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KINETIC STUDIES OF THE PHENOL SULPHOTRANSFERASE REACTION

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SUMMARY

The kinetics of the reversible sulphotransferase-catalysed reaction between *p*-nitrophenol and 3′-phosphoadenylyl sulphate to give *p*-nitrophenyl sulphate and adenosine 3′,5′-diphosphate have been investigated using a partially purified sulphotransferase from guinea-pig liver. From a study of the initial velocities of the reactions in the forward and reverse directions at different substrate concentrations, and of the inhibitory effects of the products on the forward reaction, it was concluded that the mechanism was that of a rapid equilibrium random bi bi reaction upon which was superimposed the formation of a dead-end complex of the enzyme, *p*-nitrophenol and adenosine 3′,5′-diphosphate. Studies of the specificity of the enzyme have been made. Attempts to detect a transfer reaction between *p*-nitrophenyl sulphate and 2-naphthol with adenosine 3′,5′-diphosphate as a cofactor were unsuccessful.

INTRODUCTION

The sulphotransferases form a group of enzymes which catalyse the transfer of the sulphuryl group of 3′-phosphoadenylyl sulphate to an acceptor, usually a hydroxyl-containing compound, with the formation of a sulphate ester and adenosine 3′,5′-diphosphate. Except in special cases this reaction is irreversible. None of these enzymes has been obtained in a pure state but BANERJEE AND ROY[1] described the separation from guinea-pig liver of a number of sulphotransferases one of which, a phenol sulphotransferase (3′-phosphoadenylylsulphate:phenol sulphotransferase, EC 2.8.2.1, incorrectly known as phenol sulphokinase), was obtained devoid of any other detectable type of sulphotransferase activity. This enzyme did not require added Mg^{2+} to develop its full activity, nor was it inhibited by EDTA which stabilised the enzyme, and it was therefore chosen for an investigation of the kinetics of the sulphotransferase reaction. Previous observations on the kinetics of the sulphotransferases have been made only incidentally to other investigations and the information so obtained has been insufficient to allow any conclusions to be drawn about the mechanism of the reaction. The present work, of which a preliminary account has

already appeared[2], gives the results of a detailed investigation of the kinetics of the formation of *p*-nitrophenyl sulphate together with some observations on the specificity of this phenol sulphotransferase. The principal reaction investigated, that between 3'-phosphoadenylyl sulphate and *p*-nitrophenol, is one of the few reversible sulphotransferase-catalysed reactions and in the present communication the term 'forward reaction' is taken to mean the formation of *p*-nitrophenyl sulphate and the term 'reverse reaction' to refer to the reformation of *p*-nitrophenol from the ester.

EXPERIMENTAL

Preparation of phenol sulphotransferase. This enzyme was obtained from guinea-pig liver exactly as previously described[1] and was further purified by chromatography on Sephadex G-100 at pH 7.5 in a 0.03 M EDTA–NaOH buffer containing 0.01 M mercaptoethanol. The final preparation typically had a specific activity of about 10 mμmoles nitrophenyl sulphate formed per min per mg of protein, about 4 times greater than that of the previous preparation[1].

Preparation of 3'-phosphoadenylyl sulphate. This likewise was prepared as before[1]. Many preparations were used during the course of the work and no differences were detected in the results obtained using the different specimens.

Determination of sulphotransferase activity. For routine assays the method was that described earlier[1]. The reaction mixture had a volume of 1.0 ml and the final concentrations of the reactants were: 0.1 M sodium acetate–acetic acid buffer (pH 5.6); EDTA, 3 mM; mercaptoethanol, 1 mM; *p*-nitrophenol, 1 mM; 3'-phosphoadenylyl sulphate, 0.1 mM; and protein (added last to start the reaction), approx. 150 μg/ml. After incubation at 37° for 10 min the reaction was stopped by the addition of 1 ml of methylene blue reagent[3] followed by 5 ml of chloroform. The mixture was shaken vigorously for 30 sec to extract the methylene blue salt of *p*-nitrophenyl sulphate into the chloroform which was then separated by centrifuging, dried over Na_2SO_4, and its absorbance at 650 mμ determined against a reagent blank. Control determinations were carried out by adding the enzyme to the remainder of the reaction mixture after the methylene blue reagent. The amount of *p*-nitrophenyl sulphate present was obtained from suitable calibration curves.

For the kinetic studies of the forward reaction the same general conditions and type of assay were used but the concentrations of the reactants were varied to suit the particular purpose of the experiment, details of which are given below. In the study of the inhibited reaction the enzyme was added to the previously mixed substrates and inhibitor.

In the investigation of the reverse reaction the same technique was again adopted but the substrates in this case were *p*-nitrophenyl sulphate and adenosine 3',5'-diphosphate. The results of such assays are inherently less accurate than those of the forward reaction because the reaction velocity is given by the relatively small difference between the concentration of *p*-nitrophenyl sulphate present at the beginning and at the end of the experiment.

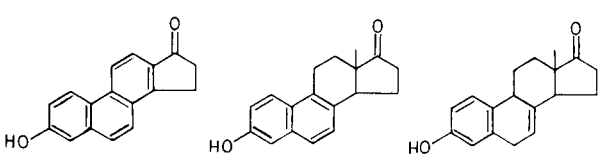
A different technique was used in attempts to study the transfer of the sulphuryl group from *p*-nitrophenyl sulphate to another phenol. The reaction mixture had a volume of 1 ml and the following composition: 0.1 M sodium acetate–acetic acid buffer (pH 5.6); EDTA, 3 mM; mercaptoethanol, 1 mM; *p*-nitrophenyl sulphate,

0.5 mM; phenol, 4 mM or 2-naphthol, 0.1 mM; the appropriate concentration of adenosine 3′,5′-diphosphate (see below) and protein, 150 μg/ml. In this case the reaction was stopped after incubation for 10 min at 37° by the addition of 2 ml of 1 M NaOH and the absorbance of the resulting solution determined at 400 mμ. The amount of *p*-nitrophenol present was determined from suitable calibration curves.

In the studies of the specificity the methylene blue technique was used because the colour yield from different sulphate esters is relatively little affected by their structure so that calibration curves need not be prepared for each individual compound. The amounts of sulphate ester (in mμmoles) required to produce an absorbance of 0.1 at 650 mμ when determined by the above method are as follows: phenyl sulphate, 20.5; 2-naphthyl sulphate, 6.6; 1-naphthyl sulphate, 6.3; *p*-nitrophenyl sulphate, 6.3; androstenolone sulphate, 6.1. Therefore, for experiments involving complex polycyclic acceptors a calibration curve prepared from androstenolone sulphate was used. It should be noted that no error would be introduced by this procedure into the calculated values of K_m but that slight errors might occur in the values for the maximum velocities reached with different acceptors. In these experiments the more complex relatively non-polar phenols were added in 0.1 ml of propylene glycol rather than in water .

Kinetic studies. The general design of the kinetic experiments was such that the results could be analysed in terms of the theory developed by CLELAND[4–6]: measurements of the initial velocities of the reaction in both directions in the presence and absence of products as inhibitors, were therefore made. Michaelis constants, apparent inhibitor constants and maximum velocities were calculated by the method of WILKINSON[7]. In the following sections the dissociation constants are given together with their 95% confidence levels.

Chemicals. The majority of the chemicals were either commercial preparations or were synthesised by standard procedures. Two specimens of adenosine 3′,5′-diphosphate (which gave identical results) were used: these were gifts from Professor J. G. BADDILEY and Dr. E. A. DAVIDSON, respectively. 2-Hydroxyphenanthrene and 15,16-dihydro-3-acetoxy-17-oxocyclopentena-[*a*]phenanthrene[8] were gifts from Dr. M. M. COOMBS. As suggested by Dr. COOMBS, the latter compound was hydrolysed to the free phenol (I) by refluxing it under N_2 in 10% KOH in 90% ethanol: the hydrolysate was diluted with water and acidified with HCl to precipitate the phenol which was collected and thoroughly washed but used without further purification. Equilenin (II) and equilin (III) were also gifts from Dr. COOMBS.



I II III

RESULTS

General properties of the enzyme. The phenol sulphotransferase could be kept for many months without significant loss of activity when stored at −25° at pH 7.4 in

TABLE I

THE EFFECT OF THE REMOVAL OF EDTA AND MERCAPTOETHANOL FROM PHENOL SULPHOTRANSFERASE BY DIALYSIS UNDER METAL-FREE CONDITIONS AGAINST BUFFERS HAVING THE COMPOSITION GIVEN BELOW

In all cases the pH of the buffer was 7.5 and the dialysis was continued for 3 days.

Composition of buffer	*Enzyme activity (mμmoles nitrophenyl sulphate per min)*
0.03 M EDTA–NaOH, 0.01 M mercaptoethanol	1.73
0.01 M Tris–acetic acid, 0.01 M mercaptoethanol	1.71
0.01 M Tris–acetic acid	1.12
0.01 M Tris–acetic acid, enzyme made 0.01 M in mercaptoethanol 10 min before assay	1.74

the presence of EDTA and of mercaptoethanol. At 5° the enzyme was less stable, the activity dropping by about 5% in a week. By dialysis under stringent metal-free conditions EDTA could be removed without loss of enzymatic activity but removal of the mercaptoethanol caused a slight, although reversible, inactivation, as is shown in Table I. Because no advantage was gained by the removal of the EDTA the enzyme was normally stored in 0.03 M EDTA–NaOH buffer (pH 7.4) containing 0.01 M mercaptoethanol.

As previously reported[1] the pH optimum for the synthesis of *p*-nitrophenyl sulphate was at pH 5.6: at this pH the enzyme is quite unstable and even at pH 6.5 a considerable amount of inactivation occurred on standing for 10 min at 37°, as shown in Table II. This instability has made further purification of the enzyme difficult and is probably at least partly the cause of the sulphotransferase reaction being of zero order for only about 10 min (ref. 1).

TABLE II

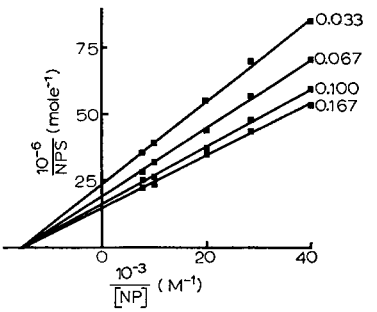
THE STABILITY OF PHENOL SULPHOTRANSFERASE AT DIFFERENT pH VALUES

The enzyme was allowed to stand in 0.01 M EDTA–NaOH buffers of the appropriate pH for 10 min at 37° before being assayed as described in the text: the activity is expressed relative to a control determination at pH 7.4.

pH	*Activity*
7.4	100
6.6	96
6.1	74
5.6	44
5.1	6.9
4.5	1.6
4.0	0.7

Kinetic studies

Forward reaction. The results of studies of the effect on the initial velocity of changes in the concentration of *p*-nitrophenol at fixed concentrations of 3'-phosphoadenylyl sulphate and to changes in the concentration of the nucleotide at fixed concentrations of the phenol are shown in Figs. 1 and 2, respectively. It is clear from



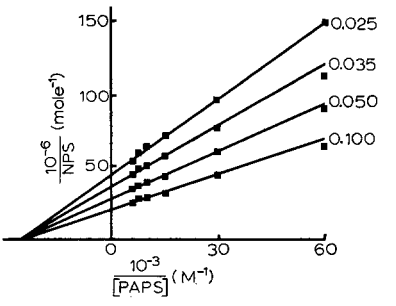
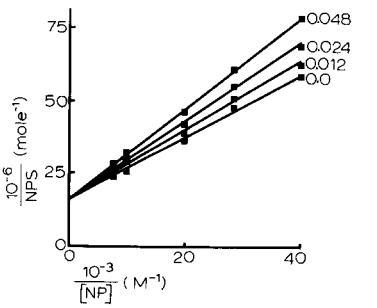


Fig. 1. The effect of varying concentrations of *p*-nitrophenol (NP) on the rate of formation of *p*-nitrophenyl sulphate (NPS) at fixed concentrations of 3'-phosphoadenylyl sulphate. The figures on the lines are the concentrations (mM) of the latter. Details of the composition of the reaction mixture in these experiments, and in those shown in all the following figures, are given in the experimental section.

Fig. 2. The effect of varying concentrations of 3'-phosphoadenylyl sulphate (PAPS) on the rate of formation of *p*-nitrophenyl sulphate (NPS) at fixed concentrations of *p*-nitrophenol. The figures on the lines are the concentrations (mM) of the latter.

those plots, both of which are families of straight lines intersecting on the abscissae, that the K_m for one substrate is independent of the concentration of the other. The values of K_m obtained from those plots were 0.070 ± 0.012 mM *p*-nitrophenol and 0.036 ± 0.010 mM 3'-phosphoadenylyl sulphate, and these same values were obtained from the secondary plots of the intercepts on the ordinates ($1/V$) of Figs. 1 and 2 against the reciprocals of the concentrations of 3'-phosphoadenylyl sulphate and of *p*-nitrophenol, respectively. Both of those secondary plots likewise gave straight lines.

Product inhibition. The inhibition of the formation of *p*-nitrophenyl sulphate by the products of the reaction, namely *p*-nitrophenyl sulphate and adenosine 3',5'-diphosphate, was studied with both of the substrates as variable substrate. The results are shown in Figs. 3–6.



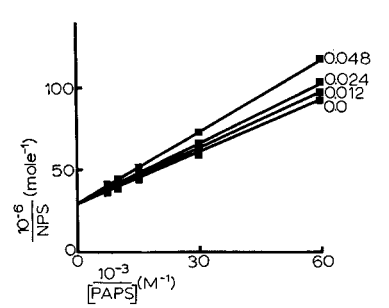


Fig. 3. The effect of different concentrations (mM, indicated by the figures on the lines) of *p*-nitrophenyl sulphate (NPS) on the rate of formation of *p*-nitrophenyl sulphate at variable concentrations of *p*-nitrophenol (NP) and at a fixed concentration (0.1 mM) of 3'-phosphoadenylyl sulphate.

Fig. 4. The effect of different concentrations (mM, indicated by the figures on the lines) of *p*-nitrophenyl sulphate (NPS) on the rate of formation of *p*-nitrophenyl sulphate at variable concentrations of 3'-phosphoadenylyl sulphate (PAPS) and at a fixed concentration (0.05 mM) of *p*-nitrophenol.

In the case of the inhibition by *p*-nitrophenyl sulphate this was, both with *p*-nitrophenol as the variable substrate and 3'-phosphoadenylyl sulphate non-saturating (0.1 mM) and with 3'-phosphoadenylyl sulphate as the variable substrate and *p*-nitrophenol non-saturating (0.05 mM), purely competitive (Figs. 3 and 4, respectively). The apparent inhibition constants, K_i', were obtained by replotting the slopes of the lines shown in these figures against the concentration of the appropriate fixed substrate. These plots were both straight lines and gave values for K_i' of 0.11 mM and 0.13 mM *p*-nitrophenyl sulphate, respectively.

With 3'-phosphoadenylyl sulphate as the variable substrate and *p*-nitrophenol non-saturating (0.05 mM), adenosine 3',5'-diphosphate was likewise a competitive

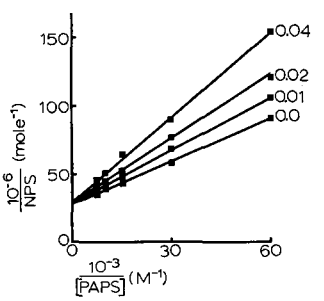


Fig. 5. The effect of different concentrations (mM, indicated by the figures on the lines) of adenosine 3',5'-diphosphate on the rate of formation of *p*-nitrophenyl sulphate (NPS) at variable concentrations of 3'-phosphoadenylyl sulphate (PAPS) and at a fixed concentration (0.05 mM) of *p*-nitrophenol.

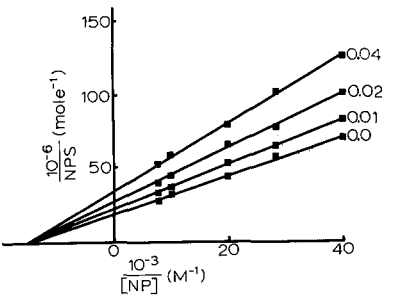


Fig. 6. The effect of different concentrations (mM, indicated by the figures on the lines) of adenosine 3',5'-diphosphate on the rate of formation of *p*-nitrophenyl sulphate (NPS) at variable concentrations of *p*-nitrophenol (NP) and at a fixed concentration (0.067 mM) of 3'-phosphoadenylyl sulphate.

inhibitor (Fig. 5) with a K_i of 0.039 mM. On the other hand, with *p*-nitrophenol as the variable substrate and with 3'-phosphoadenylyl sulphate non-saturating (0.067 mM), adenosine 3',5'-diphosphate was a non-competitive inhibitor as is shown in Fig. 6. The values of K_i' were 0.050 mM and 0.046 mM when obtained from the

TABLE III

THE VALUES OF THE MICHAELIS CONSTANTS, K_m, AND THE APPARENT INHIBITION CONSTANTS, K_i', FOR PHENOL SULPHOTRANSFERASE

Details of the experimental procedures are given in the text.

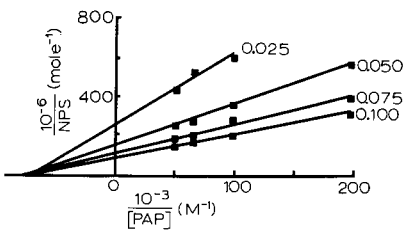
Substrate or inhibitor	K_m *(mM)*	K_i' *(mM) from experiments with varying*	
		Nitrophenol	*3'-Phosphoadenylyl sulphate*
p-Nitrophenol	0.070	—	—
3'-Phosphoadenylyl sulphate	0.036	—	—
p-Nitrophenyl sulphate	0.110	0.130	0.110
Adenosine 3',5'-diphosphate	0.014	0.050, 0.046*	0.039

* In this case alone the inhibition was non-competitive and the two values of K_i' were derived from the slopes and vertical intercepts, respectively, of the appropriate reciprocal plots.

secondary plots, which were rectilinear, of the slopes and the vertical intercepts, respectively, of this figure.

This information on the product inhibition of the formation of *p*-nitrophenyl sulphate is summarised in Table III.

Reverse reaction. The results of these studies are summarised in Figs. 7 and 8. Fig. 7 shows that the reciprocal plots obtained at fixed concentrations of *p*-nitrophenyl sulphate and at variable concentrations of adenosine 3',5'-diphosphate were straight lines intersecting on the abscissa. A replot of the intercepts on the ordinate of Fig. 7 against the reciprocal of the concentrations of *p*-nitrophenyl sulphate again gives a straight line, as shown in Fig. 8. These plots show that the two combining sites on the enzyme are independent and that the two K_m values were 0.014 mM adenosine 3',5'-diphosphate and 0.110 mM *p*-nitrophenyl sulphate, respectively.



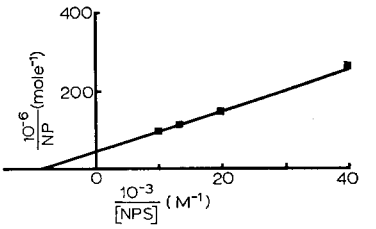


Fig. 7. The effect of varying concentrations of adenosine 3',5'-diphosphate (PAP) on the rate of formation of *p*-nitrophenol (NP) at fixed concentrations of *p*-nitrophenyl sulphate; the concentrations (mM) of the latter are shown by the figures on the lines.

Fig. 8. The replot of the intercepts on the ordinate of Fig. 7 against the reciprocals of the appropriate concentration of *p*-nitrophenyl sulphate (NPS). NP, *p*-nitrophenol.

Maximum velocities. The data summarised in Figs. 1 and 2 and 7 and 8 can also yield values for the maximum velocities of the reaction, V_1 for the forward reaction and V_2 for the reverse reaction, theoretically attainable at saturating concentrations

TABLE IV

THE VARIATION OF THE K_m VALUES OF PHENOL SULPHOTRANSFERASE WITH pH

The buffers were acetic acid–sodium acetate at and below pH 5.8 and imidazole–acetic acid above this value.

pH	*K_m (mM) for*	
	p-Nitrophenol	*3'-Phosphoadenylyl sulphate*
5.1	0.097	0.038
5.4	0.069	0.038
5.6	0.069	0.036
5.8	0.059	0.039
6.0	0.033	0.094
6.2	0.029	0.103
6.5	0.028	0.072
7.0	0.024	0.048
7.6	0.021	0.040

of both substrates. These constants were obtained from secondary plots of the type shown in Fig. 8 which itself gives a value for V_2, the intercept on the ordinate being $1/V_2$. Similar secondary plots were obtained from Figs. 1 and 2: replots of the intercepts on the ordinates of these figures (which are the reciprocals of the maximum velocities attainable at infinite concentration of one substrate but at several fixed concentrations of the other) against the reciprocals of the corresponding concentrations of the "fixed" substrate gave two straight-line graphs comparable to Fig. 8. The intercepts on the ordinates of these graphs therefore gave two independent estimates of $1/V_1$. Under the experimental conditions detailed above and at an arbitrary concentration of enzyme, the values obtained were 8.7 mμmoles/min and 1.8 mμmoles/min. respectively, for V_1 and V_2.

Effect of pH on K_m and V_1. This was studied only for the forward reaction over the pH ranges of 5.1 to 5.8 in sodium acetate–acetic acid buffers and of 6.0 to 7.6 in imidazole–acetic acid buffers, both types being at a final concentration of 0.2 M in the reaction mixture. At a constant concentration (0.1 mM) of *p*-nitrophenol and variable concentrations of 3′-phosphoadenylyl sulphate the double reciprocal plots (corresponding to those in Fig. 2) were all straight lines and the values of K_m varied only about 3-fold over the entire pH range, as shown in Table IV. With, on the other hand, a constant concentration of 3′-phosphoadenylyl sulphate (0.2 mM) and a variable concentration of *p*-nitrophenol the double reciprocal plots (corresponding

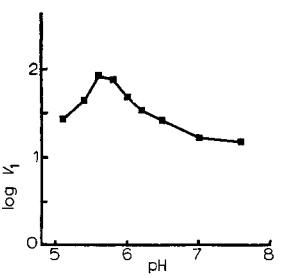


Fig. 9. The plot of log V_1 against pH. V_1 is the maximum velocity for the rate of formation of *p*-nitrophenyl sulphate calculated from the experimentally determined velocity and the known values of K_m for *p*-nitrophenol and 3′-phosphoadenylyl sulphate which are listed in Table IV. At pH values of 5.8 and below the reaction mixture contained 0.1 M acetic acid–sodium acetate buffer and at higher pH values it contained 0.1 M imidazole–acetic acid buffer.

to those in Fig. 1) were rectilinear only at pH values of 5.8 and below. At pH values greater than 6.2 substrate inhibition became increasingly obvious at concentrations of *p*-nitrophenol greater than about 0.1 mM. Again, however, the value of K_m varied only slightly over the pH range studied, as also is shown in Table IV.

The values of V_1, the maximum velocity of the forward reaction, showed a rather flat optimum at pH 5.6 to 5.8 and the plot of log V_1 against pH is shown in Fig. 9. The breaks in the curve at pH values of approx. 5.6 and 5.9 presumably reflect the pK values of ionising groups in the ternary enzyme–substrate complex but a detailed interpretation is not possible at this stage.

Specificity studies

2-Naphthol. The reaction with this substrate was studied in some detail in

order to verify its similarity to that with *p*-nitrophenol. The pH optimum for the formation of 2-naphthyl sulphate by phenol sulphotransferase was 5.6, as was that for the formation of *p*-nitrophenyl sulphate, and the effects of changes in substrate concentration were exactly comparable for the two phenols, the double reciprocal plots with 2-naphthol as substrate being similar to those shown in Figs. 1 and 2. Again, therefore, the values of the K_m for the two substrates were independent of each other and were 0.025 ± 0.004 mM 2-naphthol and 0.063 ± 0.017 mM 3'-phosphoadenylyl sulphate. As shown by Student's *t* test, the latter value was not significantly different from the value obtained with *p*-nitrophenol as the acceptor.

Other acceptors. From the above result it seemed valid to assume that phenol sulphotransferase would show similar kinetics with all substrates and that the K_m value of the phenol would be independent of the concentration of 3'-phosphoadenylyl sulphate: accordingly, other potential acceptors were tested at only one concentration of the latter. The values of K_m and V_1 for each compound were obtained

TABLE V

THE SPECIFICITY OF PHENOL SULPHOTRANSFERASE

The values of K_m and V_1 for the various substrates were obtained as described in the text, and the figures for V_1 are expressed relative to that for *p*-nitrophenol.

Substrate	*Relative* V_1	K_m *(mM)*
Phenol	0.30	2.50
p-Nitrophenol	1.00	0.070
1-Naphthol	1.02	0.025
2-Naphthol	0.95	0.025
5,6,7,8-Tetrahydro-2-naphthol	0.63	0.056
4-Nitro-1-naphthol	0.39	0.018
2-Phenanthrol	0.51	0.017
15,16-Dihydro-3-hydroxy-17-oxocyclopentena-[*a*]phenanthrene	0.13	0.015
Equilin	0.21	0.020
Equilenin	0.10	0.015
Oestrone	0.00	—

graphically from the usual double reciprocal plot and the results are shown in Table V. It is clear that all of the phenols tested were substrates for phenol sulphotransferase with the exception of oestrone.

This inability of oestrone to act as a substrate for the phenol sulphotransferase of guinea-pig liver has previously been reported[1] and these observations have now been extended by the demonstration that oestrone, under a variety of conditions does not inhibit the formation of *p*-nitrophenyl sulphate by this enzyme. This is shown by the results in Table VI.

Another potential substrate of phenol sulphotransferase is tyrosine methyl ester: this compound does inhibit the synthesis of *p*-nitrophenyl sulphate, as is also shown in Table VI. This inhibition is competitive, with an apparent K_i' of 0.02 M, which suggests that tyrosine methyl ester may well be a substrate for phenol sulphotransferase. As the sulphate ester which would be formed by this reaction does not yield a chloroform-soluble methylene blue salt this possibility could not be further investigated at this stage.

TABLE VI

THE INHIBITION OF THE SYNTHESIS OF *p*-NITROPHENYL SULPHATE BY OESTRONE AND BY TYROSINE METHYL ESTER

Concentration (mM) of		*Relative activity*
p-Nitrophenol	*Modifier*	
Oestrone		
0.100	0	1.00
0.100	0.100	0.99
0.025	0.100	1.00
Tyrosine methyl ester		
0.100	0	1.00
0.100	20	0.70

p-Nitrophenyl sulphate as a sulphate donor

Attempts were made to show the sulphotransferase-catalysed transfer of the sulphuryl group from *p*-nitrophenyl sulphate to phenol or to 2-naphthol in the presence of catalytic amounts of adenosine 3′,5′-diphosphate by a reaction similar to that previously described by GREGORY AND LIPMANN[9] and by BRUNNGRABER[10]. As shown by the results in Fig. 10 only negligible amounts of such a transfer, as measured by the appearance of *p*-nitrophenol, were detected at pH 5.6 and at concentrations of adenosine 3′,5′-diphosphate between 2 μM and 40 μM. It is clear

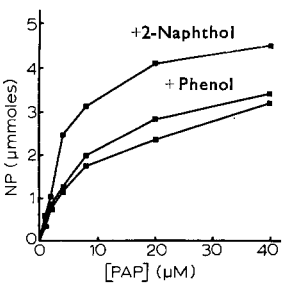


Fig. 10. The effect of the addition of phenol (4 mM) or 2-naphthol (0.1 mM) on the rate of formation of *p*-nitrophenol (NP) from *p*-nitrophenyl sulphate (0.05 mM) in the presence of varying concentrations of adenosine 3′,5′-diphosphate (PAP).

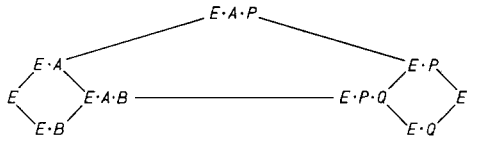
that the addition of either of those phenols scarcely increased the rate of the simple reverse reaction. Similar results were obtained with both the available preparations of adenosine 3′,5′-diphosphate either at pH 5.6, as in Fig. 10, or at pH 7.5, the pH used by GREGORY AND LIPMANN[9].

DISCUSSION

The general properties of phenol sulphotransferase need little comment except to stress the apparent instability of this enzyme. Attempts at further purification have not been successful: although preparations having a specific activity at least

ten times greater than that reported here have been obtained by chromatography on ECTEOLA-cellulose such preparations were obtained in only small amounts and were extremely unstable so that they could not be used for the work described in this paper.

The kinetic study of the forward reaction showed firstly that the values of the K_m for the two substrates, *p*-nitrophenol and 3′-phosphoadenylyl sulphate, were independent of one another; secondly that one product, *p*-nitrophenyl sulphate, acted as a competitive inhibitor with respect to both of those substrates; and thirdly that the other product, adenosine 3′,5′-diphosphate, was a competitive inhibitor with respect to 3′-phosphoadenylyl sulphate but a non-competitive inhibitor with respect to *p*-nitrophenol. Similarly, in the reverse reaction the values of K_m for the two substrates, *p*-nitrophenyl sulphate and adenosine 3′,5′-diphosphate, were independent of each other. By applying the criteria defined by CLELAND[4], it can be concluded from these results that the reaction catalysed by the phenol sulphotransferase of guinea-pig liver is a rapid equilibrium random bi bi reaction with one dead-end complex of adenosine 3′,5′-diphosphate and *p*-nitrophenol. This reaction can be represented as



where E is phenol sulphotransferase, A is *p*-nitrophenol, Q is *p*-nitrophenyl sulphate, B is 3′-phosphoadenylyl sulphate and P is adenosine 3′,5′-diphosphate. In a rapid equilibrium reaction of this type all the reactions are rapid except the interconversion of the two normal ternary complexes, $E \cdot A \cdot B$ and $E \cdot P \cdot Q$, which is rate limiting.

The velocity equation for a reaction of this type is given by

$$v = \frac{V_1 V_2 \left[AB - \frac{PQ}{K'}\right]}{K_{ia}K_b V_2 + K_b V_2 \left[1 + \frac{P}{K_i}\right] A + K_a V_2 B + V_2 AB + \frac{K_q V_1 P}{K'} + \frac{K_p V_1 Q}{K'} + \frac{V_1 PQ}{K'}}$$

where the dissociation constants are defined as follows

$$K_a = \frac{EB \cdot A}{EAB} \qquad K_b = \frac{EA \cdot B}{EAB} \qquad K_p = \frac{EQ \cdot P}{EPQ} \qquad K_q = \frac{EP \cdot Q}{EPQ}$$

$$K_{ia} = \frac{E \cdot A}{EA} \qquad K_{iq} = \frac{E \cdot Q}{EQ} \qquad K_i = \frac{EA \cdot P}{EAP}$$

and the equilibrium constant is given by

$$K' = \frac{V_1 K_p K_{iq}}{V_2 K_{ia} K_b}$$

This rather complex expression can be considerably simplified when each K_{ix} term

is equal to the corresponding K_x term, as is the case in the present situation where the K_m value of any one substrate is independent of the concentration of the other. A further simplification occurs when only initial velocities, either in the forward or the reverse direction, are considered. With these two restrictions the appropriate equations become:
for the forward reaction

$$v = \frac{V_1 AB}{K_a K_b + K_b A + K_a B + AB}$$

for the reverse reaction

$$-v = \frac{V_2 PQ}{K_p K_q + K_q P + K_p Q + PQ}$$

When one of the products is present as an inhibitor the first of these equations is transformed into the following:
for inhibition by P

$$v = \frac{V_1 AB}{K_a K_b + K_b \left[1 + \frac{P}{K_i}\right] A + K_a B + AB + \frac{K_a K_b}{K_p} P}$$

for inhibition by Q

$$v = \frac{V_1 AB}{K_a K_b + K_b A + K_a B + AB + \frac{K_a K_b}{K_q} Q}$$

Conversion of these equations into the reciprocal forms immediately makes clear the significance which must be attached to the experimentally determined values of K_m and K_i' reported above. This aspect has been discussed by CLELAND[4] and here it must suffice to say that from these figures values for the true dissociation constants of the enzyme–substrate complexes, both for the forward and the reverse reactions, can be calculated. Table VII summarises the values for those constants, some of

TABLE VII

THE DISSOCIATION CONSTANTS OF THE ENZYME–SUBSTRATE COMPLEXES OF THE PHENOL SULPHOTRANSFERASE OF GUINEA-PIG LIVER AT 37° IN 0.1 M ACETATE BUFFER (pH 5.6)

Substrate	K_s *(mM) calculated from*		
	K_m	K_i'*	K_i'**
p-Nitrophenol	0.070 ± 0.012	—	—
3′-Phosphoadenylyl sulphate	0.036 ± 0.010	—	—
p-Nitrophenyl sulphate	0.110	0.068 ± 0.008	0.034 ± 0.012
Adenosine 3′,5′-diphosphate	0.014	0.024 ± 0.002	0.019 ± 0.001***

* From experiments in which 3′-phosphoadenylyl sulphate was the variable substrate.
** From experiments in which *p*-nitrophenol was the variable substrate.
*** This value is also that for the dead-end ternary complex.

which were obtained from more than one type of experiment. It should again be pointed out that the values for *p*-nitrophenyl sulphate, especially when this is used as a substrate, are less accurate than the others because of inherent experimental difficulties.

From these values, and from those of V_1 and V_2 reported above, it is possible to calculate the value of K', the apparent equilibrium constant for the reaction between *p*-nitrophenol and 3'-phosphoadenylyl sulphate to give *p*-nitrophenyl sulphate and adenosine 3',5'-diphosphate. The appropriate definition of K' has been given above and substitution of the values for the constants gives K' equal to 3 at pH 5.6 and at 37°. This must be compared with the value of 26 obtained by GREGORY AND LIPMANN[9] for the same reaction at pH 7.8 and at room temperature. The difference between the two values is not surprising: not only do several of the components of the reaction mixture ionise over the pH range involved so that differences in K' are to be expected, but the methods of determining K' were themselves quite different. The present value is obtained from kinetic measurements, basically from the initial velocities of the enzymatic reaction in both directions, while that of GREGORY AND LIPMANN[9] was obtained by determining the amount of *p*-nitrophenol present at equilibrium which was only attained after 2 or 3 h. As the crude enzyme preparation used by these authors undoubtedly contained enzymes capable of degrading 3'-phosphoadenylyl sulphate considerable errors could have arisen. Accepting the value of 3 for K', then the corresponding value for ΔG° is only -680 cal, a very small figure which implies that the "sulphate group potential" of 3'-phosphoadenylyl sulphate is even closer to that of *p*-nitrophenyl sulphate than was thought[9].

That the phenol sulphotransferase reaction is a rapid equilibrium random bi bi reaction in which the two substrate-combining sites are completely independent is interesting because a similar situation holds with some of the phosphotransferases[11] although in others the two substrate sites are not independent[12]. This type of reaction mechanism may be characteristic of the sulphotransferases in general because in the only other examples studied, androstenolone sulphotransferase[13] and choline sulphotransferase[14], the substrate sites are certainly independent and the preliminary results which are available would not be inconsistent with these two enzymes also catalysing a rapid equilibrium random bi bi reaction.

The specificity studies which have been carried out are not sufficiently detailed to allow any conclusions to be drawn about the nature of the substrate-combining site but the fact that oestrone, alone of the phenols studied, is not a substrate suggests that only phenols having an essentially planar structure can combine with the enzyme. It must, of course, be stressed that oestrone sulphate may be formed by the enzyme but only at a rate too low to be detected by the methods used in the present study.

An aryl sulphate of particular interest is tyrosine *O*-sulphate which occurs in the urine of several mammalian species[15] and in some polypeptides[16–18] although it apparently cannot be formed directly from tyrosine[19]. Derivatives of tyrosine in which the carboxyl group is blocked and the amino group is free can, however, be sulphurylated by sulphotransferases from a number of tissues[19,20]. As shown above, tyrosine methyl ester is a competitive inhibitor of the formation of *p*-nitrophenyl sulphate by the phenol sulphotransferase of guinea-pig liver, suggesting that this enzyme might be capable of using the ester as a substrate. This has been confirmed by J. G. JONES AND P. MATTOCK (personal communication) who have shown that the

preparations of phenol sulphotransferase used in the present study can indeed sulphurylate tyrosine methyl ester. It has not, of course, been shown that only a single sulphotransferase is present in this preparation.

The apparent inability of the phenol sulphotransferase of guinea-pig liver to catalyse the transfer of the sulphuryl group from *p*-nitrophenyl sulphate to either phenol or 2-naphthol, both of which are substrates for the enzyme, is surprising in view of the catalysis of this reaction by a crude sulphotransferase from rabbit liver[9,10]. The reason for this discrepancy is not clear. Possible contributory factors are a species difference between the two enzymes, the fact that previous workers[9,10] may have been studying a mixture of several sulphotransferases, and the fact that their preparation undoubtedly contained other enzymes—such as those metabolising 3'-phosphoadenylyl sulphate—which might have had an unexpected role to play. Certainly the existence of a transferase reaction might have been predicted from the values of the several kinetic constants given above but it must be stressed that the kinetic behaviour of such a transfer would be extremely complex, the reaction sequence being a combination of two of the type shown above, so that detailed predictions on its course would be hazardous.

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