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THE SULPHATASE OF OX LIVER

XV. CHANGES IN THE PROPERTIES OF SULPHATASE A IN THE PRESENCE OF SUBSTRATE

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SUMMARY

Enzyme with modified kinetic properties has been isolated from reaction mixtures of sulphatase A (arylsulphate sulphohydrolase, EC 3.1.6.1) and its substrate, either nitrocatechol sulphate or 4-nitrophenyl sulphate. A method is described for the determination of the proportion of the modified enzyme in these preparations. The position of the equilibrium between the native and modified forms is invariant with enzyme concentration and with pH between pH 5 and 7. Sulphate displaces the equilibrium in favour of the native enzyme primarily through the formation of a native enzyme-sulphate complex.

The modified enzyme has the same sedimentation coefficients as native sulphatase A at pH 5 and 7.5 and does not contain any detectable 4-nitrocatechol or nitrocatechol sulphate.

The apparently inactive modified enzyme is strongly activated by SO_4^{2-}, but only slightly activated by 4-nitrocatechol. Phosphate and pyrophosphate, inhibitors of the native enzyme, also activate the modified enzyme. The low activity of the modified enzyme was found to be due to a powerful substrate inhibition which is decreased in the presence of SO_4^{2-}. A reaction mechanism which explains the anomalous kinetics of sulphatase A has been proposed.

INTRODUCTION

ROY[1] first noted that the kinetics of the reaction catalysed by sulphatase A (arylsulphate sulphohydrolase, EC 3.1.6.1) of ox liver was not that of a simple enzyme reaction and subsequently[2] showed that the progress curves for the hydrolysis of nitrocatechol sulphate by the enzyme were anomalous. Examples of progress curves determined at that time are shown in Fig. 1. BAUM *et al.*[3] and BAUM AND DODGSON[4] made a detailed study of the kinetics of the reaction catalysed by sulphatase A from human liver which showed similar anomalies. They suggested a mechanism in which a modified form of sulphatase A was slowly produced during its reaction with sub-

strate. They further postulated that the modified enzyme contained a second site which could bind either substrate to form an inactive complex (of the type ES_2) or a sulphate ion to give a complex (of the type EIS) which could break down to form reaction products. ANDERSEN[5] also concluded that the sulphatase A of ox liver was converted into a modified form during the catalytic reaction but, on the basis of experiments carried out in the presence of Sr^{2+}, suggested that 4-nitrocatechol was the product responsible for the increased velocity in the later stages of progress curves of the type shown in Fig. 1. These early observations, which were made with only partly purified preparations of sulphatase A, all showed that one or both of the reaction products were involved in the increased velocity during the later stages of the reaction although they had no effect on the enzyme in the absence of substrate. Sulphate can also act as an inhibitor of sulphatase A[1].

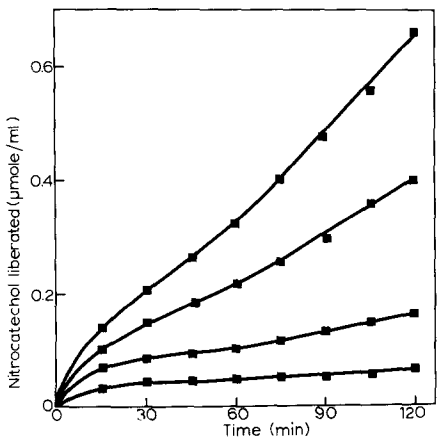


Fig. 1. Progress curves for the hydrolysis of 3 mM nitrocatechol sulphate in 0.125 M acetate buffer (pH 5) at 37° by a crude preparation of sulphatase A. The relative enzyme concentrations in the reactions are, from bottom to top of the figure, 1, 2, 3 and 4.

The sulphatase A of ox liver has now[6] been obtained in a homogeneous form and some of its physical properties[7,8] are pertinent in view of the early suggestion[1] that the kinetic anomalies might arise through the polymerization of the enzyme. In uni-univalent buffers, ionic strength 0.1, sulphatase A exists as a monomer (mol. wt. 107 000) at pH values greater than 6.5 and as a tetramer at pH values less than 5.5 and at protein concentrations greater than about 5 μg/ml. At intermediate pH values the enzyme exists as an equilibrium mixture of polymeric forms. At pH 5, but at protein concentrations of less than about 5 μg/ml, the tetramer dissociates to give lower polymers of which the monomer is probably dominant.

The present paper describes the isolation of a modified form of sulphatase A from reaction mixtures containing the enzyme and its substrate and shows how the properties of this form can account for the anomalous kinetics, as exemplified by the progress curves shown in Fig. 1.

MATERIALS AND GENERAL METHODS

Enzyme

Sulphatase A was prepared from ox liver as previously described[6] except that the final chromatography at pH 5 was carried out on Sephadex G-200 rather than on Sephadex G-100.

Substrate

Dipotassium 2-hydroxy-5-nitrophenyl sulphate was prepared by the method of Roy[9], reconverted to the monopotassium salt and crystallised from 75% ethanol until it was free from SO_4^{2-} and contained less than 0.1% of 4-nitrocatechol.

Determination of sulphatase A activity

In all cases the substrate was monopotassium 2-hydroxy-5-nitrophenyl sulphate and the reaction temperature was 37°.

In most experiments the course of the reaction was followed by titration of the liberated H^+ with 0.007 M carbonate-free NaOH in a pH-stat (PHM26-TTT11-SBR2-SBU assembly Radiometer Ltd., Copenhagen). The reaction mixture had a volume of 10 ml and contained 0.1 M NaCl and 0.01% bovine serum albumin: the pH, enzyme concentration and substrate concentration were varied as required. The reaction was started by the addition of not more than 0.2 ml of substrate solution to the remainder of the reaction mixture which had been equilibrated for 5 min. It was found that the presence of bovine serum albumin was necessary to prevent an irreversible inactivation which was otherwise caused by prolonged stirring of dilute solutions of sulphatase A. Initial velocities were measured from the slopes of the pH-stat recordings during the second minute of the reaction: unavoidable irregularities made the slope at earlier times difficult to determine.

For some purposes a spectrophotometric method was used to determine the liberated 4-nitrocatechol. In these assays the reaction mixture was buffered with sodium acetate–acetic acid buffers at pH 5 or 6 and with imidazole–HCl buffers at pH 7. The reaction was started by adding a small volume of enzyme and after incubation for the appropriate period a suitable sample (0.05–0.25 ml) was diluted in 0.2 M KOH and the extinction measured at 510 nm (ε at 510 nm = 12 600 for 4-nitrocatechol).

Recovery of enzyme from reaction mixtures

A suitable volume (usually 5 ml) of reaction mixture was applied to a column (1.5 cm × 24 cm) of Sephadex G-25 equilibrated at 2° with 0.1 M NaCl containing 0.5 mM sodium acetate (pH 6) and eluted with the same buffer. The column had previously been calibrated with bovine serum albumin so that the elution volume of protein was accurately known and a 10 ml fraction of the eluate containing the enzyme was collected. This was dialysed against several changes of the above buffer for 24 h at 2°. Control experiments showed that 85% of the enzyme applied to the column was recovered by this technique, even at the low enzyme concentrations used in these experiments.

In some cases a large column (5 cm × 60 cm) was used to handle up to 100 ml

of reaction mixture. The eluate was in this case concentrated by ultrafiltration through a Diaflo UM10 membrane at 2°.

Ultracentrifugation

Sedimentation coefficients were measured at 2° in aluminium-filled epon double sector cells in an An-E rotor in the Spinco Model E ultracentrifuge at speeds of 48 000 or 52 000 rev./min. Sedimenting boundaries were analysed by the method of BALDWIN[10].

RESULTS

Isolation of a modified form of sulphatase A

Sulphatase A was incubated for 30 min at 37° in a reaction mixture containing 0.1 M NaCl, 0.1 M sodium acetate (pH 6) and 0.05 M nitrocatechol sulphate. Such reactions are subsequently called preparative reactions and their typical progress curves, determined spectrophotometrically, are similar to those of Fig. 1. The sulphatase A was recovered from the reaction mixture by the method already described and Fig. 2 shows the progress curves for the hydrolysis of nitrocatechol sulphate by this enzyme and by a similar concentration of native sulphatase A. Addition of 0.05 ml of 0.2 M K_2SO_4 (to give a concentration of 1 mM SO_4^{2-}, ignoring the small amount produced by the hydrolysis of the substrate) 30 min after the start of the reaction causes the reaction velocity to increase. The increase is not instantaneous and only after about 10 min is the maximum rate attained. During the preparative

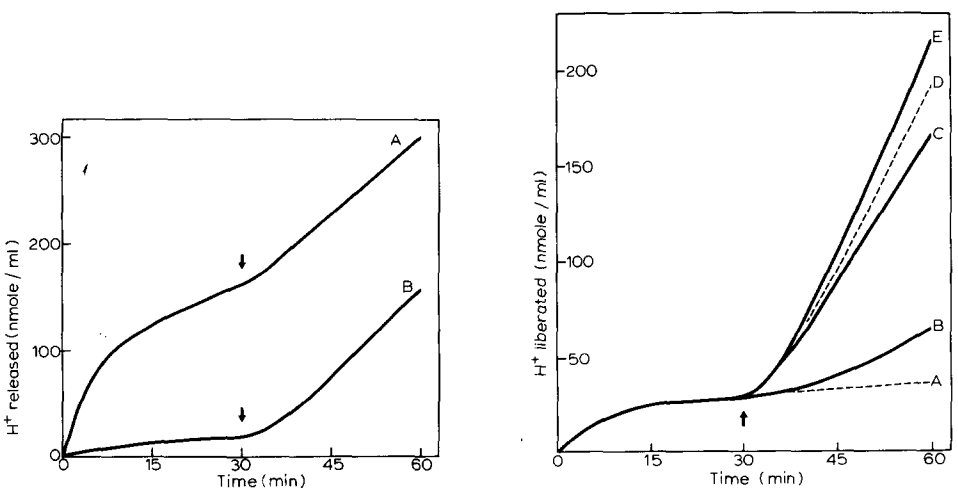


Fig. 2. Progress curves for the hydrolysis of 1 mM nitrocatechol sulphate by (A) native sulphatase A and (B) modified sulphatase A recovered from a preparative reaction. Assay in the pH-stat at pH 6 and 37°. At 30 min 10 μmoles of K_2SO_4 were added to both reaction mixtures (volume 10 ml). Protein concentration 0.25 μg/ml in both cases.

Fig. 3. Progress curves for the hydrolysis of 1 mM nitrocatechol sulphate by modified sulphatase A. At 30 min the following additions were made: A, none; B, 8.6 μmoles 4-nitrocatechol; C, 10 μmoles K_2SO_4; E, 8.6 μmoles 4-nitrocatechol and 10 μmoles K_2SO_4. Curve D shows the summation of the separate effects of 4-nitrocatechol and K_2SO_4 for comparison with curve E.

reaction the enzyme has been modified so that, compared with the native enzyme, its initial velocity is almost zero but its velocity in the presence of 1 mM SO_4^{2-} is hardly altered.

It should be noted that in experiments such as these shown in Fig. 2 the time of addition of K_2SO_4 is not critical. In the absence of SO_4^{2-} the slow rate of hydrolysis of nitrocatechol sulphate by modified sulphatase A is maintained for several hours and even after 4 h the addition of K_2SO_4 causes the characteristic increase in velocity.

Fig. 3 shows the effect of 4-nitrocatechol on modified sulphatase A. It is clear that this, the second reaction product, also activates the modified enzyme. Its effect is less than that of an equivalent concentration of SO_4^{2-} and has been ignored in subsequent experiments.

When the substrate in the preparative reaction is 0.1 M 4-nitrophenyl sulphate rather than nitrocatechol sulphate a similarly modified sulphatase A is produced. The progress curve of the latter, with nitrocatechol sulphate as substrate, was indistinguishable from curve B in Fig. 2.

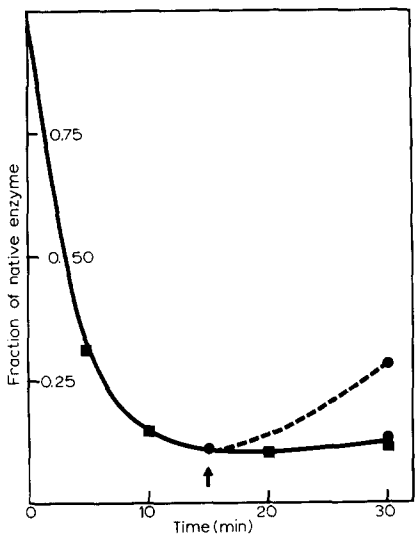
Phosphate and pyrophosphate ions can replace SO_4^{2-} as activators of the modified enzyme although they, like SO_4^{2-}, are inhibitors of native sulphatase A. The effects of these anions have not been further investigated because of the complications likely to arise by the unavoidable presence of two activating species, the relative amounts of which must vary during the reaction because of the production of SO_4^{2-}.

Estimation of amount of modified enzyme present

The fraction of modified sulphatase A present in any preparation may be obtained from the ratio of the initial rate to the final rate (between 20 and 30 min after the addition of K_2SO_4) under the conditions specified in Fig. 2. If it is assumed that the modified enzyme is inactive in the absence of SO_4^{2-} then the initial rate is proportional to the concentration of native sulphatase A in the enzyme used to start the reaction. The final rate is proportional to the concentration of native plus modified sulphatase A in the ratio determined by the standard conditions of the assay. When native sulphatase A is used to start the reaction both rates are linearly related to the enzyme concentration and the ratio of initial to final rates is 3.45 (range in five experiments, over a 3-fold variation in enzyme concentration, 3.37–3.67) so that the fraction of modified sulphatase A in any preparation is obtained by multiplying the ratio of initial to final rates by the factor 0.29 (*i.e.* 1/3.45). On this basis the enzyme used to give curve B in Fig. 2 contained 91% of modified enzyme.

Equilibrium between native and modified enzyme

Figs. 4 and 5 show the results of experiments in which the proportion of modified enzyme was determined at various times during the preparative reaction. At an enzyme concentration of 1 μg/ml (progress curve similar to lowest curve in Fig. 1) the fraction of native enzyme falls to 0.1–0.15 within 15 min and remains at this value (Fig. 4). At an enzyme concentration of 10 μg/ml (progress curve similar to uppermost curve in Fig. 1) the fraction of native enzyme falls and then rises again (Fig. 5). To eliminate the effects of considerable amounts of SO_4^{2-} produced during the preparative reaction at the higher enzyme concentration Ba^{2+} was included in the reaction and the results are also shown in Fig. 5. Fig. 4 shows the converse



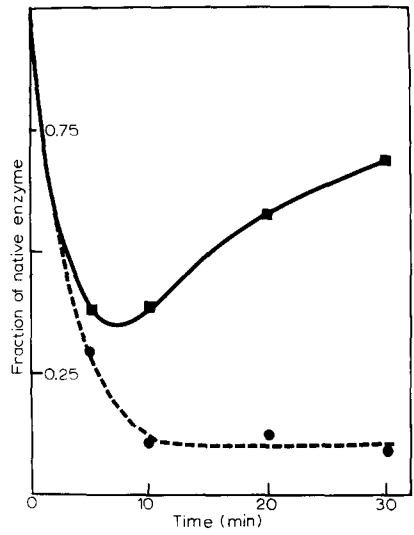


Fig. 4. Time course of the disappearance of native sulphatase A in a preparative reaction containing 1 μg enzyme/ml. The different symbols refer to two different experiments in one of which a sample of the preparative reaction was made 25 mM K_2SO_4 after 15 min incubation (- - - -).

Fig. 5. Time course of the disappearance of native sulphatase A in a preparative reaction containing 10 μg enzyme/ml. ■, standard preparative reaction; ●, preparative reaction containing 50 mM $BaCl_2$.

experiment in which SO_4^{2-} was added to a preparative reaction containing sulphatase A at a concentration of 1 μg/ml. These experiments establish that there is an equilibrium between the native and the modified forms of sulphatase A and that one of the factors governing the position of this equilibrium is the concentration of SO_4^{2-}, an increased concentration of SO_4^{2-} favouring an increased concentration of the native enzyme. It must be stressed that in these experiments no distinction is drawn between the various forms of native and modified sulphatase A which are present in the preparative reaction. The native enzyme, for example, can exist as free enzyme, enzyme–substrate and enzyme–sulphate complexes, but these are dissociated during the chromatography of the enzyme. A similar argument must apply to the various forms of the modified enzyme. That a similar change in the proportion of native to modified enzyme occurred during the assay of the latter, as in Fig. 2, was readily shown. The enzyme isolated just prior to the addition of SO_4^{2-} contained 15% of the native form while that isolated 30 min after the addition of SO_4^{2-} contained 45% of the native form.

The effect of varying pH and protein concentration on the equilibrium between the two forms of enzyme is shown in Fig. 6. Although there is an apparent dependence of the position of this equilibrium on protein concentration at pH 5 and 6, but not at pH 7, this effect is abolished by the inclusion of Ba^{2+} in the preparative reaction. Again the position of the equilibrium in the absence of Ba^{2+} can be related as above to the amount of SO_4^{2-} produced during the preparative reaction. The rate of disappearance of native enzyme is, however, pH-dependent. The half-time for this reaction—that is, the time for the rate of hydrolysis of nitrocatechol sulphate to

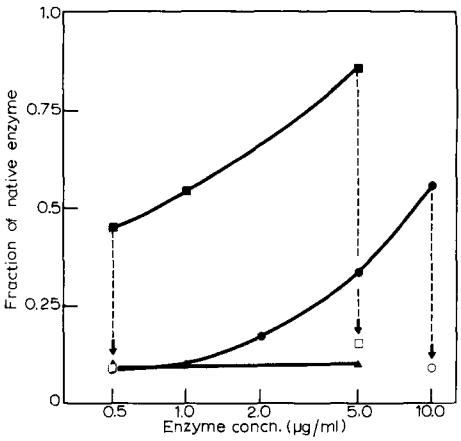


Fig. 6. The effect of enzyme concentration and pH of the preparative reaction on the proportion of native sulphatase A remaining after 30 min. ■, pH 5; ●, pH 6; ▲, pH 7 under standard preparative conditions; □, pH 5; ○, pH 6 in preparative reactions containing 50 mM $BaCl_2$.

drop to half its initial rate when Ba^{2+} is present—varies with pH. The values are 5.5 min, 2.0 min and 1.2 min at pH 5.0, 6.0 and 7.0, respectively.

Reconversion of modified to native sulphatase A

Modified sulphatase A reverted to native sulphatase A (defined as showing the kinetic properties of the native enzyme) only extremely slowly at 2° and in the pH range 5 to 7.5. After two weeks under these conditions the proportion of native enzyme increased by only a few percent, as shown by an increased initial rate and a constant final rate in assays as described in Fig. 2. At 37° however, the conversion of modified to native sulphatase was more rapid (Table I). The only point to stress is that the addition of 0.1 M K_2SO_4 made no significant difference to the rate of this conversion.

PHYSICAL PROPERTIES OF THE MODIFIED ENZYME

The large scale preparation used for this investigation contained about 50%

TABLE I

RECONVERSION OF MODIFIED TO NATIVE SULPHATASE A

The proportion of native enzyme was determined as described in the text. Initially, the modified preparation contained 16% native enzyme. Temperature of reaction 37°.

Conditions	*Native enzyme (%) present after*	
	4 h	*17 h*
0.1 M NaCl, 0.5 mM acetate (pH 6)	56	110
0.1 M NaCl, 0.5 mM acetate, 0.1 M K_2SO_4 (pH 6)	43	—
1 M KCl, 0.5 mM acetate (pH 6)	35	80
0.1 M NaCl, 1 M acetate (pH 8)	46	100

of native enzyme. The absorption spectrum in the region 220–450 nm showed no detectable difference from that of the normal enzyme: because of experimental difficulties small differences would have gone undetected but the modified enzyme contained less than one equivalent of 4-nitrocatechol or nitrocatechol sulphate.

The modified sulphatase A sedimented at pH 7.5 in 0.1 ionic strength barbitone buffer[6] with a sedimentation coefficient of 6 S and at pH 5 in 0.1 ionic strength acetate buffer[6] with a sedimentation coefficient of 14 S. These values are indistinguishable from those of native sulphatase A. The sedimenting boundary appeared symmetrical and the homogeneity with respect to sedimentation coefficient, irrespective of the presence of two enzyme forms, was shown by boundary analysis.

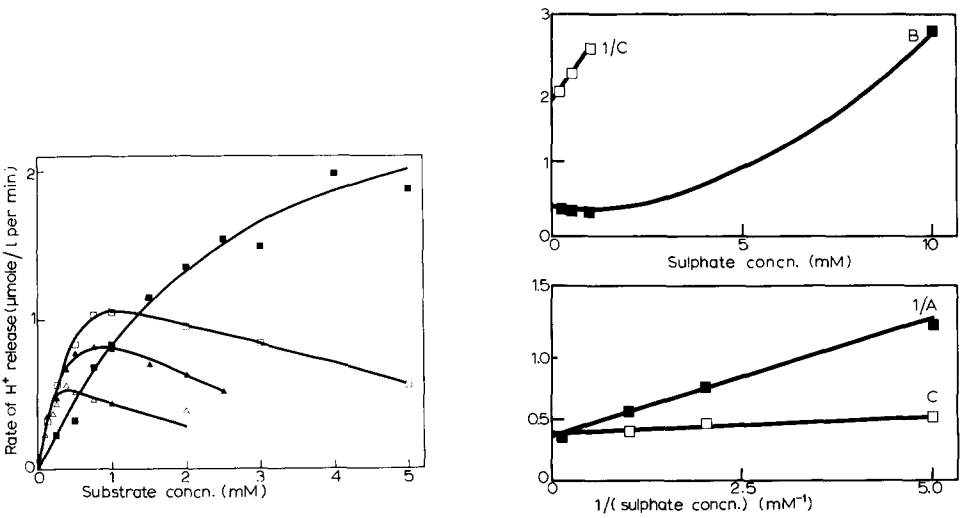


Fig. 7. The effect of varying substrate concentration on the rate of hydrolysis of nitrocatechol sulphate by modified sulphatase A at different concentrations of K_2SO_4. The concentrations of the latter refer to the amount added (see text for details). Assay in pH-stat at pH 6 and 37°. Amount of K_2SO_4 added (per 10 ml reaction mixture); 100 μmoles (■); 10 μmoles (□); 5 μmoles (▲); 2 μmoles (△).

Fig. 8. The variation with sulphate concentration of the coefficients A, B and C in the expression $v = As/(B + s + Cs^2)$. Estimates of the coefficients were obtained from the data in Fig. 7. The ordinates are in arbitrary units and the graphs are intended only to show the form of the relationships.

Kinetic properties of the modified enzyme

These were determined under the general conditions of Fig. 2 but with varying conditions of pH, substrate concentration and sulphate concentration. In all cases the enzyme was incubated with the substrate for 30 min before the required amount of K_2SO_4 was added: the rate was then measured after a further 30 min. The amount of SO_4^{2-} produced during the assay has been ignored in the present treatment of the data and in Fig. 7 the concentration of SO_4^{2-} refers only to that added after 30 min incubation. Because the enzyme concentration was kept low in these experiments the amount of SO_4^{2-} produced during the first 30 min incubation amounted in the least favourable case to about 7% of that actually added: the amount produced during the second 30 min was similar.

The optimum pH for the reaction was approx. 5.7, similar to that for native sulphatase A. The effects of substrate and SO_4^{2-} concentration are shown in Fig. 7. Modified sulphatase A is subject to powerful substrate inhibition which becomes apparent at lower substrate concentrations as the concentration of SO_4^{2-} is decreased. At high concentrations (10 mM) of SO_4^{2-} the substrate inhibition apparently disappears and an inhibition due to SO_4^{2-} at low substrate concentrations becomes apparent.

The data in Fig. 7 were fitted graphically[11] to the standard rate equation for substrate inhibition (Eqn. 1)

$$v = \frac{As}{B + s + Cs^2} \quad (1)$$

and Fig. 8 shows the variations of the coefficients A, B and C with the concentration of SO_4^{2-}.

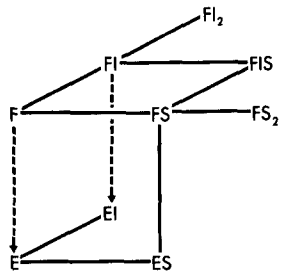
DISCUSSION

The method described above allows the separation of a stable modified form of sulphatase A which is produced during the reaction of the enzyme with a substrate, either nitrocatechol sulphate or 4-nitrophenyl sulphate. Its success depends upon the rapid removal at low temperatures of the substrate and reaction products.

The position of the equilibrium between the native and modified forms of the enzyme is independent of the concentration of the enzyme (Fig. 6), a finding which shows that the modification cannot be associated with a polymerization or depolymerization reaction. This is confirmed by the indistinguishable sedimentation coefficients at pH 5 and 7.5 of the native and modified forms of the enzyme. Therefore the modified enzyme must be similar in size to the native enzyme and still retain the latter's ability to polymerize. These results would appear to finally disprove the suggestion by ROY[1] that the anomalous kinetics of sulphatase A were caused by the polymerization of the enzyme.

The modified enzyme shows little, if any, activity in the absence of SO_4^{2-} but quite rapidly regains its activity when these ions are added (Fig. 2). The effect of 4-nitrocatechol is similar to that of SO_4^{2-} but quantitatively is less significant (Fig. 3): in what follows only the effect of SO_4^{2-} will be considered but the lesser effect of 4-nitrocatechol must not be forgotten. The observation (Fig. 8) that the coefficient A in equation 1 is dependent upon the concentration of SO_4^{2-} shows that an enzyme species which yields reaction products must contain SO_4^{2-}. Further, the modified enzyme shows strong substrate inhibition, the extent of which is dependent upon the concentration of SO_4^{2-}, being less the higher the concentration of SO_4^{2-} (Fig. 7). Finally, although SO_4^{2-} is necessary for the activity of the modified form of the enzyme it acts as an inhibitor at high concentrations, an effect which is particularly obvious at low substrate concentrations. These observations suggest the formation of the enzyme complexes FS_2, FI_2 and FIS where F is the modified enzyme, S nitrocatechol sulphate and 4-nitrophenyl sulphate and I sulphate, nitrocatechol, phosphate and pyrophosphate. These properties must be contrasted with those of the native enzyme which does not show substrate inhibition and is purely competitively inhibited by SO_4^{2-}: therefore although complexes such as ES and EI (where E is native sulphatase A) exist, ES_2, EI_2 and EIS do not.

The results presented here confirm the reaction scheme proposed by BAUM AND DODGSON[4] for human sulphatase A and can be represented as follows:



This scheme differs from that of BAUM AND DODGSON[4] mainly in its being more precise, and the basis of both is the formation of F which has a second site capable of binding substrate, products or other anions. No evidence for the direct conversion of E to F nor of EI to FI has been obtained in this or earlier[3,4] work. The reaction F to E does proceed although at a slower rate (half-times of several hours) than the other reactions in the scheme. It is not clear whether FI can form EI but the observation that the rate of conversion of F to E in the presence of SO_4^{2-} is not significantly different from that in the absence of SO_4^{2-} (Table I) suggests that these two reactions proceed at comparable rates and can be ignored in kinetic experiments.

Both the inactivation caused by the conversion of the native enzyme to the modified enzyme and the subsequent activation of the latter by SO_4^{2-} are slow reactions with half-times of 2–3 min. Therefore one or more of the reactions involving the enzymically inactive species must be slow. It seems a reasonable, but not a necessary, assumption that similar reactions will control the rate in both directions. In the above scheme the simplest assumption, and the one we adopt is that FS_2 is the inactive enzyme species and the reaction $FS + S \rightleftarrows FS_2$ is rate-limiting in both directions. Another possible rate-limiting reaction is the intercoversion $ES \rightleftarrows FS$: however, although this could account for the slow inactivation in the early stages of the reaction it could not account for the slow reactivation by SO_4^{2-} and another reaction, presumably one leading to FIS, would have to be rate-limiting under these conditions.

On the basis of the above scheme under equilibrium conditions (*i.e.* at least 15 min after the addition of SO_4^{2-}) and assuming that only ES and FIS can give reaction products (with velocity constants k_1 and k_2, respectively) the following velocity equation (Eqn. 2) was derived. If FS also gives the reaction products then Eqn. 2 would be of the same form but the coefficient in the numerator would be more complex. In this expression the constants K_1–K_9 are the dissociation constants of

$$v = \frac{\left[\frac{k_1K_3K_6 + k_2i}{K_3K_6 + K_6 + i}\right]es}{\left[\frac{K_3K_6}{K_3K_6 + K_6 + i}\right]\left[K_1 + \frac{K_4}{K_3} + \left(\frac{K_1}{K_2} + \frac{K_9}{K_3K_6}\right)i + \frac{K_9}{K_3K_6K_8}i^2\right] + s + \left[\frac{K_6}{K_5(K_3K_6 + K_6 + i)}\right]s^2} \quad (2)$$

ES, EI, FS (to ES), FS (to F), FS_2, FIS (to FS), FI, FI_2 and FIS (to FI), respectively. At constant SO_4^{2-} the equation is of the same form as Eqn. 1, and can

account for the observed variation of the coefficients A, B and C with the concentration of SO_4^{2-}.

We propose that complex progress curves of the form shown in Fig. 1 are explicable by the above reaction sequence, as follows (see also refs. 3 and 4). The enzyme–substrate complex *ES*, can undergo two reactions: one to give the reaction products and the native enzyme and another to form *FS* which in turn slowly forms FS_2, an inactive enzyme species. These reactions will lead to a slow substrate-dependent inactivation of sulphatase A. The subsequent behaviour will depend upon the concentration of SO_4^{2-} which is present. If this be low, either because of a low total enzyme concentration or because of the presence of Ba^{2+} in the reaction mixture, then FS_2 remains the dominant species and the rate of product formation falls to zero after about 10 min. At high concentrations of SO_4^{2-}, the situation is complicated by two opposing effects. Firstly, SO_4^{2-} can slowly displace the equilibrium between the *E*-containing species and *F*-containing species in favour of the former (Figs. 4 and 5), due presumably to the formation of greater amounts of *EI* than of *FI* and FI_2. This must result in a decrease in the concentration of *ES* which will tend to reduce the reaction velocity. Secondly, SO_4^{2-} can allow the formation of FIS by a reaction dependent on the slow formation of *FS* from FS_2, leading to an increased reaction velocity. The net effect of increasing SO_4^{2-}, which is observed to increase the reaction velocity, will therefore depend on the relative magnitudes of the individual rate constants of these reactions.

There appear to be some discrepancies between the present findings and those of ANDERSEN[5] who found that in the presence of Sr^{2+} the activation of the modified form of sulphatase A was due to 4-nitrocatechol and not to SO_4^{2-}. A possible explanation of this finding is that the solubility of $SrSO_4$ is, unlike that of $BaSO_4$, not negligible so that ANDERSEN's experiments may have been carried out in a constant but low (about 0.5 mM) concentration of SO_4^{2-}. A further complication is caused by the rather powerful absorption of sulphatase A by $BaSO_4$, an absorption which probably accounts for ANDERSEN's claim that sulphatase A is irreversibly inhibited by Ba^{2+}. This absorption is prevented by the presence of bovine serum albumin in the reaction mixture, as in the present experiments, and certainly there is no evidence from either the present work or that of BAUM *et al.*[3] and BAUM AND DODGSON that Ba^{2+} irreversibly inhibits sulphatase A.

REFERENCES

1 A. B. ROY, *Biochem. J.*, 55 (1953) 653.
2 A. B. ROY, *Experientia*, 13 (1956) 32.
3 H. BAUM, K. S. DODGSON AND B. SPENCER, *Biochem. J.*, 69 (1958) 567.
4 H. BAUM AND K. S. DODGSON, *Biochem. J.*, 69 (1958) 573.
5 S. O. ANDERSEN, *Acta Chem. Scand.*, 13 (1959) 120, 884, 1671.
6 L. W. NICHOL AND A. B. ROY, *J. Biochem. Tokyo*, 55 (1964) 643.
7 L. W. NICHOL AND A. B. ROY, *Biochemistry*, 4 (1965) 386.
8 L. W. NICHOL AND A. B. ROY, *Biochemistry*, 5 (1966) 1379.
9 A. B. ROY, *Biochem. J.*, 68 (1958) 519.
10 R. L. BALDWIN, *J. Phys. Chem.*, 63 (1959) 1570.
11 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.