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THE SULPHATASE OF OX LIVER

XXI: KINETIC STUDIES OF THE SUBSTRATE-INDUCED INACTIVATION OF SULPHATASE A

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Summary

The theoretical basis is given for methods of determining the apparent velocity constant, k^*, for the substrate-induced inactivation of sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) and the initial velocity, v_o, of the catalytic reaction. The expression is of the same form as the empirical relationships previously used but the significance of the various terms is clearly established.

The method has been applied to the characterisation of the inactivation occurring during the hydrolysis of a number of substrates and it has been shown that k^* varies with s_o in a hyperbolic relationship described by k, a velocity constant at infinite substrate concentrations and by K, a constant analogous to the Michaelis constant. Although K varies considerably for different substrates, and is consistently less than the corresponding K_m, k is almost constant at 0.23 min^{-1}. It is therefore suggested that the inactivation of the enzyme does not proceed through an enzyme · substrate complex but through the enzyme · SO_4^{2-} complex produced during the catalytic reaction.

The effects of several variables on these parameters are described.

Introduction

It has long been known that sulphatase A, functioning either as an aryl-sulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) or as a cerebroside sulphatase, becomes inactivated during its catalytic cycle and may, under appropriate conditions, be subsequently reactivated by the products of the reaction, particularly by SO_4^{2-} [1—6]. No quantitative studies have been made of this inactivation although semiquantitative observations suggested that the

rate of inactivation is less when the substrate is 4-methylumbelliferone sulphate or ascorbate 2-sulphate [7] than when it is the commonly used nitrocatechol sulphate. A more detailed investigation of the effects of different substrates was required and this in turn required the measurement of the velocity constants for the substrate-induced inactivation of the enzyme. The inactivation occurs only in the presence of the substrate so that its rate can be measured only from the decreasing rate of the catalysed reaction. Further, the enzyme can be reactivated by the reaction products [2—4], and this effect is detectable as early as 5 min after the start of the reaction (as can be seen by comparing progress curves in KCl and $BaCl_2$), so that only the initial stages of the reaction can be used to measure the rate of inactivation. The half-time for the inactivation is, under usual conditions, of the order of 3 min [5,6,8] which means that precise values of the velocity constant of the inactivation are very difficult to obtain because the reaction can be followed for only one half-time or less. Other practical problems [8] increase the difficulties.

Experimental

Substrates

Potassium aryl sulphates were prepared by standard methods and were recrystallised from water or from 70% ethanol, both containing 0.01 M $KHCO_3$ to prevent the autocatalytic decomposition of the more labile esters. The final products were free of the parent phenol and of K_2SO_4 (less than 0.1% of the latter).

Enzyme assays

Sulphatase A was prepared from ox liver as before [9]. In most cases the reaction was followed in a pH-stat (assembly PHM26-TT11-SBR2-ABU12; Radiometer, Copenhagen). The standard conditions were: volume, 10 ml; temperature, 37°C; pH 5.6; μ adjusted to 0.1 with KCl; sodium acetate, 0.5 mM (except with nitrocatechol sulphate). Changes in ionic strength were made by altering the concentration of KCl. The reaction was started by adding 10—25 μl of enzyme solution, pH about 7.5, and the reaction was followed by titration with 0.015 M NaOH. Readings were normally taken at 8-s intervals between 1 and 3 min from the start of the reaction. Some problems in this type of assay are considered in the Appendix.

In some experiments with nitroquinol sulphate (potassium 4-hydroxy-2-nitrophenyl sulphate) as substrate the reaction was followed by a continuous spectrophotometric method [10]. The standard conditions were: volume, 3 ml; temperature, 37°C; pH 5.6; μ, adjusted to 0.1 with sodium acetate-acetic acid buffer. Higher ionic strengths were obtained by adding NaCl. Readings were normally taken at intervals of 10 s between 0.5 and 3 min from the start of the reaction.

For longer incubation times, samples (0.5 ml) were withdrawn at 2 min intervals from 10 ml of reaction mixture (pH 5.6 in sodium acetate-acetic acid buffer, μ = 0.1, 37°C) and added to 0.5 ml of 0.3 M NaOH. The absorbance of the resulting solutions was read at the appropriate wavelength (2-nitrophenol, ϵ_{415} = 4720, 4-nitrocatechol, ϵ_{510} = 12 600; nitroquinol, ϵ_{535} = 4100).

In all these assays the enzyme concentration was less than 1 μg/ml: exact concentrations are specified below.

Theory

Laidler and Bunting [11] have pointed out that it is difficult to handle the general case of an enzyme inactivated by its substrate because the steady-state approximation cannot be applied to the concentration of the enzyme · substrate complex. However, in the special case of the substrate-induced inactivation of sulphatase A it may be possible to make this approximation because the rate of inactivation is only about 10^{-4} of that of the catalytic reaction. Therefore, at any instant the concentration of active enzyme can, in so far as the catalytic reaction is concerned, be considered constant because

$$\frac{de}{dt} << \frac{ds}{dt}$$

The fundamental model is the generally accepted one [2—6] that the inactive form of the enzyme, F, arises from the enzyme · substrate complex. The generalising assumption is made that K_m need not equal K, a Michaelis constant for the substrate-induced inactivation. This implies that the inactivation reaction is kinetically distinct from the catalytic reaction because, if it were not, the same Michealis constant would apply to both processes once a steady-state had been reached. However, previous work [4] has suggested that the interconversion of E and F is slowly reversible in the presence of substrate so that a slow interconversion of ES and FS must be considered. The model is therefore the following:

$$\begin{array}{l} E + S \rightleftharpoons ES \rightarrow E + \text{products} \\ \qquad\quad \Big\Vert \text{(slow reactions)} \\ E + S \rightleftharpoons FS \rightarrow F\ (+ \text{products?}) \end{array}$$

This implies that after the elapse of sufficient time for the equilibrium between ES and FS to be established then a single Michaelis constant would describe both the hydrolytic reaction and the inactivation.

On this basis Eqns. 1 and 2 can be written with k_3 and k being velocity constants for the formation from the appropriate enzyme · substrate complexes of the reaction products and inactive enzyme respectively.

$$-\frac{ds}{dt} = \frac{k_3 e \cdot s}{K_m + s} \tag{1}$$

$$-\frac{de}{dt} = \frac{ke \cdot s}{K + s} \tag{2}$$

From Eqn. 2 an expression for $e \cdot s$ may be obtained: substitution of this in Eqn. 1 gives

$$-\frac{ds}{dt} = -\frac{k_3}{k} \cdot \frac{(K + s)}{(K_m + s)} \cdot \frac{de}{dt}$$

which rearranges to

$$\frac{K_m + s}{K + s}\,ds = \frac{k_3}{k}\,de$$

Integration, with the boundary condition that at $t = 0$, $s = s_o$ and $e = e_o$ gives

$$e = e_o - \frac{k}{k_3}(s_o - s) - \frac{k}{k_3}(K_m - K)\ln\frac{K + s_o}{K + s}$$

which rearranges to

$$e = e_o - \frac{k}{k_3}(s_o - s) - \frac{k}{k_3}(K_m - K)\ln\left[1 + \frac{s_o - s}{K + s}\right]$$

But $(s_o - s)/(K + s)$ must be small at the early stages of the reaction so that $\ln[1 + (s_o - s)/(K + s)]$ may be replaced by $(s_o - s)/(K + s)$ to give

$$e = e_o - \frac{k}{k_3}(s_o - s) - \frac{k}{k_3}(K_m - K)\cdot\frac{s_o - s}{K + s}$$

which simplifies to

$$e = e_o - \frac{k(s_o - s)(K_m + s)}{k_3(K + s)} \tag{3}$$

This expression for e can be substituted in Eqn. 1 to give

$$-\frac{ds}{dt} = \frac{k_3 s\left[e_o - \dfrac{k(s_o - s)(K_m + s)}{k_3(K + s)}\right]}{K_m + s}$$

so that

$$-dt = \frac{(K_m + s)(K + s)ds}{k_3 e_o s(K + s) - ks(s_o - s)(K_m + s)}$$

$$= \frac{[KK_m + (K + K_m)s + s^2]ds}{s[(k_3 K e_o - kK_m s_o) + (k_3 e_o - ks_o + kK_m)s + ks^2]}$$

$$\therefore -\int dt = KK_m\int\frac{ds}{s(a + bs + cs^2)} + (K + K_m)\int\frac{ds}{a + bs + cs^2} + \int\frac{sds}{a + bs + cs^2}$$

where

$a = k_3 e_o K - kK_m s_o$, $b = k_3 e_o - ks_o + kK_m$ and $c = k$.

Integration with the same boundary conditions as above gives

$$-t = \frac{KK_m}{a}\ln\frac{s}{s_o} + \left[\frac{a - cKK_m}{2ac}\right]\ln\frac{a + bs + cs^2}{a + bs_o + cs_o^2}$$

$$+ \left[\frac{2ac(K + K_m) - b(cKK_m + a)}{2ac\sqrt{q}}\right]\ln\frac{(2cs + b - \sqrt{q})(2cs_o + b + \sqrt{q})}{(2cs + b + \sqrt{q})(2cs_o + b - \sqrt{q})} \tag{4}$$

where $q = (b^2 - 4ac)$. At the early stages of the reaction s is close to s_o so that

the first term can be neglected. The coefficients of the second and third terms may be expanded by substituting the appropriate values for a, b and c, and simplified by making the justifiable assumption that terms containing k_3e_o are small compared to those in K, K_m and s so that $\sqrt{q} = k(K_m + s_o)$. Proceeding in this way, the coefficients of the last two terms of Eqn. 4 simplify to $(K + s_o)/2ks_o$ so that it may be written

$$-t = \frac{K + s_o}{2ks_o}\left[\ln\frac{a + bs + cs^2}{a + bs_o + cs_o^2} + \ln\frac{(2cs + b - \sqrt{q})(2cs_o + b + \sqrt{q})}{(2cs + b + \sqrt{q})(2cs_o + b - \sqrt{q})}\right]$$

The terms in the brackets are expanded by inserting the appropriate values of a, b, c and q to give

$$-t = \frac{K + s_o}{2ks_o}\left[\ln\frac{k_3e_o(K + s) - k(K_m + s)(s_o - s)}{k_3e_o(K + s_o)} + \ln\frac{k_3e_o(K + s_o) - k(K_m + s_o)(s_o - s)}{k_3e_o(K + s_o)}\right] \tag{5}$$

As s is close to s_o, $(K + s)$ and $(K_m + s)$ can be replaced by $(K + s_o)$ and $(K_m + s_o)$ respectively so that

$$-t = \frac{K + s_o}{2ks_o} \cdot 2\ln\frac{k_3e_o(K + s_o) - k(K_m + s_o)(s_o - s)}{k_3e_o(K + s_o)}$$

$$= \frac{K + s_o}{ks_o}\ln\frac{k_3e_o(K + s_o) + k(K_m + s_o)(s_o - s)}{k_3e_o(K + s_o)} \tag{5a}$$

$$\therefore \frac{k_3e_o(K + s_o) + k(K_m + s_o)(s_o - s)}{k_3e_o(K + s_o)} = e^{-k^*t}$$

where

$$k^* = k \cdot \frac{s_o}{K + s_o} \tag{6}$$

$$\therefore (s_o - s) = \frac{k_3e_o(K + s_o)}{k(K_m + s_o)}[1 - e^{-k^*t}] = \frac{V_o^*}{k}[1 - e^{-k^*t}] \tag{7}$$

where

$$V_o^* = V_o\frac{K + s_o}{K_m + s_o} \tag{8}$$

Substituting the value of k^* from Eqn. 6 gives

$$(s_o - s) = \frac{k_3e_os_o}{k^*(K_m + s_o)}[1 - e^{-k^*t}] = \frac{v_o}{k^*}[1 - e^{-k^*t}] \tag{9}$$

Eqns. 7 and 9 are of the same form as the empirical relationship used previously [8]. If $K_m = K$, the term outside the brackets becomes $V_o/k = v_o/k^*$ and k^* is $k \cdot s_o/(K_m + s_o)$. By expanding the exponential Eqn. 9 becomes

$$(s_o - s) = v_ot - \frac{v_ok^*}{2!} \cdot t^2 + \frac{v_o^2k^{*2}}{3!} \cdot t^3 \ldots$$

which is of the same form as the empirical power series in time previously used for the evaluation of some kinetic data [12]. It also shows that when $k = 0$, Eqn. 9 simplifies to that for a zero-order reaction.

It also follows, by differentiating Eqn. 9, that the variation with time of the velocity of the catalytic reaction is given by

$$-\frac{ds}{dt} = v_o \cdot e^{-k^* t}$$

As pH-stat recordings, for reasons inherent in the instrumentation [13], show irregularities in their initial stages and cannot be extrapolated through the origin, empirical constants must be added to Eqns. 7 and 9 to give Eqns. 7a and 9a from which the various coefficients can be obtained by

$$(s_o - s) = \frac{V_o^*}{k}[1 - e^{-k^* t}] + C \qquad (7a)$$

$$= \frac{v_o}{k^*}[1 - e^{-k^* t}] + C \qquad (9a)$$

suitable computation, either by fitting the exponentials directly or by the method of Guggenheim [14] where two series of observations are taken, u_1, u_2 ... at times t_1, t_2 ... and u_1', u_2' ... at times $t_1 + \tau$, $t_2 + \tau$ ... (u is $(s_o - s)$). Suitable manipulation of Eqns. 7 and 9 gives

$$\ln(u' - u) = -k^* t + \ln\left[\frac{V_o^*}{k}(1 - e^{-k^* \tau})\right] \qquad (7b)$$

and

$$\ln(u' - u) = -k^* t + \ln\left[\frac{v_0}{k^*}(1 - e^{-k^* \tau})\right] \qquad (9b)$$

In both cases the slopes of plots of $\ln(u' - u)$ against t gives k^* and from the intercepts on the ordinates it is possible to calculate v_o, the initial velocity of the enzyme reaction, or V_o^*, a function of V_o (Eqn. 8).

Computations

Eqns. 7a and 9a were fitted by a least-squares procedure on a Univac 1100 computer. At least 15 experimental points were used. Eqns. 7b and 9b were fitted by a least-squares procedure in a PDP8 computer: at least 9 values of $(u' - u)$ were used and τ was about 1 min.

Eqn. 6 shows that k^* is related to k by a Michaelis relationship so that the latter, and K, were obtained from values of k^* at several values of s_o. The computations were made by the method of Wilkinson [15], as were those of V_o and K_m from v_o.

Eqn. 3 was fitted by data obtained from the usual progress curves. At any time, t, $(s_o - s)$ is directly obtained from the recording: as s_o is known, s may be calculated. The slope of the progress curve at time t is a measure of the concentration of the enzyme, e, and this may be calculated using the known specific activity of the enzyme at the appropriate substrate concentration. The slope of the progress curve at time t was obtained from a least-squares fit of five values of $(s_o - s)$ at times t_{-2}, t_{-1}, t, t_{+1} and t_{+2}.

Results

Validity of the method

The assumptions made in simplifying Eqn. 4 to Eqn. 5 were justified by computations using the appropriate values of the kinetic constants for nitrocatechol sulphate. The maximum error caused by the simplifications was about 5% and with other substrates, which are used at higher concentrations, the error was much less: with 25 mM nitroquinol sulphate it was about 1% and with 100 mM 4-nitrophenyl sulphate it was about 0.1% The simplifications are therefore justifiable.

Some evidence for the validity of the earlier stages in the derivation of Eqn. 4 is given by Fig. 1 which shows the plot of e against $(s_o - s)\ (K_m + s)/(K + s)$, according to Eqn. 3. As predicted, the points fit a straight line drawn using the known values of e_o and s_o, and the values of K and K_m in Table I. The plot, which is somewhat 'noisy' because of the errors in determining the point slope of the progress curve (see previous section), is independent of s_o (Eqn. 3): 10 experiments, over a 10-fold range of nitroquinol sulphate concentrations gave a value for e_o, the intercept on the ordinate, of 0.64 ± 0.01 μg/ml and for k/k_3, the slope, of $0.69 \pm 0.05 \times 10^{-5}$. The expected values were 0.63 μg/ml and 0.69×10^{-5} respectively.

The values of k^* and v_o obtained from Eqns. 7 and 9 are quite precise. Ten replicate determinations in the pH-stat, with nitrocatechol sulphate as substrate, gave mean values for k^* and v_o of 0.24 ± 0.01 min^{-1} and 81.7 ± 2.4 $\mu mol \cdot l^{-1} \cdot min^{-1}$ by direct fitting of the exponential (Eqn. 9a) and 0.24 ± 0.02 min^{-1} and 81.3 ± 2.8 $\mu mol \cdot l^{-1} \cdot min^{-1}$ by the Guggenheim method (Eqn. 9b). It is obvious that the values obtained by fitting the same data to the two forms of Eqn. 9 are indistinguishable. The precision of the methods fall as k^* falls and the progress curves approximate more and more closely to straight lines.

Computations were also made to assess the interval over which the methods are valid. These showed that provided the measurements spanned more than

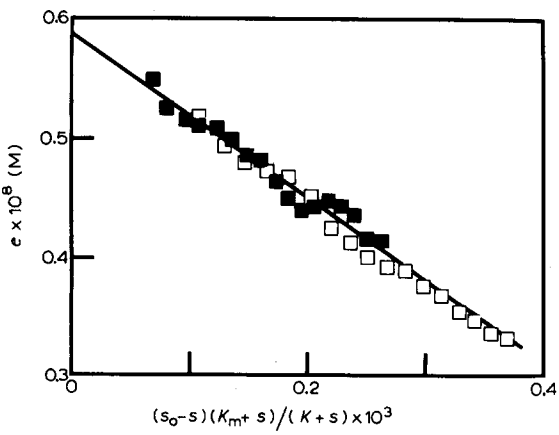

Fig. 1. Relationship between the enzyme concentration, e, and the substrate concentration, s, plotted according to Eqn. 3 in the text. pH-stat assay with nitroquinol sulphate, 23.9 mM (□) or 2.39 mM (■). Values of K_m and K from Table I. Enzyme concentration, 0.63 μg/ml.

TABLE I

KINETIC CONSTANTS FOR THE HYDROLYSIS OF SULPHATE ESTERS BY SULPHATASE A AND FOR THE SUBSTRATE-INDUCED INACTIVATION OF THE ENZYME

All determinations made at pH 5.6, $\mu = 0.1$, 37°C. Values were computed by the method of Wilkinson [15] and are given ± the standard error.

	K_m (mM)	V_o (μmol · mg^{-1} · min^{-1})	K (mM)	k (min^{-1})
Nitrocatechol sulphate	1.0 ± 0.2	234 ± 19	0.34 ± 0.15	0.22 ± 0.02
Nitroquinol sulphate	5.92 ± 0.29	243 ± 4	2.56 ± 0.40	0.23 ± 0.01
2-Nitropyridyl 3-sulphate	5.88 ± 0.28	252 ± 5	1.29 ± 0.22	0.22 ± 0.01
2-Nitrophenyl sulphate	12.3 ± 0.9	270 ± 10	2.52 ± 0.30	0.23 ± 0.01
Ascorbate 2-sulphate	24.3 ± 3.3	90 ± 8	5.35 ± 1.14	0.22 ± 0.02
4-Methylumbelliferone sulphate	41.0 ± 4.2	48 ± 4	9.56 ± 0.95	0.28 ± 0.01
2-Naphthyl sulphate	40.6 ± 3.2	37 ± 1	10.6 ± 2.6	0.26 ± 0.03
3-Nitrophenyl sulphate	104 ± 17	225 ± 2	25.9 ± 6.6	0.28 ± 0.03
4-Nitrophenyl sulphate	223 ± 32	277 ± 30	52.8 ± 15.2	0.30 ± 0.01

about 1 min then the values of k^* and v_o were independent both of the interval between readings and the starting point.

Initial velocities

Effect of enzyme concentration. This can be studied only over a rather restricted range in the pH-stat, but with nitroquinol sulphate as substrate in the spectrophotometric assay at pH 4.6, 5.0 or 5.6, k^* was independent of enzyme concentration over the useful range, 0.05 to about 2 μg/ml. Over the same range, v_o varied directly with the concentration of enzyme. Sulphatase A exists as a monomer-tetramer equilibrium and the weight-fraction of monomer changes from 0.98 to 0.16 over this concentration range at pH 5.0 [12]: these results therefore show that the monomer and tetramer are inactivated at very similar rates by the substrate. They also confirm the previous observations [12] that these two forms have similar catalytic activities.

The effect of pH. The effect of changes in pH on k^* and v_o with nitroquinol sulphate as substrate is shown in Fig. 2. There are obvious differences between the reactions in KCl (pH-stat assay) and in sodium acetate (spectrophotometric assay), but in both cases these two parameters vary in a similar way, except at low pH. In general, the variation in k^* is much less than that in v_o. Similar curves to those in Fig. 2 were found with nitrocatechol sulphate (optimum pH, 5.6) and with ascorbate 2-sulphate (optimum pH, 4.8), both measured in the pH-stat. Detailed studies of the effect of pH are impracticable because, as already noted, of the existence of sulphatase A as a polymerising system [12]. At the concentration used in these assays, about 1 μg/ml, it exists as a tetramer at low pH values and as a monomer at high pH values so that the interpretation of the effects of changes in pH is very complex.

The sharp increase in k^* at low pH values (Fig. 2), which also occurs with nitrocatechol sulphate and ascorbate 2-sulphate, is partly due to the instability of sulphatase A in dilute solutions at low pH [12] but is primarily due to a reaction involving the substrate. As previously noted [16], there are differences between the inactivation occurring at pH 4.6 and at 5.6. The enzyme inacti-

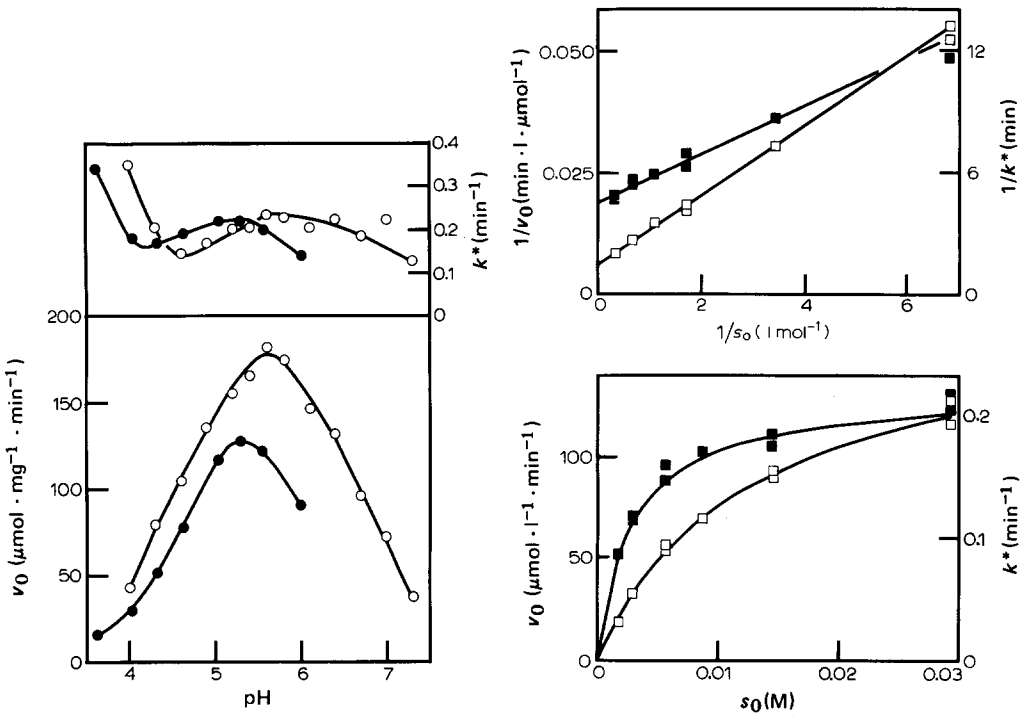

Fig. 2. The effect of pH on v_0 and k^* for the hydrolysis of nitroquinol sulphate, 15 mM. ○, pH-stat assay, enzyme concentration 0.67 μg/ml; ●, spectrophotometric assay, enzyme concentration 1.7 μg/ml.

Fig. 3. The effect of varying concentration of 2-nitrophenyl sulphate on v_0 (□) and k^* (■) at pH 5.6. Results are plotted directly in A and as reciprocals in B. pH-stat assay, enzyme concentration 0.6 μg/ml. In A the curves are computed from the values of k, K, V_0 and K_m in Table I; in B the lines are those obtained by the method of Wilkinson [15].

vated through its reaction with nitrocatechol sulphate at pH 4.6 is not reactivated by SO_4^{2-} either at that pH or at pH 5.6: it is, on the contrary, inhibited by SO_4^{2-} as is the initial velocity of native sulphatase A.

Effect of substrate concentration. In general, k^* decreases with decreasing substrate concentration, as predicted by Eqn. 6. Fig. 3 shows the relationships, at pH 5.6, between k^* and v_0 and the initial concentration, s_0, of the substrate (2-nitrophenyl sulphate) both as direct plots and in the corresponding double reciprocal forms. From such data K_m, V_0, K and k can be calculated and values of these constants for a number of substrates are listed in Table I. It is clear that K is consistently less than K_m: the ratio K/K_m varies from 0.20 to 0.43, with a mean of 0.28. There is much less variation in k than in the other parameters listed in Table I and only the values for methylumbelliferone, 3-nitrophenyl and 4-nitrophenyl sulphates are significantly different from the mean of the other values, 0.23 min^{-1}. The situation with 3-nitrophenyl sulphate will be considered later, but it should be noted that it was not practicable to use the other two substrates in concentrations much greater than K_m so that these results are not highly significant.

With 3-nitrophenyl sulphate the situation was complicated by the fact that at concentrations greater than about 50 mM the rate of inactivation greatly

increased and the use of any of the forms of Eqn. 9 gave values for v_o which were too high to be compatible with the pH-stat recordings. The reason for this behaviour has not been established but 3-nitrophenyl sulphate is quite surface-active, with a critical micelle concentrations of about 5 mM, so that the inhibition may be similar to that brought about by high concentrations of sodium dodecyl sulphate [17].

Fig. 4 shows the relationship between V_o^* and s_o (Eqn. 8) again for 2-nitrophenyl sulphate. As predicted, there is a linear relationship between V_o^* and $(K + s_o)/(K_m + s_o)$: the slope of the line gives a value of 172 μmol · l^{-1} · min^{-1} for V_o (Eqn. 8) compared with a value of 168 μmol · l^{-1} · min^{-1} obtained from the variation of v_o with s_o (Fig. 3).

At high concentrations of 2-nitrophenyl sulphate or of nitroquinol sulphate there was little change in k^* or v_o. The results for the former are shown in Table II. The highest value of s_o which could be used, still keeping the ionic strength at 0.1, was only about 8 × K_m. Similar experiments with nitrocatechol sulphate, which has a much lower K_m, were not practicable because the high buffer capacity of this substrate (pK 6.5) precluded its use in the pH-stat at concentrations greater than about 5 mM.

At pH 4.6 the effect of varying substrate concentration was more complex and k^* increased slightly with decreasing substrate concentration, at least with ascorbate 2-sulphate or 2-nitrophenyl sulphate, the only two substrates tested. The initial velocity apparently varied in a normal fashion with s_o but the recip-

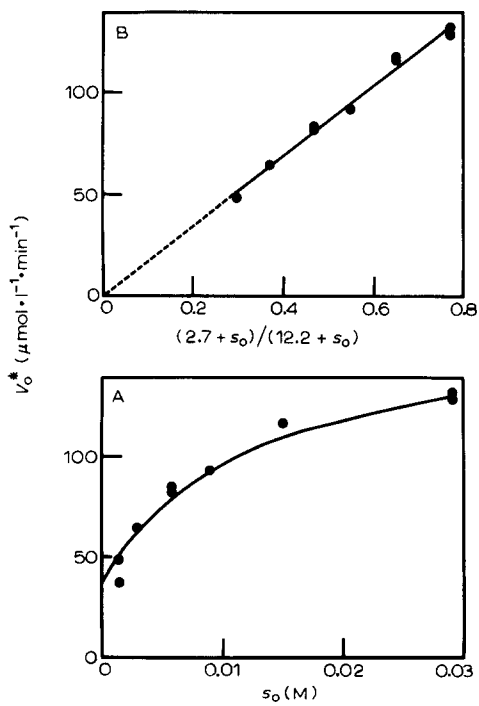

Fig. 4. Data of Fig. 3 plotted to show the variation of V_o^* (from Eqn. 7a) with the initial concentration (s_o) of 2-nitrophenyl sulphate plotted directly (A) or according to Eqn. 8 (B).

TABLE II

THE EFFECT OF HIGH CONCENTRATIONS OF 2-NITROPHENYL SULPHATE ON THE SUBSTRATE-INDUCED INACTIVATION OF SULPHATASE A

pH 5.6, μ = 0.1, 37°C, enzyme concentration 0.5 μg/ml. The two values are from duplicate determinations.

s_o (mM)	v_o (μmol · l^{-1} · min^{-1})	k^* (min^{-1})
20	101, 101	0.20, 0.20
40	119, 120	0.22, 0.22
60	125, 127	0.21, 0.22
80	132, 128	0.22, 0.21
100	131, 125	0.21, 0.20

rocal plot was non-linear as previously noted with ascorbate 2-sulphate [7] and nitrocatechol sulphate [12] at pH 4.5.

Effect of ionic strength. Changes in ionic strength change K_m and K in similar ways: KCl behaves as an inhibitor with a K_i of about 0.3 and 0.6 M when measured from v_o and k^* respectively. Both constants therefore increase with increasing ionic strength and the extrapolated values, at zero ionic strength, of K_m and K were 4.2 and 2.2 mM nitroquinol sulphate respectively (compare with Table I). Any effect on k was small: at concentrations of 0.1, 0.25, 0.5 and 1 M KCl its values were 0.23 ± 0.01, 0.23 ± 0.02, 0.19 ± 0.01 and 0.18 ± 0.01 min^{-1} respectively. The net effect is a considerable drop in k^* with increasing ionic strength, from 0.21 min^{-1} at μ = 0.1 to 0.14 min^{-1} at μ = 1.0, both at 25 mM nitroquinol sulphate.

Effect of sulphate concentration. The effect of the reaction product, SO_4^{2-}, on the substrate-induced inactivation of sulphatase A was difficult to study, first because of the relatively low rate of the hydrolytic reaction and second because of the drop in k^* which made the progress curves more nearly linear. Some results with nitroquinol sulphate are given in Table III. These are consistent with SO_4^{2-} behaving as a competitive inhibitor both of the hydrolytic reaction and of the substrate-induced inactivation of sulphatase A with K_i values of about 2 mM and 1 mM SO_4^{2-} respectively.

Temperature. The effect of varying temperature was investigated only with nitroquinol sulphate, in the continuous spectrophotometric assay. Over the

TABLE III

EFFECT OF SO_4^{2-} ON THE APPARENT KINETIC CONSTANTS

Substrate: nitroquinol sulphate. Assay in pH-stat at pH 5.6, μ = 0.1. Enzyme concentration, 0.6 μg/ml. The apparent constant (± standard error) were computed by the method of Wilkinson [15].

SO_4^{2-} (mM)	K_m (mM)	V_o (μmol · l^{-1} · min^{-1})	K (mM)	k (min^{-1})
0.0	6.07 ± 0.28	185 ± 3	1.72 ± 0.25	0.22 ± 0.01
0.419	7.53 ± 0.56	181 ± 4	2.32 ± 0.44	0.22 ± 0.01
1.03	11.2 ± 0.6	197 ± 5	3.80 ± 0.90	0.22 ± 0.02
2.11	14.0 ± 0.7	180 ± 5	6.66 ± 0.95	0.21 ± 0.01
3.05	15.3 ± 1.1	170 ± 5	9.42 ± 0.96	0.18 ± 0.01
4.10	19.9 ± 1.3	175 ± 5	8.16 ± 1.9	0.18 ± 0.01

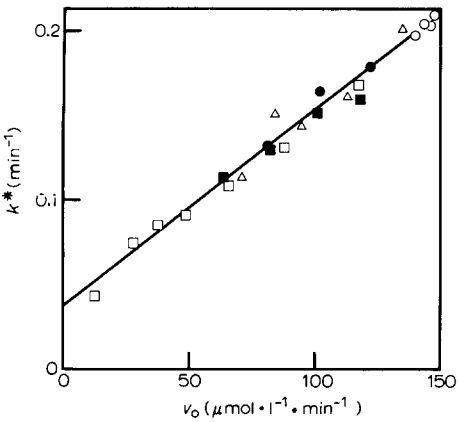

Fig. 5. The relationship between v_0 and k^* for the hydrolysis of nitroquinol sulphate (15 mM) at pH 5.6 in different concentrations of organic solvents: ●, dioxane; □, dimethylformamide; ■, dimethylsulphoxide and △, ethanol. The open circles show the relationship in water. Spectrophotometric assay: enzyme concentration 1 μg/ml. The concentrations of the solvents increased in steps of 5% (v/v) but are not shown on the Figure because the response varied from solvent to solvent.

temperature range 24—45°C, k^* increased 4-fold whereas v_0 increased only 2.5-fold. The values of k^* and v_0 gave good fits of the Arrhenius relationship with values of 56 kJ/mol and 33 kJ/mol respectively for the substrate-induced inactivation and the catalytic reaction.

Effect of organic solvents. Again this was investigated only with nitroquinol sulphate and it must be stressed that no account was taken of any change in pH caused by the addition of organic solvents to the buffer solution. The results are summarised in Fig. 5 which shows that the addition of dimethylsulphoxide, dimethylformamide, dioxane or ethanol to the reaction mixture decreased both v_0 and k^*, and that there was a linear relationship between these constants although the line did not obviously extrapolate through the origin. The response varied slightly from solvent to solvent and the enzyme was particularly sensitive to dioxane of which concentrations greater than about 15% (v/v) gave a sharp increase in k^* with little change in v_0. This presumably reflected the pronounced effect of dioxane on the structure of sulphatase A [17].

Bound sulphatase A. A sample (1.4 mg) of sulphatase A was coupled to 200 mg of CNBr-Sepharose 4B as described by the manufacturers and this preparation was used in the normal assay in the pH-stat. It was not possible to obtain reliable values for v_0 because of the difficulty in pipetting suspensions of the enzyme but apparently reliable values of k^* were obtained. With substrate concentrations ranging from 0.13 to 5 mM nitrocatechol sulphate there was no significant variation in k^*, the mean value being 0.14 min^{-1} for 16 observations. This value of k^* must approximate to k, and therefore K must be much less than 0.13 mM, and so much less than the corresponding value for the unbound enzyme (Table I).

Prolonged incubation times

Experiments involving long incubation times were technically difficult in the

pH-stat because with useful values of v_o care had to be taken that too extensive hydrolysis of the substrate, with the concomitant increase in concentration of reaction products, did not occur. Also, the great drop in enzyme activity during relatively prolonged reaction times (see, for example, Fig. 2 or ref. 4 or Fig. 1 of ref. 5) made it difficult to achieve useful reaction rates in the later stages. In such experiments with 2-nitrophenyl sulphate k^* and v_o were determined as usual from data between 1 and 3 min after the start of the reaction and k_4^* and v_4 were determined from data obtained between 4 and 6 min after the start of the reaction. Both sets of data were then used to compute K_m, K and k. The results for the longer times are given in Table IV from which it is clear that after the reaction has proceeded for 4 min K_m and K are indistinguishable. This change is not due to the accumulation of SO_4^{2-} because identical results were obtained when the ionic strength was maintained at 0.1 with $BaCl_2$ so that the concentration of SO_4^{2-} was effectively zero throughout.

Some determinations were also carried out as described by Stinshoff [5], with an incubation time of 20 min. Values of v_o and k^* (from Eqn. 9a) were used to compute K_m, K and k as usual. The results are also given in Table IV and again there is no difference between K_m and K measured under these conditions.

Discussion

The mechanism which is the basis of the equations used in the present work will be considered later but the present methods for determining k^*, the apparent velocity constant for the substate-induced inactivation of sulphatase A, are quite soundly based in theory and are of the same form as the empirical methods used previously [8]. Both methods, using either Eqn. 9a or 9b, avoid errors introduced [8] by the use [5,6] of linear approximations [18] to Eqns. 7 and 9, and the Guggenheim treatment has the added advantage that the precise zero time of the reaction need not be known. Values of v_o, the initial velocity of the enzyme reaction, can also be obtained by either method but for this precise zero times are required. It must be stressed that the derivation of Eqns. 7 and 9 assumes that s remains close to s_o and the treatment would be invalid is this condition were not met. Only with nitrocatechol sulphate, normally used at a concentration of less than 5 mM, is care required to meet this requirement when using the relatively insensitive assay in the pH-stat.

It must also be stressed that the methods will give a value for k^* whatever be the mechanism of the inactivation of the enzyme. As already pointed out, the constant determined at pH 4.5 pertains to a different reaction than that at pH 5.6. It is therefore important to show that the reaction involved is substrate-induced, by demonstrating that it does not occur in the absence of substrate.

The principal results are those in Table I which show that when data from short reaction times are used to obtain v_o and k^* then the corresponding values of K_m and K for any given substrate are different. On the other hand, the constant k, the velocity constant for the inactivation of the enzyme at infinite substrate concentration, varies little with different substrates. It is also independent of V_o, and so presumably of the velocity constant of the reaction leading from the enzyme · substrate complex to reaction products. Proof of the

constancy of k would require more precise determinations than can be made at present but the results suggest that the rate-limiting step in the formation of the inactive enzyme may involve not an enzyme · substrate complex, as previously suggested [2—6], but rather an enzyme · SO_4^{2-} complex derived from the former during the catalytic cycle. If this be the case, two conclusions can be drawn: one, that the reaction leading to F must yield at least the phenolic reaction product otherwise E · SO_4^{2-} could not be produced and two, that the latter complex must be different from that formed when SO_4^{2-} acts as a competitive inhibitor and so reduces k^*. Such a difference is not unexpected because, as far as is known, the sulphatase reaction is irreversible and certainly SO_4^{2-} has no action on the enzyme in the absence of the substrate. The results in Table II, and the similar results with nitroquinol sulphate, are important in showing that k^* does not increase as the substrate concentration is increased above the optimum. This effectively disproves the suggestion by Nicholls and Roy [4] that the rate-limiting step in the inactivation of sulphatase A is the formation of a complex of the type FS_2.

The values of K_m given in Table I differ from values previously reported. The difference arises from the fact that in the present work true values of v_o were used to obtain K_m and the ionic strength was held constant at 0.1. It should be noted that to obtain meaningful values of K_m it is essential to measure v_o. If the amount of reaction product formed in a given time is simply taken as the reaction velocity then the apparent K_m is a function of time. This was shown by using Eqn. 9 to compute the amounts of product produced at various reaction times and these to compute K_m, the apparent value of which fell exponentially with time. Using the appropriate constants for nitrocatechol sulphate (Table I), the computed values of K_m after 15 and 30 min incubation periods were 0.42 and 0.28 mM respectively, compared with the experimental value, from v_o, of 1.0 mM. Similar changes have been found experimentally in the present work and have been noted previously by Stinshoff (Table IV of ref. 5).

The effect of temperature needs little comment but the fact that the Arrhenius activation energy of the substrate-induced inactivation of sulphatase A is greater than that for the catalytic reaction explains previous observations [19,20] that the 'anomalous' kinetics of sulphatase A become less apparent as the temperature is decreased.

With longer reaction times the interpretation of the results is less clear. Nevertheless, such experiments suggest (Table IV) that the values of K_m and K become identical under these conditions. This is also suggested from the data of Stinshoff (Table III of ref. 5) which gives, using the nomenclature of this paper, values of 0.59 ± 0.05 mM and 0.59 ± 0.06 mM respectively for K_m and K.

These observations suggest that the mechanism of the substrate-induced inactivation of sulphatase A is somewhat different from that previously considered. The most important observation is that in the initial stages of the reaction K_m and K are not identical. As there is nothing to suggest that the two processes involve different forms of the enzyme — for example, no differences have been noted in different preparations of sulphatase A and the inactivation can go to completion — they must involve kinetically distinct reactions, presumably at different sites on the enzyme. Evidence for this is given by Fig. 5 which implies that it should theoretically be possible to achieve inactivation in

TABLE IV

VALUES OF K_m, K AND k MEASURED OVER PROLONGED INCUBATION TIMES

The first values for 2-nitrophenyl sulphate were obtained from measurements in the pH-stat: all other values were obtained by spectrophotometric measurements. pH 5.6, μ = 0.1, 37°C. Substrate and enzyme concentrations: 2-nitrophenyl sulphate, 30 mM, 0.5 μg/ml and 0.2 μg/ml; nitroquinol sulphate, 35 mM, 0.2 μg/ml; nitrocatechol sulphate, 5 mM, 0.08 μg/ml.

Substrate	Incubation (min)	K_m (mM)	K (mM)	k (min^{-1})
2-Nitrophenyl sulphate	4—6	5.9 ± 0.1	5.3 ± 0.6	0.20 ± 0.01
	0—20	11.1 ± 0.8	7.7 ± 0.7	0.19 ± 0.01
Nitroquinol sulphate	0—20	7.3 ± 0.2	7.5 ± 0.7	0.21 ± 0.01
Nitrocatechol sulphate	0—20	0.84 ± 0.06	0.77 ± 0.08	0.25 ± 0.01

the absence of any hydrolytic reaction because k^* is finite when v_o is zero. However, as K_m and K become identical over longer reaction times and, as already pointed out, the inactivation can go to completion, an interconversion of the participating forms of the enzyme is implied. The results in Table I suggest that it is an enzyme · SO_4^{2-} complex which is involved in the inactivation step and those in Table II show that the rate-limiting step cannot involve the formation of an FS_2 complex. The picture which emerges is of two processes which are independent in the early stages of the reaction but which become interdependent as the reaction proceeds and an equilibrium between them is slowly established. This can be represented by the following scheme in which the first reaction is the normal hydrolytic reaction and the second substrate-induced inactivation.

$$\begin{array}{l} \qquad\qquad\qquad\quad \phi \cdot OH \\ \qquad\qquad\qquad\quad + \\ E + S \rightleftharpoons ES \rightleftharpoons \ldots \rightleftharpoons E \cdot SO_4^{2-} \rightleftharpoons E + SO_4^{2-} \\ \qquad\qquad\qquad\quad \Big\| \quad \text{(slow reactions)} \\ E + S \rightleftharpoons E'S \rightleftharpoons \ldots \rightleftharpoons E' \cdot SO_4^{2-} \rightleftharpoons F\ (+ SO_4^{2-}\ ?) \\ \qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad \phi \cdot OH \end{array}$$

There is still no information on the nature of the change in the enzyme which causes it to lose its activity although it seems to be presumed that it is conformational [6]. Perhaps the finding that sulphatase A bound to Sepharose is less rapidly inactivated than the soluble enzyme supports this view but much further work is required in this area. This will be facilitated by the availability of a soundly-based method allowing the determination of the kinetic constants of both the catalytic reaction and the inactivation.

Appendix

The assay of sulphatase A in the pH-stat

The use of the pH-stat in biochemistry was extensively discussed by

Jacobsen et al. [13] in 1958 and the following brief treatment must be read in conjunction with this work. These authors derived an expression describing the drift in pH with time in a reaction controlled by a pH-stat functioning in the Δt mode, that is, in the way in which a Radiometer Titrator (Radiometer Ltd., Copenhagen) functions with the proportional control operative, as is normally the case. This expression (Eqn. 18 of ref. 13) is given in Eqn. 10 using the symbols of Jacobsen et al. [13] to facilitate cross-referencing. In Eqn. 10,

$$\mathrm{pH} = \mathrm{pH}_p + \mathrm{e}^{-Pt/BC} \int_0^t \left[\frac{\mathrm{dpH}}{\mathrm{d}t}\right]_0 \mathrm{e}^{Pt/BC}\,\mathrm{d}t \tag{10}$$

pH is the actual pH of the reaction mixture at time t, pH_p is the value set on the titrator, P is a proportionality constant governing the rate of addition of reagent within the proportional band of the titrator and BC is the buffer capacity of the system. The form of $(\mathrm{dpH}/\mathrm{d}t)_0$, the change in pH in the uncontrolled system, varies with the nature of the reaction and is particularly simple in those enzyme reactions which are of zero order in which case $(\mathrm{dpH}/\mathrm{d}t)_0$ is constant. Jacobsen et al. [13] also showed in their Eqn. 15 that

$$\left[\frac{\mathrm{dpH}}{\mathrm{d}t}\right]_0 = -BS \cdot \frac{\mathrm{d}N_{\mathrm{H}^+}}{\mathrm{d}t}$$

where $\mathrm{d}N_{\mathrm{H}^+}/\mathrm{d}t$ is the rate of production of H^+ ions in the uncontrolled system. In the case of the sulphatase A reaction $\mathrm{d}N_{\mathrm{H}^+}/\mathrm{d}t = -\mathrm{d}s/\mathrm{d}t = v_0\mathrm{e}^{-k^*t}$ so that

$$\left[\frac{\mathrm{dpH}}{\mathrm{d}t}\right]_0 = -\frac{v_0 \cdot \mathrm{e}^{-k^*t}}{BC}$$

Substitution of this into Eqn. 10 followed by integration and rearrangement gives Eqn. 11 where M_{H^+} is the amount of NaOH added by the pH-stat and ω is given by Eqn. 12, in which $\Delta\mathrm{pH}_m$ is the proportional band setting on the titrator and x is the ratio of the maximum rate of addition of NaOH (burette speed) to v_0.

$$M_{\mathrm{H}^+} = -\frac{v_0}{k^*}(1 - \mathrm{e}^{-k^*t}) + \frac{v_0}{k^* - \omega}(\mathrm{e}^{-k^*t} - \mathrm{e}^{-\omega t}) \tag{11}$$

$$\omega = \frac{x \cdot v_0}{BC \cdot \Delta\mathrm{pH}_m} \tag{12}$$

Again in a zero order reaction x is constant: in a reaction of any other order it is not. In a first order reaction x can be taken as approximately constant in the early stages of the reaction, providing it is initially of a reasonably high value, but in the case of the sulphatase A reaction it cannot be considered constant at any stage and in this case ω can be shown to have the form of Eqn. 13 in which $(\mathrm{d}M_{\mathrm{H}^+}/\mathrm{d}t)_{\max}$ is the burette speed. Substitution;

$$\omega = -\frac{(\mathrm{d}M_{\mathrm{H}^+}/\mathrm{d}t)_{\max} \cdot \mathrm{e}^{-k^*t}}{BC \cdot \Delta\mathrm{pH}_m} \tag{13}$$

of this value for ω in Eqn. 11 gives the expression for the rate of addition of NaOH to a sulphatase A reaction under control in a pH-stat: that is, for the

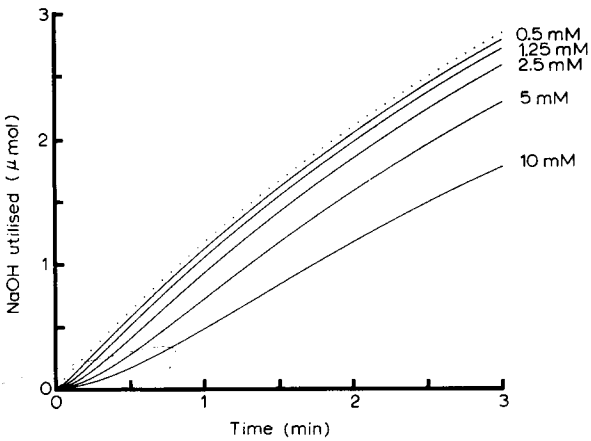

Fig. 6. Computer-simulated curves for the hydrolysis by sulphatase A of nitrocatechol sulphate in the pH-stat at pH 5.6, using a value of 130 μmol l^{-1} min^{-1} for v_0 and 0.23 min^{-1} for k^*. The different lines are computed keeping the above parameters constant and varying the buffer capacity by changing the substrate concentration, as indicated on the Figure. The same effect would be obtained by keeping the nitrocatechol sulphate concentration at 0.5 mM and having acetate of the indicated concentration present in the reaction mixture. The dots show the theoretical curve for the same reaction.

amount of substrate hydrolysed in time t. In this expression the first term on the right hand side is the theoretical expression describing the sulphatase A reaction (Eqn. 9) and the second term arises from the mode of action of the pH-stat. It is obviously highly dependent upon ω, that is, upon the buffer capacity of the system, BC.

With 2-nitrophenyl sulphate as substrate in the presence of 0.5 mM sodium acetate at pH 5.6 (standard assay conditions given in the accompanying paper) the line drawn from Eqn. 11, using the experimentally determined values of k^* and v_0 (Eqn. 9a), gives a good fit to the experimental points and differs only slightly from the corresponding theoretical curve computed from Eqn. 9.

The situation with nitrocatechol sulphate is quite different. This is routinely used at a concentration of 3 mM and as it has a pK of 6.5 it has a considerable buffer capacity in the pH range used in studies of sulphatase A activity. In Fig. 6 are shown computed curves (Eqn. 11) for different values of ω, all the other parameters being held constant. The great increase in sigmoidality with decreasing ω (increasing BC) is obvious, as is also the difference between the theoretical and the pH-stat curves. The experimental values agree well with the latter. With concentrations of nitrocatechol sulphate up to about 2.5 mM the difference is effectively constant after the reaction has proceeded for 1 min so that reliable values of k^* and v_0 can be obtained from such data because only C in Eqn. 9a would be altered. This is certainly not the case with higher concentrations of nitrocatechol sulphate where Δ does not become constant until after much longer times and the use of Eqn. 9a would give erroneous results.

It is obvious that great care must be taken when using nitrocatechol sulphate as a substrate in pH-stat assays of sulphatase A: it can be used in routine assays when the conditions are strictly standardised but if any alteration in these should change the buffer capacity then serious difficulties could arise. Nitrocatechol sulphate is therefore not the substrate of choice for kinetic studies of

sulphatase A in the pH-stat. In these, 2-nitrophenyl sulphate or nitroquinol sulphate are preferable substrates because they have no buffering action in the pH range of interest and they have useful values of K_m and V_o (Table I). The latter substrate has the added advantage that it can also be used in continuous spectrophotometric assays of the enzyme [10].

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