REVIEW ARTICLE

The anomalous kinetics of sulphatase A*

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Introduction

Sulphatase A was first separated in a crude form from an acetone powder of ox liver (Roy, 1953a) and was clearly an arylsulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) because it catalysed the reaction:

where R-OH is a phenol. Rather later it was shown (Mehl & Jatzkewitz, 1964, 1968) that its physiological role was the hydrolysis of galactose 3-sulphate residues in cerebroside sulphate and other sulphated galactolipids.

It can also hydrolyse ascorbate 2-sulphate (Roy, 1975), monosaccharide sulphates (Roy & Turner, 1982), some steroid sulphates (Waheed & Van Etten, 1980a) and, apparently, adenosine 3',5'-monophosphate (Uchida *et al.*, 1981). The hydrolysis of sulphate esters occurs by fission of the O-S bond (Spencer, 1958, 1959; Roy & Turner, 1982; Marker & Roy, 1983).

Roy (1953a) noted that the extent of the hydrolysis of nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) by a partially purified sulphatase A from ox liver was not, when measured over 1 h, linearly related to the enzyme concentration, e_0 , although it was so related to e_0 raised to the power of $\frac{3}{2}$ (Roy, 1953b). It was suggested that this was caused by the polymerization of sulphatase A to a species more catalytically active than the monomer. Dodgson & Spencer (1956), using a similarly purified enzyme from human liver, showed this hypothesis to be untenable and that the 'anomalous' behaviour was caused by the enzyme reaction being not, in general, of zero order. At high enzyme concentrations it approximated to this, but at low concentrations the reaction almost stopped within about 15 min. Roy (1957) subsequently showed the same effects with the ox enzyme.

More detailed studies of the human enzyme (Baum & Dodgson, 1958; Baum *et al.*, 1958) showed that the progress curves for the hydrolysis of nitrocatechol sulphate, at pH 5.0 and 37 °C, comprised three stages: stage I, lasting about 15 min, during which the initial velocity rapidly decreased; stage II, a period of very slow hydrolysis; and stage III, during which the reaction velocity increased to a more-or-less constant value which was, however, always less than the initial one. The relative lengths of the stages depended upon the enzyme concentration, as shown in Fig. 1. Baum & Dodgson (1958) concluded that this behaviour was caused by interactions between sulphatase A and its substrate such that during stage I the enzyme was slowly converted to an inactive form which persisted through stage II and was reactivated in stage III by the reaction products (4-nitrocatechol and

Fig. 1. Effect of enzyme concentration on the shapes of the progress curves for the hydrolysis of nitrocatechol sulphate by the sulphatase A of ox liver at pH 5.6, ionic strength 0.1 in KCl, 37 °C

The reaction was followed in a pH-stat and the reaction volume was 10 ml. The points are experimental and the lines were drawn using eqn. 11 in the text. The enzyme concentration corresponding to the upper curve was $0.56 \mu\text{g} \cdot \text{ml}^{-1}$ and this was sequentially decreased by a factor of 2 to give the lower curves.

sulphate, P and Q) provided that the substrate was also present. Certain other anions, such as phosphate, pyrophosphate and selenate, could replace sulphate in stage III. All such activating anions are competitive inhibitors of stage I. The activity during stage III, unlike the initial activity, was subject to substrate inhibition and was also inhibited by high concentrations of sulphate or of the above anions. This, according to Baum & Dodgson (1958), showed the exposure of a second substrate-binding site during stage I. To explain these observations they proposed Scheme 1A, in which the salient points are the formation by the modified enzyme, F, of the unproductive complexes FS_2 and FQ_2 and the productive complex FQS .

In Scheme 1A the major activating role was ascribed to sulphate and quantitative data with added second product, 4-nitrocatechol, were difficult to obtain because the enzyme activity was followed by the spectrophotometric determination of this phenol. No such limitation was placed upon the work of Andersen (1959a,b,c) who followed the hydrolysis of nitrocatechol

* A tribute to Professor K. S. Dodgson, who died on 9 January 1986.

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Scheme 1. Models for the substrate-induced modification of sulphatase A: A, Baum & Dodgson (1958); B, Andersen (1959c); C, Roy (1985); D, O'Fagain *et al.* (1982)

E, native sulphatase A; F, substrate-modified sulphatase A; S, nitro catechol sulphate; P, 4-nitro catechol; Q, sulphate. —, Rapid reactions and rapidly attained equilibria; ----, reactions with half-times of several minutes; reactions with half-times of hours. Arrow-heads indicate apparently irreversible reactions.

sulphate in a pH-stat using a titrant containing equivalent amounts of base, substrate and BaCl_2 or SrCl_2 so that not only was the pH held constant but the substrate concentration was kept at the initial value and the sulphate was removed as BaSO_4 or SrSO_4 . Under these conditions, and at pH 5.0 and 20 °C, the progress curves were biphasic and described by eqn. 1 in which the coefficients *A*, *B* and *C* are functions of velocity and Michaelis constants and *p* is the amount of product formed in time *t*:

$$p = A \cdot t + B(1 - e^{-Ct}) \quad (1)$$

This was the first quantitative treatment of the sulphatase A reaction and it showed, for example, that the parameter *C*, an apparent rate constant for the modification of the enzyme, varied with substrate concentration.

At 40 °C, Andersen (1959c) observed the three-stage progress curves described by Baum & Dodgson (1958) and to explain these eqn. 2 was derived from Scheme 1B:

$$t + \alpha(1 - e^{-\lambda t}) + \beta \cdot \ln(1 - e^{-\lambda t}) = \gamma \cdot p + \beta \cdot \ln p - \delta \quad (2)$$

The coefficients α , β , γ , δ and λ are again functions of rate and Michaelis constants, and quantitative treatment of the experimental data showed that λ , an apparent rate constant for the modification of the enzyme, was invariant with enzyme concentration: Scheme 1B clearly

differs considerably from that of Baum & Dodgson (1A) but the experimental conditions were very different because the concentration of sulphate was kept close to zero so that the activation in stage III must have been caused by 4-nitrocatechol.

Factors influencing v_0

Determination of v_0 . It is clear from the progress curves shown in Fig. 1 that only by using short reaction times can reliable estimates of v_0 be obtained. Stinshoff (1972) showed that during the first few minutes of the reaction there was a linear relationship between $1/p$ and $1/t$: the slope of the line was $1/v_0$ and its intercept on the ordinate was $1/p_\infty$, where p_∞ is the amount of product which would be produced at infinite time provided no reactivation of the enzyme occurred. The latter parameter is of little interest in itself but Stinshoff (1972) showed that $t_{1/2}$, the half-time for the inactivation of the enzyme, was given by $(\sqrt{2}-1)p_\infty/v_0$. Roy (1972), by making some extreme simplifications, derived these relationships on the assumption that the inactivation of the enzyme occurred by a slow first-order reaction from the enzyme-substrate complex. Subsequently it was shown (Nicholls *et al.*, 1974), using model data, that although the Stinshoff method gave satisfactory estimates of v_0 it did not do so for p_∞ which was required for the estimation of $t_{1/2}$. Any estimates of the latter which are so obtained (e.g. Waheed & Van Etten, 1980b; Waheed *et al.*, 1985) must be viewed with caution.

A more detailed theoretical treatment, which again assumed that the substrate-induced modification of sulphatase A occurred through the enzyme-substrate complex and that the substrate concentration could be considered constant (Roy, 1978), gave eqn. 3, in which k^* is the apparent rate constant for the substrate-induced modification, and predicted that k^* would vary with substrate concentration according to eqn. 4. In the latter, k is the rate constant for the modification at infinite substrate concentration and K a constant analogous to K_m : obviously these could be obtained from measurements of k^* at several substrate concentrations. Waley (1980) showed by a completely different treatment that the apparent rate constant for the inactivation of an enzyme by a suicide substrate should vary in a hyperbolic fashion with substrate concentration.

$$p = \frac{v_0}{k^*}(1 - e^{-k^*t}) \quad (3)$$

$$k^* = \frac{ks_0}{K + s_0} \quad (4)$$

Eqn. 3, or minor variants of it (Roy, 1978), therefore provide the method of choice for the determination of v_0 for the sulphatase A reaction. When v_0 is so measured, the 'anomalous' kinetics of sulphatase A are not apparent except in so far as the substrate-induced modification, governed by k^* , occurs. In particular, v_0 is linearly related to the enzyme concentration over the range 0.05–2 $\mu\text{g/ml}$ at pH 4.6, 5.0 and 5.6 while k^* is independent of enzyme concentration over the same range (Roy, 1978). By using methylumbelliferone sulphate as substrate the range of enzyme concentration could be extended from 0.02 to 50 $\mu\text{g/ml}$ and the same linear relationship was found between v_1 (velocity 1 min after the start of the reaction) and enzyme concentration. This relationship was somewhat unexpected because ox liver

sulphatase A exists as a monomer-tetramer system (Nichol & Roy, 1965; Jerfy *et al.*, 1976) and at pH 5 the weight-fraction of the monomer varies from 0.98 to 0.16 over the concentration range 0.05–2 $\mu\text{g/ml}$, so that the two species must have very similar activities. O'Fagain *et al.* (1982) have also shown, with rat liver sulphatase A, that k^* is independent of enzyme concentration.

Effect of substrate concentration. With ox liver sulphatase A at pH 5.6, 37 °C and ionic strength 0.1, both v_0 and k^* varied in the expected hyperbolic fashion with substrate concentration. Values of the derived parameters (K_m and V , and K and k) are given in Table 1 from which it can be seen that K was consistently less than K_m . The most interesting point in Table 1 is the relative constancy of k which ranged only from 0.22 to 0.30 min^{-1} (mean $0.25 \pm 0.03 \text{ min}^{-1}$) whereas the other three parameters varied widely. Further, the three highest values of k in Table 1 are relatively unreliable because the corresponding values of K_m were such that V could not be reached experimentally. With the two substrates which could be used at concentrations high relative to K_m , nitroquinol sulphate (4-hydroxy-2-nitrophenyl sulphate) and 2-nitrophenyl sulphate, there was no fall in v_0 or k^* at high substrate concentrations ($s_0 > 10K_m$).

The constancy of k was unexpected. If the formation of the substrate-modified enzyme did proceed through the different enzyme-substrate complexes then k might have been expected to vary from substrate to substrate, probably much as V varied. As this was not the case, it seemed possible that the modification of the enzyme occurred not through an enzyme-substrate complex but through an enzyme species common to the reaction with all substrates, most probably the enzyme-sulphate complex which is the last in the catalytic cycle (see below).

At pH 4.6 the effect of substrate concentration was different and k^* was invariant ($0.23 \pm 0.02 \text{ min}^{-1}$) between 0.5 and 50 mM-2-nitrophenyl sulphate. Between 0.5 and 25 mM-ascorbate 2-sulphate at pH 4.8, k^* was likewise invariant. The variation of v_0 with s_0 was apparently hyperbolic for both substrates but the corresponding reciprocal plots were non-linear: as the tetrameric form of sulphatase A is dominant at these pH values, negative co-operativity effects might have been important (Jerfy *et al.*, 1976).

There have been few comparable studies with other sulphatases A, but both Stinshoff (1972) and Lee & Van Etten (1975a), with the enzymes from human kidney and

rabbit liver, respectively, found that $t_{1/2}$ for the substrate-induced modification varied with substrate concentration. Unfortunately, as has already been pointed out, the significance of $t_{1/2}$ as obtained by these authors is doubtful and further treatment of their data is not justifiable.

In contrast, O'Fagain *et al.* (1982) found that the apparent rate constant for the substrate-induced modification of rat liver sulphatase A was independent of substrate concentration over the range 0.02–48 mM-nitrocatechol sulphate at pH 5.9. As this behaviour is similar to that of the ox enzyme below pH 5, but not at pH 5.6, it would be useful to have information about the behaviour of the rat enzyme at higher pH values.

Effect of pH. The effect of pH on the sulphatase A reaction is inherently complex. Apart from its usual effects on v_0 through changes in K_m and V , analogous changes in k^* through K and k are possible. Further complications are likely to be caused by the influence of pH and enzyme concentration on the polymeric state of the enzyme: low pH values favour the tetrameric form of ox (Nichol & Roy, 1965; Jerfy *et al.*, 1976) and human (Draper *et al.*, 1976) sulphatase A, and of the dimeric form of rabbit sulphatase A (Waheed & Van Etten, 1979a). To further complicate the issues, the most commonly used substrate, nitrocatechol sulphate, has a pK of 6.4 so that the proportion of it existing as the singly-charged anion, which is probably the form binding to the enzyme (O'Fagain *et al.*, 1983), will vary considerably over the pH range of interest. In light of this, it is hardly surprising that in the early work of Roy (1953b) and of Baum *et al.* (1958) rather complex pH curves were found.

Plots of v_0 against pH are, in contrast, unimodal. With nitrocatechol and nitroquinol sulphates (Roy, 1978) or glucose 3-sulphate (Roy & Turner, 1982) the optimum pH was 5.6 while with ascorbate 2-sulphate it was about 4.7 (Roy, 1975). The low value for the latter substrate presumably reflects the presence therein of an ionizing group with a pK of 2.8 (Bond *et al.*, 1972). In the few studies which have been made, there was a general parallelism between the effects of pH on v_0 and k^* (Roy, 1978; Butler & Mantle, 1984).

Lee & Van Etten (1975a) found bimodal pH curves for rabbit liver sulphatase A when the velocity was measured by the amount of 4-nitrocatechol liberated from its sulphate over a period of 3 min: this rate must differ

Table 1. Kinetic constants for the catalytic and substrate-induced modification reactions of ox liver sulphatase A

The parameters pertain to eqns. 3 and 4 in the text and were determined at pH 5.6, ionic strength 0.1.

Substrate	K_m (mM)	V_0 ($\mu\text{mol} \cdot$ $\text{mg}^{-1} \cdot \text{min}^{-1}$)	K (mM)	k (min^{-1})
Nitrocatechol sulphate	1.0	230	0.34	0.22
Nitroquinol sulphate	5.9	240	2.6	0.22
2-Nitropyridinyl 3-sulphate	5.9	250	1.3	0.22
2-Nitrophenyl sulphate	12	270	2.5	0.23
Ascorbate 2-sulphate	24	90	5.4	0.22
4-Methylumbelliferone	41	48	9.6	0.28
2-Naphthyl sulphate	41	37	11	0.26
3-Nitrophenyl sulphate	100	230	26	0.28
4-Nitrophenyl sulphate	220	280	53	0.30

from v_0 , and will differ from it by differing amounts at different pH values because of the pH-dependent changes in k^* . More detailed studies of this system were subsequently made (Lee & Van Etten, 1975b) using both nitrocatechol sulphate and 4-nitrophenyl sulphate as substrates. With the former, v_0 was obtained by the Stinshoff method while with the latter it was estimated by drawing tangents to the progress curves at zero time. The shapes of the simple plots of v_0 against pH were not commented on but the derived logarithmic plots of V_0 , K_m and K_m/V_0 showed no abnormalities. They showed ionizing groups with pK values of 6.1 and 4.3 in the enzyme and enzyme-substrate complex, respectively: the former could be that of the histidyl residue which is essential for the activity of sulphatase A (Jerfy & Roy, 1969, 1974; Lee & Van Etten, 1975b).

In similar studies with rat liver sulphatase A, using nitrocatechol sulphate as substrate, O'Fagain *et al.* (1983) showed that the enzyme contained two ionizing groups important for its activity. The pK values were 4.5 and 5.8, and the latter changed to 7.3 in the presence of substrate. It should be noted that the effect of pH on v_0 was not reported in this study. From the effect of pH on the inactivation rate constant (k_s in Scheme 1D), Butler & Mantle (1984) suggested that the same ionizing groups were involved in the substrate-induced modification.

Effect of temperature. The classical work of Baum *et al.* (1958) and of Andersen (1959a,c) showed that the 'anomalous' kinetics of sulphatase A were temperature-dependent, becoming less apparent as the temperature was lowered, a fact utilized in a recent method for the determination of sulphatase A in human tissues at a reaction temperature of 0 °C (Lee-Vaupel & Conzelman, 1987). The temperature effects reflect the lower activation energy for the catalytic than for the modification reaction: values are 33 and 56, 56 and 160, 49 and 76, and 47 and 87 kJ·mol⁻¹ for the ox (Roy, 1978), rabbit (Waheed & Van Etten, 1980b) and rat and human (O'Fagain, 1981) enzymes, respectively.

Effect of inhibitors. Of obvious interest is sulphate, a reaction product, which has long been known to be an inhibitor of sulphatase A, although apparently not a powerful one (Roy, 1953b). The inhibition is purely competitive, not only with respect to v_0 but also to k^* : the values of K_i with respect to these two parameters are 2 and 1 mM-sulphate, respectively, at pH 5.6 (Roy, 1978). Assuming that the sulphatase reaction is kinetically unibi, then competitive inhibition by sulphate shows this to be the second reaction product to be released from the enzyme.

Detailed studies by O'Fagain *et al.* (1983) with rat liver sulphatase A have shown that the inhibitory species is HSO_4^- , not SO_4^{2-} . The same seems to be the case with ox liver sulphatase A (Roy, 1985a): the apparent values of K_i at pH 5.6, 5.0 and 4.5 were 2, 0.5 and 0.2 mM, respectively, in terms of total sulphate but when expressed in terms of HSO_4^- (pK 1.9) the values of K_i were indistinguishable with a mean of 0.4 μM - HSO_4^- . The value for rat liver sulphatase A was 0.39 μM .

Sulphite has usually been considered to be a much more powerful inhibitor of sulphatase A than sulphate (Roy, 1953b; Lee & Van Etten, 1975a) with a K_i of about 1 μM . However, when the comparison is made between HSO_4^- and HSO_3^- then the inhibitory effects of the two

ions are very similar because sulphite exists almost entirely as HSO_3^- (pK 7.2) over the above pH range.

Other inhibitors, comparable in action to sulphate, are thiosulphate, selenate, borate, arsenate, phosphate and pyrophosphate. With phosphate it has again been shown that the inhibitory species is the singly-charged anion, H_2PO_4^- (O'Fagain *et al.*, 1983). It is interesting to note that polyanions and nucleotides are also competitive inhibitors of rat liver sulphatase A (O'Fagain *et al.*, 1983).

Isolation of the substrate-modified enzyme

Baum & Dodgson (1958) showed that a preparation of human liver sulphatase A containing a considerable proportion of the substrate-modified form could be recovered from reaction mixtures in stage II, but the techniques were not then available to exploit this approach. Nicholls & Roy (1971) used gel filtration to separate the ox liver enzyme from its substrate (nitrocatechol or 4-nitrophenyl sulphates) and the reaction products, so obtaining a preparation containing about 90% of the modified enzyme. By allowing the latter to react with further substrate and repeating the isolation it was possible to obtain a preparation containing about 98% of the modified form (Prosser & Roy, 1980). The method of preparation is not ideal because the strong absorption of aromatic compounds to gel-filtration media means that the enzyme is in more prolonged contact with sulphate than with the substrate or the phenolic product, an unsatisfactory situation because of the known effects of sulphate on the equilibria between the native and substrate-modified forms of sulphatase A (Nicholls & Roy, 1971; O'Fagain *et al.*, 1982). This may explain why sulphatase A recovered by such methods generally seems to contain less of the modified form than would be expected from rate measurements made during the preparation (O'Fagain *et al.*, 1982; Roy, 1985a). Another approach, utilizing the strong binding of sulphatase to concanavalin A, seems to have some advantages (Roy, 1985a).

Similar methods have been used by Waheed & Van Etten (1979b, 1980b) to obtain substrate-modified rabbit sulphatase A: Ba^{2+} was included in the reaction mixtures to eliminate sulphate and so drive the equilibrium in favour of the substrate-modified enzyme. This procedure was not possible with the ox enzyme which was firmly absorbed by the precipitated BaSO_4 from which it could not be eluted, although it remained active. It is interesting to note that the rat liver enzyme is not absorbed to precipitated BaSO_4 under these conditions (O'Fagain *et al.*, 1980).

Physical properties of the modified enzyme. No difference between the native and substrate-modified forms of ox liver sulphatase A could be found in any of the physical properties investigated: sedimentation coefficient, molecular mass, tetramerization as a function of pH, or u.v., c.d. and intrinsic fluorescence spectra (Nicholls & Roy, 1971; Prosser & Roy, 1980; Prosser *et al.*, 1980). Any structural difference between the two forms must therefore be small, perhaps only conformational.

The rabbit enzyme has not been investigated in such detail, but Waheed & Van Etten (1980b) stated that in this case the substrate-induced modification was accompanied by a change in the c.d. spectrum which

indicated that about 76% of the helical structure of the enzyme had been lost. Differences in the intrinsic fluorescence spectrum were in keeping with this. Such a large structural change seems incompatible with the previous observation (Lee & Van Etten, 1975a) that the spontaneous reversion of the modified to the native enzyme (see below) is accompanied by only a small structural change.

Reversion to the native enzyme. Although the substrate-modified sulphatases A of ox and rabbit liver are stable for weeks at 0 °C they partially regain their activities at higher temperatures, presumably by reversion to the native enzymes. Data are given in Table 2, at pH 7.5 for the ox enzyme (Roy, 1985a) and at pH 5.5 (presumably) for the rabbit enzyme (Lee & Van Etten, 1975a). With the former enzyme at pH 5.6 the rate of reversion was an order of magnitude less, but accurate data could not be obtained. The data in Table 2 give activation energies of 130 and 89 kJ·mol⁻¹, respectively, for the spontaneous reversion of the ox and rabbit sulphatase A to their native forms, while the entropy of activation for the latter reaction was -33 kJ·mol⁻¹·K⁻¹ (Lee & Van Etten, 1975a). It was this low value which led to the suggestion that the reversion reaction involved only a small conformational change: as mentioned above, the reported changes in the c.d. spectra seem incompatible with this.

Prosser & Roy (1980) and Roy (1985a) showed that 2.5 mM-4-nitrocatechol doubled the rate of reversion of the substrate-modified ox enzyme to the native form ($k = 8.2 \text{ min}^{-1}$ at 37 °C and pH 7.5). This did not occur if 2.5 mM-sulphate was simultaneously present, and the latter had no effect on its own, nor did 2-nitrophenol.

Chemistry of the substrate-modified enzyme. Stinshoff (1972) suggested that useful information might be obtained by using [¹⁴C]nitrocatechol [³⁵S]sulphate for the preparation of substrate-modified sulphatase A: while this experiment has never been carried out, the use of nitrocatechol [³⁵S]sulphate has indeed provided valuable data. With either rabbit (Waheed & Van Etten, 1979b, 1980b) or ox (Prosser & Roy, 1980) sulphatase A this substrate gave a modified enzyme containing ³⁵S, presumably as ³⁵SO₄²⁻ or some simple derivative thereof although its nature has not yet been established. The substrate-modified rabbit enzyme contained two atoms of ³⁵S per molecule (M_r 140 000) whereas the ox enzyme

contained only one atom per molecule (M_r 107 000). There is no evidence for the presence of either substrate or the phenolic reaction product in substrate-modified sulphatase A (Nicholls & Roy, 1971) but their absence should be confirmed by using substrates containing ¹⁴C.

The presumed ³⁵SO₄²⁻ was firmly bound to the substrate-modified enzyme because it remained associated with it during gel-filtration, dialysis and ion-exchange chromatography. It is therefore surprising that Prosser & Roy (1980) found, with the ox enzyme, that the label was exchanged, with no change in the activity of the modified enzyme, by dialysis against 0.01 M-K₂SO₄. Conversely, when the unlabelled substrate-modified enzyme was dialysed against K₂³⁵SO₄ solution the label was incorporated into the enzyme (Prosser 1981). Prosser & Roy (1980) also showed that when the amount of substrate-modified enzyme in the preparation was decreased, either by spontaneous reactivation at 37 °C or by reaction with substrate in presence of sulphate, ³⁵S was lost so that the amount remaining in the residual substrate-modified enzyme remained at one atom per molecule.

It had been hoped that the ³⁵S bound to the modified enzyme would provide a label for the active site of sulphatase A, but when the protein was denatured the radioactivity was lost. It therefore appears unlikely that the presumed ³⁵SO₄²⁻ is present as an ester because, in general, denaturation would not cause fission of such a bond. Further, a sulphate ester would not be expected to undergo the exchange reaction described above. It may therefore be that the sulphate is trapped in the substrate-modified enzyme by some conformational change which may, in turn, be stabilized by the sulphate. Much more information is needed in this area and, until this is obtained, it seems unwise to adopt the term 'covalent modification' (Waheed & Van Etten, 1979b) for the substrate-induced change in sulphatase A.

Activation of the substrate-modified enzyme. The fundamental observations are again those of Baum & Dodgson (1958) who showed that the enzymically-inactive substrate-modified form of human liver sulphatase A regained its activity in the presence of substrate and sulphate or certain other anions. They therefore postulated that the complex FQS in Scheme 1A was a productive one, in the sense that it broke down to give the reaction products. Nicholls & Roy (1971) subsequently showed that under these conditions the equilibria between the native and substrate-modified forms of ox sulphatase A were displaced in favour of the former, a finding confirmed by Prosser & Roy (1980). A preparation containing about 90% of substrate-modified sulphatase A was incubated with nitrocatechol sulphate and sulphate and the enzyme re-isolated: it was almost entirely in the native form. This being so, it seems unnecessary to postulate, as did Baum & Dodgson (1958) that the complex FQS (Scheme 1A) is a productive one in the above sense: it may simply convert to the native enzyme, or complexes of the native enzyme, more rapidly than do F, FS or FQ.

With the rabbit enzyme, the results are surprisingly different. As already noted, Waheed & Van Etten (1980b) concluded that the formation of substrate-modified rabbit sulphatase A was accompanied by a large increase in the random structure of the protein. When this modified form was activated, by incubating with nitrocatechol sulphate and sulphate, and the protein isolated

Table 2. Rate constants for the spontaneous reversion of substrate-modified to native sulphatases A

Constants were measured at pH 7.5 for the ox enzyme (Roy, 1985a) and pH 5.5 for the rabbit enzyme (Waheed & Van Etten, 1975a).

<i>t</i> (°C)	$10^8 \times k \text{ (min}^{-1}\text{)}$	
	Rabbit	Ox
25	0.078	0.35
30	0.11	—
37.5	5.2	3.3
43	12	—
45	—	10
50	17	—

it had lost essentially all its secondary structure (Waheed & Van Etten, 1980b). One can only agree with the statement that the substrate-modified sulphatase A of rabbit liver must be a very fragile molecule, yet this does not seem to be in accord with the earlier observation that it reverts, at least partly, to the native enzyme on heating at 50 °C (Lee & Van Etten, 1975a).

This activation of the modified enzyme by sulphate in the presence of substrate is a relatively slow reaction (Nicholls & Roy, 1971; Stinshoff, 1972; Lee & Van Etten, 1975a) but quantitative data are few. However, Roy (1985a) showed that the apparent rate constants for the activation were of the same order of magnitude as those for the substrate-induced inactivation and, further, that again there was a hyperbolic relationship between these constants and the concentration of sulphate.

The reaction during stage III

Any explanation of the typical three-phase progress curves of the sulphatase A reaction (Fig. 1) requires information about stage III. Stage I is defined by v_0 and k^* , the determination of which has already been considered, and stage II is one of low, or even zero, activity. Stage III, on the other hand, can be a period of relatively high activity and can be prolonged. The basic information was provided by Baum & Dodgson (1958): that the activity of sulphatase A during stage III is subject both to substrate inhibition and to inhibition by high concentrations of those anions which activate at lower concentrations. The latter two effects are related: the optimum substrate concentration is a function of the sulphate concentration, and the lower the latter, the lower the former (Nicholls & Roy, 1971).

As already noted, the effect of sulphate on v_0 is highly pH-dependent because of the ionization of the ligand, HSO_4^- . If the functional enzyme during stage III were simply native sulphatase A then similar pH effects would be expected. The effect of sulphate on the velocity during stage III is shown in Fig. 2: the optimum concentration of sulphate decreases markedly as the pH is lowered

(Roy, 1985a). This explains the apparent inability of sulphate to activate the modified enzyme at pH 4.5 (Roy, 1979, 1980): the concentration of sulphate used in these experiments, 3 mM, was beyond the optimum at that pH. Also shown by Fig. 2 is that the data fitted eqn. 5 which is of the same form as that describing v_0 for an enzyme subject to substrate inhibition (v_t is the velocity, at constant substrate concentration, of the sulphatase A reaction during stage III, V_t is the corresponding maximum velocity, i is the concentration of sulphate and K_1 and K_2 are Michaelis-type constants). The parameters V_t , K_1 and K_2 were computed by fitting experimental data to eqn. 5 and their values used to draw the lines in Fig. 2:

$$v_t = \frac{V_t i}{K_1 + i + i^2/K_2} \quad (5)$$

The activation by phosphate during stage III fitted an equation of the same form as eqn. 5 but with different values of the parameters (Roy, 1985a).

Although equations of the form of eqn. 5 describe the effects of both substrate (Nicholls & Roy, 1971) and sulphate (Roy, 1985a) during stage III of the reaction, this does not prove the formation of the complexes FS_2 and FQ_2 in Scheme 1A. All it shows is that the ligands S and Q must each enter the reaction sequence at least at two separate points. The Baum–Dodgson model (Scheme 1A) can therefore be considerably simplified to Scheme 1C yet still account for the experimental observations. There is therefore no need to invoke the formation of a second binding site during the substrate-induced modification. The existence of the complex FQS still seems a necessary postulate because the activation of the modified enzyme requires both sulphate and substrate: it is not necessary, however, that this complex directly yields the reaction products. As already noted, it need only revert to the native enzyme more rapidly than F, FS or FQ. The only point requiring further consideration is the nature of the species giving rise to the modified enzyme: in Scheme 1C this is presumed to be the enzyme–substrate complex but, as has been noted above, there is evidence to suggest that it may proceed through an enzyme–sulphate complex. This will be considered again later.

Sulphatase A as a hysteretic enzyme

O'Fagain *et al.* (1982) considered the progress curves of reactions catalysed by partially purified sulphatases A from rat or ox liver and from human placenta: the substrate was nitrocatechol sulphate at pH 5.9 and 37 °C. Substrate-modified rat sulphatase A was not activated by sulphate so that two models were necessary. Both are encompassed in Scheme 1D which is applicable in its entirety to the ox and human enzyme: with the rat enzyme FQ is not formed so that K is infinitely large. In Scheme 1D, as in Scheme 1C, only the native enzyme is considered to yield a productive complex and in the theoretical treatment it was assumed that the slow reactions involving the substrate-modified forms of the enzyme (F and FQ) did not disturb the steady state of the native species. In the form pertaining to the rat enzyme, in which FQ is not formed, Scheme 1D gives rise to eqn. 6 (which is of the same form as eqn. 1):

$$p = \frac{k_2 es}{K_m + s} \left[\frac{k_4}{k_3 + k_4} \cdot t + \frac{k_3}{(k_3 + k_4)^2} (1 - e^{-(k_3 + k_4)t}) \right] \quad (6)$$

Fig. 2. Effect of sulphate concentration on the final velocity (stage III) of the hydrolysis of nitrocatechol sulphate by the sulphatase A of ox liver at pH 5.6 (●), 5.0 (■) and 4.5 (▲), ionic strength 0.1 and 37 °C

The points are experimental and the lines drawn by fitting the data to eqn. 5 in the text.

Table 3. Constants for the slow reactions involved in the substrate-induced modification of sulphatases A

The data are taken from O'Fagain *et al.* (1982) and the significance of the constants is shown in Scheme 1D.

	k_3 (min ⁻¹)	k_4 (min ⁻¹)	k_5 (min ⁻¹)	K (mM)
Rat liver	0.795	0.0134	—	—
Ox liver	0.2337	0.0033	0.720	60
Human placenta	0.5187	0.0034	—	—

The first term on the right is v_0 . In eqn. 6, the apparent rate constant for the substrate-induced modification is $(k_3 + k_4)$ and so independent of substrate concentration, as was found experimentally. An alternative treatment of Scheme 1D, however, does predict an effect of substrate concentration on the rate of inactivation. More experimental data under a wider range of conditions are required.

Two expressions were derived from the complete version of Scheme 1D in which FQ is formed: eqn. 7 when the reaction was started by the addition of the native enzyme and eqn. 8 when started with the substrate-modified enzyme. The coefficients A , B and C are functions of rate constants, K and sulphate concentration, and are different in the two expressions:

$$p = v_0[A \cdot t + B(1 - e^{-C \cdot t})] \quad (7)$$

$$p = v_0[A \cdot t + B(e^{-C \cdot t} - 1)] \quad (8)$$

The experimental data fitted these equations satisfactorily and gave the values in Table 3 for the constants in Scheme 1D. Again the apparent rate constant for the substrate-induced modification is $(k_3 + k_4)$: the value for the ox enzyme from the data in Table 3 is 0.24 min⁻¹, in good agreement with that of 0.22 min⁻¹ for k in Table 1. Unfortunately, the complete progress curves of reactions catalysed by ox or human sulphatase A, which are activated by sulphate, could not be described by combining eqns. 7 and 8.

A different approach was made by Roy (1985a), using as a model Scheme 1C which is analogous to the hysteretic systems described by Frieden (1970) and should be described by his general expression, eqn. 9. This is not directly applicable to the sulphatase A system because v_r , the final velocity, varies with the concentration of the reaction product, sulphate.

$$p = v_r \cdot t - \frac{1}{k^*} (v_r - v_0) (1 - e^{-k^* \cdot t}) \quad (9)$$

The relationship between v_r and sulphate concentration, at fixed substrate concentration, has been given in eqn. 5. This has been put in a more convenient form in eqn. 10, in which $V_{rel.}$ is V_r/v_0 : V_r is not a maximum velocity with respect to substrate concentration but with respect to sulphate concentration at a fixed substrate concentration. Combining eqns. 9 and 10, replacing i by p , which is appropriate when the only sulphate present is that produced during the reaction, and rearranging, gives eqn. 11 which should describe the progress curve of a reaction catalysed by sulphatase A:

$$v_r/v_0 = \frac{V_{rel.} i}{K_1 + i + i^2/K_2} \quad (10)$$

$$p = \left[\frac{V_{rel.} p}{K_1 + p + p^2/K_2} \cdot t - \frac{1}{k^*} \left(\frac{V_{rel.} p}{K_1 + p + p^2/K_2} - 1 \right) (1 - e^{-k^* \cdot t}) \right] v_0 \quad (11)$$

It was used to draw, by an iterative procedure (Roy, 1985a) the lines in Fig. 1. The fit of the experimental data is satisfactory, considering the simplifications which are involved. These are: 1, the substrate concentration cannot be considered constant at the highest enzyme concentrations; 2, no account is taken of any effect of the second reaction product, 4-nitrocatechol; and 3, the spontaneous reversion of the substrate-modified to the native enzyme is ignored.

The kinetics of the sulphatase A of kangaroo liver (Roy, 1971) differ from those of the ox enzyme in that the apparent equilibrium constant for the inactivation reaction is about 10, compared with about 200 for the ox enzyme (see below) and although its substrate-modified form is activated by sulphate, in the presence of substrate, there is no evidence for any inhibition at high concentrations of the latter. Eqn. 5 is therefore not applicable, but a corresponding equation of a different form was derived which, when combined with eqn. 10, gave one of a similar form to eqn. 11 which satisfactorily accounted for the behaviour of the enzyme (Roy, 1985b).

Is the reaction ES → FS reversible?

When sulphatase A reacts with its substrate in the presence of Ba²⁺ then the concentration of sulphate in solution is effectively zero so that the species EQ, FQ and FQS in Schemes 1A and 1C cannot exist. With nitrocatechol sulphate as substrate under these conditions the reaction velocity did not fall to zero but to a constant value of about 0.5% of v_0 ; with 2-nitrophenyl sulphate, on the other hand, the final velocity was indistinguishable from zero (Roy, 1985a). The reaction ES → FS must therefore be essentially irreversible with the latter substrate and FS can have no residual sulphatase activity. With nitrocatechol sulphate, the residual activity must therefore be due to native enzyme in the equilibrium mixture of the native and substrate-modified species and the apparent equilibrium constant must be about 200. The apparent rate constant for the substrate-induced modification, k , is 0.27 min⁻¹ (Table 1) and must equal $k_f + k_r$ (the rate constants for the appropriate forward and reverse reactions), so that k_r must be about 10⁻³ min⁻¹. This is similar to that for the spontaneous reversion of substrate-modified to native enzyme and so could indicate that the apparent equilibrium between these, when nitrocatechol sulphate is the substrate, may simply reflect the balance between the two different reactions ES → FS and F → E. As has been pointed out, the latter reaction is accelerated by 4-nitrocatechol in the absence of sulphate so that the irreversibility with 2-nitrophenyl sulphate as substrate would simply reflect the lack of effect of 2-nitrophenol on the spontaneous reversion of the modified to native enzyme. Once again, much further information is needed to resolve this problem.

Cerebroside sulphatase activity

The study of the cerebroside sulphatase activity of sulphatase A is complicated by the insolubility in water of the substrates, sulpholipids containing galactose 3-sulphate residues. Although human sulphatase A can hydrolyse dispersions of cerebroside sulphate when the buffer concentration is less than 10 mM-formate (Stinshoff & Jatzkewitz, 1975), in general the reaction mixtures must contain either the appropriate activator protein (Mehl & Jatzkewitz, 1964; Conzelman & Sandhoff, 1987a,b), which forms a 1:1 complex with the sulpholipid to give the physiological substrate, or a bile salt, such as taurodeoxycholate, to form mixed micelles (Porter *et al.*, 1972; Jerfy & Roy, 1973).

Kinetic studies of the latter systems are fraught with difficulties and only two points need be made here. First, the inhibition of the cerebroside sulphatase activity by sulphate is noncompetitive, either in low buffer concentrations (Stinshoff & Jatzkewitz, 1975) or in the presence of bile salts (Porter *et al.*, 1972; Jerfy & Roy, 1973). Second, there is no evidence for a substrate-induced modification of sulphatase A when this is functioning as a cerebroside sulphatase (Jerfy & Roy, 1973; Roy, 1979). The noncompetitive inhibition by sulphate has been explained in terms of the micellar nature of the substrate (Jerfy & Roy, 1973; Stinshoff & Jatzkewitz, 1975) but a simpler explanation is possible (Roy, 1979). Because of the considerable chemical differences between the substrates for the arylsulphatase and cerebroside sulphatase activities of sulphatase A there could be significant kinetic differences between the two activities, notwithstanding the fact that it is the O-S bond of both substrates which is split (Spencer, 1958, 1959; Marker & Roy, 1983). If the binding of the cerebroside sulphate, and of the cerebroside formed on its hydrolysis, involve hydrophobic interactions, which are unlikely to participate in the binding of aryl sulphates or phenols, then sulphate could be the first-released product, not the second. In this case the inhibition by sulphate would be noncompetitive, not competitive as in the arylsulphatase reaction, and further, an enzyme-sulphate complex would not be formed during the catalytic cycle.

Which enzyme species is modified?

The usual assumption in models (Scheme 1) of the substrate-induced modification of sulphatase A is that of Baum & Dodgson (1958): that the modification proceeds from the enzyme-substrate complex. This assumption was queried by Roy (1978) on the grounds that the rate constant for the substrate-induced modification, at infinite substrate concentration, varied little from substrate to substrate (Table 1). This seemed to argue against the inactivation proceeding through different enzyme-substrate complexes and suggested that an enzyme species common to all substrates must be the key compound. The obvious possibility was the enzyme-sulphate complex which, from kinetic evidence, must be the last intermediate in the catalytic cycle.

Further evidence for this is scanty but there are two points which give some support to the hypothesis. First, the substrate-induced modification does not occur during cerebroside sulphatase activity: as noted above, it is possible that an enzyme-sulphate complex is not formed in this system so that a substrate-induced modification

would not be expected. Second, the substrate-modified enzyme contains sulphate, or some simple derivative thereof, derived from the substrate (Waheed & Van Etten, 1980b; Prosser & Roy, 1980), but it does not contain the phenolic reaction product which must therefore have dissociated from the enzyme. This could happen concurrently with the modification, whatever that be in chemical terms, in which case the nature of the reacting species becomes obscure.

Only one point argues against the involvement of the enzyme-sulphate complex. Sulphate, in the absence of substrate, does not allow the formation of substrate-modified sulphatase A but it is a competitive inhibitor, with respect to v_0 , of its arylsulphatase activity and so must form an enzyme-sulphate complex. If this cannot yield the modified enzyme, then either it must be different from that produced during the catalytic cycle or the latter is indeed not involved in the modification reaction. The existence of two different 'enzyme-sulphate' complexes is not impossible and the apparent inconsistency may simply reflect the lack of detailed information about the reaction mechanism. The complex formed when sulphate acts as an inhibitor of v_0 is presumably a true enzyme-sulphate complex: that formed during the catalytic cycle may be a transient enzyme-SO₃ complex which is capable of reacting not only with water to give sulphate but with a nucleophile in the enzyme to give the modified form of the latter (Waheed & Van Etten, 1980b; O'Fagain *et al.*, 1982; Butler & Mantle, 1984).

More detailed studies of the catalytic reaction itself are needed. First, to clarify the nature of the sulphate in the modified enzyme, and second, to clarify the mechanism of the arylsulphatase reaction. Is a sulphated enzyme formed as an intermediate or is the reaction a concerted one, as suggested by O'Fagain *et al.* (1983), which simultaneously forms HSO₄⁻ and the phenolic product?

This uncertainty makes little difference to the previous work. The models in Scheme 1 can be adapted to allow the modification to proceed through an enzyme-sulphate complex (see, for example, Roy, 1985) and this would in no way detract from the validity of the pioneering work of Ken Dodgson and his group in this area.

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