor. The K_{is} value for adenosine 3',5'-bisphosphate inhibition with respect to adenosine 3'-phosphate 5'-sulphatophosphate did not depend on the type, but did depend on the concentration of the sulphate acceptor used. The K_{is} value decreased when the concentration of the sulphate acceptor was raised.

Fig. 5. Inhibition by adenosine 3',5'-bisphosphate of the sulphotransferase reaction with adenosine 3'-phosphate 5'-sulphatophosphate as the variable substrate

Plots of reciprocal initial velocities, expressed in arbitrary fluorimetric units, against reciprocal adenosine 3'-phosphate 5'-sulphatophosphate concentrations at different constant concentrations (μM) of the inhibitor adenosine 3',5'-bisphosphate: $\circ$, 0; Δ , 10; $\square$, 20; $\bullet$, 30. The concentration of the sulphate acceptor was kept constant at 0.05 mM. Lines were constructed by the method of the direct linear plot (Eisenthal & Cornish-Bowden, 1974).

Dead-end inhibition. The use of dead-end inhibitors can assist in deducing a kinetic mechanism, and these inhibitors are particularly important where the reverse reaction cannot be studied and/or the product-inhibition studies are limited. ADP and *p*-anisidine were found to be appropriate. Dead-end complexes were formed, as neither ADP nor *p*-anisidine can serve as substrates. ADP showed competitive inhibition with respect to the sulphate donor and non-competitive inhibition with respect to either sulphate acceptor. Inhibition by *p*-anisidine was uncompetitive with respect to the sulphate donor and non-competitive with respect to either sulphate acceptor.

Fig. 6. Inhibition by adenosine 3',5'-bisphosphate of the sulphotransferase reaction with 4-hydroxy-3-methoxyphenylethylene glycol as the variable substrate

Plots of reciprocal initial velocities, expressed in arbitrary fluorimetric units, against reciprocal concentrations of 4-hydroxy-3-methoxyphenylethylene glycol at different constant concentrations (μM) of the inhibitor adenosine 3',5'-bisphosphate: $\circ$, 0; Δ , 10; $\square$, 20; $\bullet$, 30. The concentration of the sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) was kept constant at 0.05 mM. Lines were constructed by the method of the direct linear plot (Eisenthal & Cornish-Bowden, 1974).

Table 2. Apparent inhibition constants for adenosine 3',5'-bisphosphate

The apparent inhibition constants were calculated from replots of the slopes and vertical intercepts of double-reciprocal plots as described by Cleland (1963b). Slope and vertical intercepts were calculated from K_m and V values obtained from the direct linear plots (Eisenthal & Cornish-Bowden, 1974). All replots were linear. Values are means $\pm$ s.e.m. The numbers of experiments are indicated in parentheses. The concentration of the non-varied substrate was kept at 0.05 mM.

Sulphate acceptor		4-Hydroxy-3-methoxyphenylethylene glycol		4-Methylumbelliferone	
Inhibition constant	Variable substrate	Sulphate donor	Sulphate acceptor	Sulphate donor	Sulphate acceptor
K_{is}		3 ± 2 (2)	7 ± 2 (4)	1.1 ± 0.3 (5)	9.7 ± 0.2 (3)
K_{ii}		∞	13 ± 3 (4)	∞	14.3 ± 0.4 (3)

Heat inactivation of the enzyme and protection by its substrates

At 46°C the partially purified enzyme quickly lost its activity in the absence of its substrates. The decrease was $64 \pm 5\%$ after an 8 min preincubation period. However, addition of 0.375 mM-adenosine 3'-phosphate 5'-sulphatophosphate to the enzyme before inactivation had a protective effect on the enzyme, the decrease in enzyme activity now being $26 \pm 1\%$. Such a protective action was not observed by the addition of either sulphate acceptor in concentrations up to 2 mM. The decrease in enzyme activity could be fully restored by the extra addition of 2 mM-dithiothreitol after preincubation, indicating that a thiol group was involved in the inactivation process. Moreover, when dithiothreitol was included during the whole procedure, the enzyme appeared to be stabilized considerably. The values mentioned are means $\pm$ S.E.M. for two separate experiments.

Discussion

Several studies with rat and rabbit brain as the enzyme source have been performed in which the properties of phenol sulphotransferase and the kinetic parameters of the sulphotransferase reaction *in vitro* have been presented (Foldes & Meek, 1973; Wong, 1975; Pennings *et al.*, 1976a). We sought to clarify the mechanism of the rat brain sulphotransferase reaction. For this purpose the enzyme has been purified 10-fold (Table 1).

Meek & Neff (1973) showed that phenol sulphotransferase from rat brain is active *in vitro* towards 4-hydroxy-3-methoxyphenylethylene glycol. We (Pennings *et al.*, 1976a) have presented evidence that this enzyme could be assayed with the artificial substrate 4-methylumbelliferone as well. We now present additional evidence that both substrates are sulphated by the same enzyme, as the specific activities of the enzyme assayed with these substrates increase equally during the purification procedure. Moreover, other results obtained with 4-hydroxy-3-methoxyphenylethylene glycol as the sulphate acceptor are similar to those obtained with 4-methylumbelliferone as the acceptor. However, the pH optimum for the sulphotransferase reaction and the Michaelis constants for the sulphate acceptors (K_m^B) are dependent on the acceptor used.

In contrast with freshly prepared homogenates, partially purified phenol sulphotransferase is activated and stabilized by the addition of 1 mM-dithiothreitol, indicating the presence of thiol groups. Obviously, the thiol groups are reversibly oxidized during the purification procedure. Foldes & Meek (1973) observed no effect of 0.1–1.0 mM-mercaptoethanol, a less powerful thiol reducing agent, on partially purified phenol sulphotransferase. In

agreement with their findings is the absence of an effect of Mg^{2+} on enzyme activity.

Our results indicate, as do those of others (Foldes & Meek, 1973; Wong, 1975), that the pH optimum of the brain sulphotransferase reaction can be acceptor-dependent. The apparent pH optimum is also dependent, however, on the concentration of the acceptor. This is caused by substrate inhibition, which is most apparent between pH 7.0 and 8.0 and at acceptor concentrations over 0.05 mM (Figs. 3 and 4). Substrate inhibition by the acceptor has also been observed by Banerjee & Roy (1968) and Foldes & Meek (1973) for the guinea-pig liver and rat brain phenol sulphotransferase reactions respectively. However, these workers did not study the type of substrate inhibition.

The value of 7.8 for the pH optimum for the sulphation of 4-hydroxy-3-methoxyphenylethylene glycol is identical with that reported by Wong (1975), but different from that of pH 6.4 reported by Foldes & Meek (1973). The discrepancy with the latter authors may be explained by the difference in the buffers used.

The initial-velocity experiments yield two series of intersecting plots (Figs. 2 and 3). These intersecting plots rule out a Ping Pong mechanism and are consistent with a sequential mechanism. The reaction mechanism may be of the ordered or random type. As a special case of the ordered sequential mechanism, a Theorell–Chance mechanism is possible in which the concentration of the ternary complexes approaches zero.

Under steady-state conditions the initial velocity of sequential mechanisms can be described by the equation:

$$v = \frac{V[A][B]}{K_s^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B]} \quad (1)$$

where v is the initial velocity, A and B are the first and second substrates and V and K values are kinetic constants (Cleland, 1963a, 1970). The cross-over point of the double-reciprocal plot has a horizontal co-ordinate of $-1/K_s^A$ when A is the substrate being varied, and $-K_m^A/(K_s^A K_m^B)$ when B is varied. The vertical co-ordinate in either plot is $(1 - K_m^A/K_s^A)/V$ and the ratio of K_m^A to K_s^A therefore determines the vertical position of the cross-over point. It must be emphasized that equality of K_m^A and K_s^A has no diagnostic value as far as distinguishing mechanisms. Eqn. (1) is algebraically symmetrical with respect to A and B. Product- and dead-end-inhibition studies are therefore required to distinguish the two substrates.

A truly rapid-equilibrium random mechanism must be excluded on grounds that such a mechanism would not show substrate inhibition at all, unless one substrate had some affinity for the part of the binding site designed for the other, and the

Scheme 1. *Reaction scheme for the sulphotransferase reaction*

Schematic illustration of the forward reaction. The enzyme is denoted as E; 4-methylumbelliferone as MU; 4-hydroxy-3-methoxyphenylethylene glycol as MHPG; 4-methylumbelliferone sulphate as MUS; 4-hydroxy-3-methoxyphenylethylene glycol sulphate as MHPG-S; and adenosine 3',5'-bisphosphate as PAP. The formation of dead-end complexes (E·B·Q) is indicated.

inhibition should be of the competitive type. In an ordered Bi Bi mechanism linear uncompetitive substrate inhibition (Fig. 4) is to be expected when the second substrate (B) to add to the enzyme (E) can also form a dead-end complex with E·Q, Q being the second product to leave the enzyme (Scheme 1). The findings that the K_s^A for adenosine 3'-phosphate 5'-sulphatophosphate and the K_{is} for the competitive inhibition of adenosine 3',5'-bisphosphate with respect to adenosine 3'-phosphate 5'-sulphatophosphate are independent of the type of sulphate acceptor used (Table 2) are in accordance with such a mechanism in which adenosine 3'-phosphate 5'-sulphatophosphate (A) is the first substrate and the sulphate acceptor (B) the second.

The results of the product-inhibition studies (Table 2 and Figs. 5 and 6) give no indication of a certain mechanism. They rule out, however, the ordered Bi Bi mechanism in which the sulphate acceptor is the first substrate and adenosine 3'-phosphate 5'-sulphatophosphate the second, as adenosine 3',5'-bisphosphate would then be expected to exhibit non-competitive inhibition with respect to adenosine 3'-phosphate 5'-sulphatophosphate, rather than the competitive inhibition that is obtained. A Theorell-Chance mechanism with such an order of addition of substrates cannot be excluded by the product-inhibition studies, but is ruled out by the dead-end-inhibition studies; as ADP would be expected to show uncompetitive inhibition with respect to the sulphate acceptor, rather than the non-competitive inhibition that is seen. The substrate-inhibition data, too, are inconsistent with such a mechanism.

The inhibition by *p*-anisidine with respect to adenosine 3'-phosphate 5'-sulphatophosphate rules out a random mechanism, as non-competitive

inhibition would be expected, rather than the uncompetitive inhibition that is observed.

The only remaining possibilities, which are consistent with all kinetic data, are the ordered sequential Bi Bi reaction mechanism or the Theorell-Chance reaction mechanism, in which the order of addition and release of substrates and products is as shown in Scheme 1. These mechanisms differ only in that the steady-state concentration of the central complexes approaches zero in the Theorell-Chance mechanism. The existence of central complexes can only be inferred from careful analysis of kinetic constants or from first-product- or alternate-first-product-inhibition studies. The apparent irreversibility of the reaction and the inability to use 4-methylumbelliferone sulphate or 4-hydroxy-3-methoxyphenylethylene glycol sulphate as product inhibitors do not permit us to evaluate the importance of the central complexes.

At first sight the non-competitive inhibition by *p*-anisidine with respect to the sulphate acceptor does not accord with this mechanism, as competitive inhibition is expected from the dead-end combination of *p*-anisidine with E-adenosine 3'-phosphate 5'-sulphatophosphate complexes. However, we may as well expect the dead-end combination of *p*-anisidine with E-adenosine 3',5'-bisphosphate complexes. The latter binding predicts uncompetitive inhibition, as the point of combination of *p*-anisidine comes after the point of combination of the variable substrate, i.e. the sulphate acceptor. Therefore the inhibition by *p*-anisidine with respect to the sulphate acceptor must be expected to be linear non-competitive and this is in agreement with the results. The increase in the slope results from the dead-end combination of *p*-anisidine with E-adenosine 3'-

phosphate 5'-sulphatophosphate complexes and the increase in the intercept results from the dead-end combination of *p*-anisidine with E-adenosine 3',5'-bisphosphate complexes. The inhibition is linear, as combination of *p*-anisidine with E-adenosine 3'-phosphate 5'-sulphatophosphate complexes cannot increase the steady-state concentration of E-adenosine 3',5'-bisphosphate complexes, and vice versa.

With adenosine 3'-phosphate 5'-sulphatophosphate as the variable substrate the same sort of reasoning leads to the prediction of two effects that both increase the intercept, one from the combination of *p*-anisidine with E-adenosine 3'-phosphate 5'-sulphatophosphate complexes and one from the combination of *p*-anisidine with E-adenosine 3',5'-bisphosphate complexes. Therefore the inhibition of *p*-anisidine with respect to the sulphate donor adenosine 3'-phosphate 5'-sulphatophosphate is expected to be linear uncompetitive, in agreement with the results.

The dead-end combination of sulphate acceptor with E-adenosine 3',5'-bisphosphate complexes has another consequence besides uncompetitive substrate inhibition. It is also responsible for the observed decrease in the apparent K_{is} value for adenosine 3',5'-bisphosphate inhibition with respect to adenosine 3'-phosphate 5'-sulphatophosphate when the sulphate-acceptor concentration is increased. In the presence of adenosine 3',5'-bisphosphate, E-adenosine 3',5'-bisphosphate complexes are drawn off into dead-end complexes as the concentration of the sulphate donor is increased. Thus the apparent inhibition by adenosine 3',5'-bisphosphate is enhanced and as a consequence the apparent K_{is} value is decreased.

The heat-inactivation experiments indicate that adenosine 3'-phosphate 5'-sulphatophosphate can bind to the enzyme in the absence of the sulphate acceptor and do not contradict the ordered Bi Bi reaction mechanism, as proposed above.

We emphasize that the experiments were carried out at pH 7.4 and that the results are valid only at this pH. In this respect Banerjee & Roy (1968) reported that the mechanism of the reaction catalysed by phenol sulphotransferase from guinea-pig liver was a rapid-equilibrium random mechanism, but the experiments were carried out at pH 5.6, the apparent pH optimum for the enzyme with *p*-nitrophenol as the sulphate acceptor. These authors also observed substrate inhibition by the sulphate acceptor and found it to be dependent on the pH:

it was not observed at pH 5.6, but became increasingly obvious at pH values greater than 6.2. At first glance a comparison with our results suggests that phenol sulphotransferase from rat brain differs from the guinea-pig liver enzyme. However, the reaction mechanism may depend on the pH and the difference in pH optimum can be explained by a difference in the sulphate acceptor used. Further work is necessary to clarify these points.

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