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The effect of pH on the kinetics of arylsulphatases A and B

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The effect of pH on the kinetics of rat liver arylsulphatases A and B is very similar and shows that two groups with pK values of 4.4–4.5 and 5.7–5.8 are important for enzyme activity. Substrate binding has no effect on the group with a pK of 4.4–4.5; however, the pK of the second group is shifted to 7.1–7.5 in the enzyme–substrate complex. An analysis of the effect of pH on the K_i for sulphate inhibition suggests that HSO_4^- is the true product. A model is proposed that involves the two ionizing groups identified in the present study in a concerted general acid–base-catalysed mechanism.

Several sulphatases have been described that catalyse the desulphation of a variety of sugar sulphate substrates. The existence of many of these enzymes was inferred from clinical syndromes where increased excretion and/or accumulation of sulphated sugar derivatives was evident (see, e.g., McKusick *et al.*, 1978, for review).

Recently, several of these enzymes, e.g. *N*-acetylglucosamine 6-sulphate sulphatase (Basner *et al.*, 1979), *N*-acetylgalactosamine 6-sulphate sulphohydrolase (Lim & Horwitz, 1981) and glucosamine *ON*-disulphate *O*-sulphohydrolase (Weissman *et al.*, 1980) have been purified. Two lysosomal enzymes, arylsulphatases A and B, have been known for many years, since their activity is easily measured by using 2-hydroxy-5-nitrophenyl sulphate as substrate. Arylsulphatase A has recently been shown to catalyse the desulphation of lipid-linked galactose 3-sulphate (Mehl & Jatzkewitz, 1968; Yamato *et al.*, 1974; Fluharty *et al.*, 1974), whereas arylsulphatase B catalyses the desulphation of *N*-acetylgalactosamine 4-sulphate (O'Brien *et al.*, 1974; Fluharty *et al.*, 1975). None of the sulphatases are particularly abundant proteins and the assays using sugar sulphates as substrates are time-consuming, so that few mechanistic studies have been reported. We have analysed the pH-dependence of arylsulphatases A and B with 2-hydroxy-5-nitrophenyl sulphate in an attempt to identify functionally important ionizing groups in these two proteins.

Materials and methods

Materials

2-Hydroxy-5-nitrophenyl sulphate and 4-nitrophenyl sulphate were obtained from Sigma. All other reagents used were of the highest purity available. Deionized distilled water was used throughout.

Partial purification of rat liver arylsulphatases A and B

Rat liver arylsulphatases A and B were partially purified as previously described (O'Fagain *et al.*, 1982).

Assay of arylsulphatase activity

Arylsulphatase activity was measured by monitoring the production of 4-nitrocatechol and 4-nitrophenol from 2-hydroxy-5-nitrophenyl sulphate and 4-nitrophenyl sulphate respectively. Assays were conducted at 30°C in a final volume of 0.5 ml containing buffer [0.33 M-sodium acetate/acetic acid, pH 4–6; 0.33 M-Mes (4-morpholine-ethanesulphonic acid)/NaOH, pH 5.6–7.1; 0.2 M-Tris/acetate, pH 6.2–8.0] and substrate dissolved in the appropriate buffer (and titrated if necessary). The reaction was normally started by the addition of 10 μl of the enzyme and terminated by the addition of 1 M-NaOH. The 4-nitrocatechol produced was measured by its A_{515} ($\varepsilon = 12\,600$ litre·mol^{-1}·cm^{-1}; Waheed & Van Etten, 1979) and the 4-nitrophenol by the A_{400} ($\varepsilon = 18\,200$ litre·mol^{-1}·cm^{-1}; Dodgson & Spencer, 1953).

Treatment of data

All initial rate data were plotted in double-reciprocal form. The slopes (K_m/V) and intercepts ($1/V$) of each line were estimated by fitting the data to eqn. (1) by the method of Wilkinson (1961):

$$v = \frac{V[S]}{K_m + [S]} \qquad (1)$$

When Na_2SO_4 was used as a reversible inhibitor, the slope replots were fitted to a straight line by linear regression and the K_i was obtained by extrapolating

to the inhibitor-concentration axis. The V and V/K_m values obtained at different pH values were fitted by a least-squares procedure to eqn. (2):

$$f = \frac{\bar{f}}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \quad (2)$$

where f is the measured kinetic parameter, $\bar{f}$ is the 'pH-corrected' parameter, H is the hydrogen-ion concentration and K_1 and K_2 are dissociation constants.

Results

The pH-stability of both enzymes was demonstrated by pre-incubating them for various periods at the extremes of pH used in the kinetic experiments before assaying activity at pH 5.9. In these and subsequent experiments, initial rates were measured over a few minutes. This is particularly important for arylsulphatase A, which exhibits a slow reversible substrate-dependent inactivation (O'Fagain *et al.*, 1982). As some of the data sets (e.g. the variation of the K_i for sulphate with pH) took several days to complete, the pH examined on the first day was repeated on completion of the experiment. These experiments showed that K_m values were unaffected. Decreases in V not exceeding 10% were encountered with arylsulphatase B, whereas arylsulphatase A remained completely stable.

For both arylsulphatases A and B, two pK values of 4.4–4.5 and 7.1–7.5 are apparent in the V data (Figs. 1*a* and 2*a*). Two pK's are also seen in the V/K_m data, yielding values of 4.4 and 5.8 for arylsulphatase A and 4.5 and 5.7 for arylsulphatase B (Figs. 1*b* and 2*b*). The pK of 7.5 in Fig. 1(*a*) is a least-squares estimate, although any pK above 7 would equally well fit the data shown in Figs. 1(*a*) and 1(*c*). Similarly, the pK of 7.1 used in the theoretical continuous curve for Fig. 2(*c*) is derived from the points in Fig. 2(*a*) and used in Fig. 2(*c*), although any pK above 7 would equally well fit the data of Fig. 2(*c*). It is only in Fig. 3(*c*) that the alkaline pK is readily apparent in the K_m data. It should be noted that pK_i etc. are molecular pK values and not group pK values (see, e.g., Dixon, 1976). The results of similar experiments using 4-nitrophenyl sulphate as substrate for arylsulphatase B are shown in Fig. 3. The effect of pH on the K_i for sulphate against arylsulphatase A and B is shown in Fig. 4. Despite a pronounced 'buffer effect', it is clear that lowering the pH enhances the inhibition by sulphate. For both enzymes the inhibition by sulphate remained strictly competitive over the entire range of pH studied. When a similar experiment was conducted with orthophosphate as a competitive inhibitor of arylsulphatase B (O'Fagain *et al.*, 1980), the K_i was independent of pH over the range 4.5–5.9. The K_i values obtained were 35 μM, 24 μM and 47 μM at pH values of 4.5, 5.2 and 5.9 respectively.

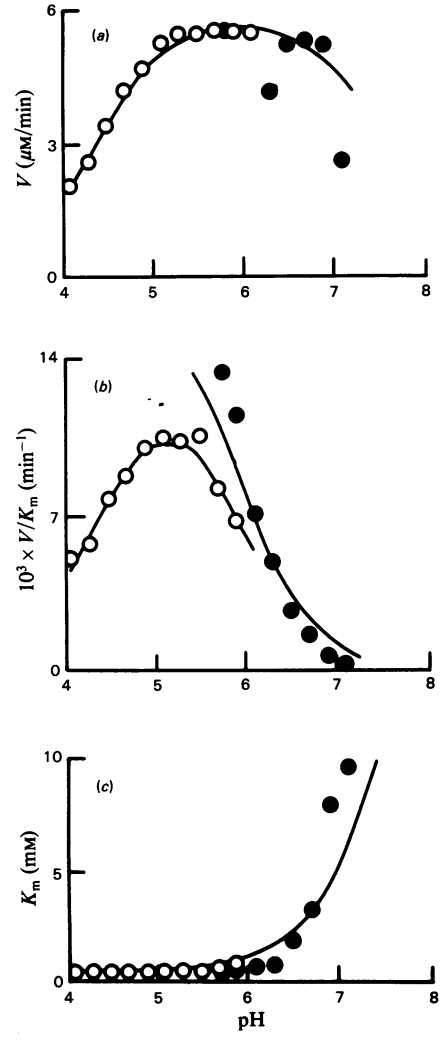


Fig. 1. *Effect of pH on the kinetics of arylsulphatase A* The effect of pH on the kinetic parameters V and V/K_m using 2-hydroxy-5-nitrophenyl sulphate as substrate are shown in (*a*) and (*b*) respectively. Data points are shown for acetate (○) and Mes (●) buffers. The continuous lines are least-squares fits to eqn. (2), where K_1, K_2 and $\bar{f}$ are, for (*a*), 4×10^{-5} M, 3×10^{-8} M and 6, and for (*b*), 4×10^{-5} M, 1.5×10^{-6} M and 1.45×10^{-2} respectively. The effect of pH on K_m is shown in (*c*) to point out that K_m is not sensitive to the ionizations characterized by K_1 and K'_1. The experiments illustrated in Figs. 1, 2 and 3 were conducted with 1 mg of partially purified enzyme.

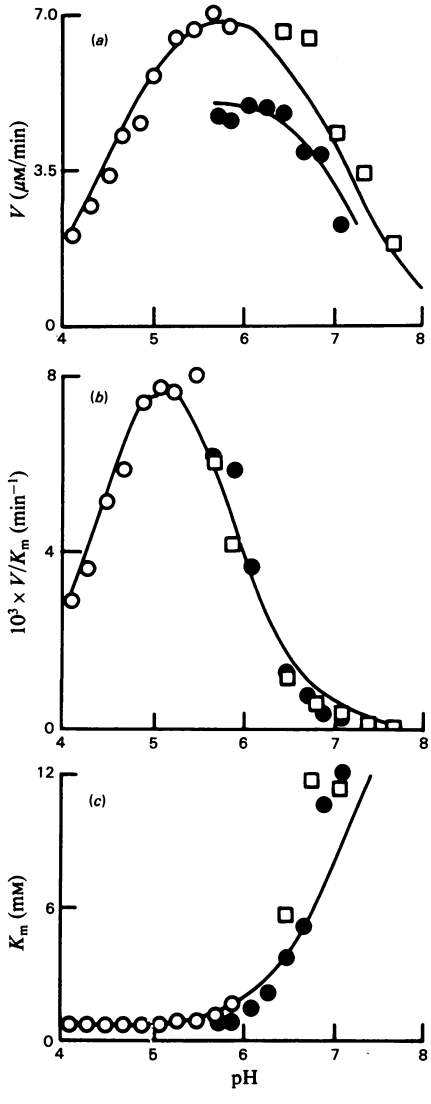


Fig. 2. *Effect of pH on the kinetics of arylsulphatase B*
The effect of pH on the kinetic parameters V and V/K_m using 2-hydroxy-5-nitrophenyl sulphate as substrate are shown in (*a*) and (*b*) respectively. Data points are shown for acetate (○), Mes (●) and Tris (□) buffers. The continuous lines are least-squares fits to eqn. (2), where K_1, K_2 and $\bar{f}$ are, for (*a*), 3×10^{-5}M, 8×10^{-8}M and 7.5, and for (*b*), 3×10^{-5}M, 2×10^{-6}M and 1.15×10^{-2} respectively. The effect of pH on K_m is shown in (*c*) to point out that K_m is not sensitive to the ionizations characterized by K_1 and K'_1.

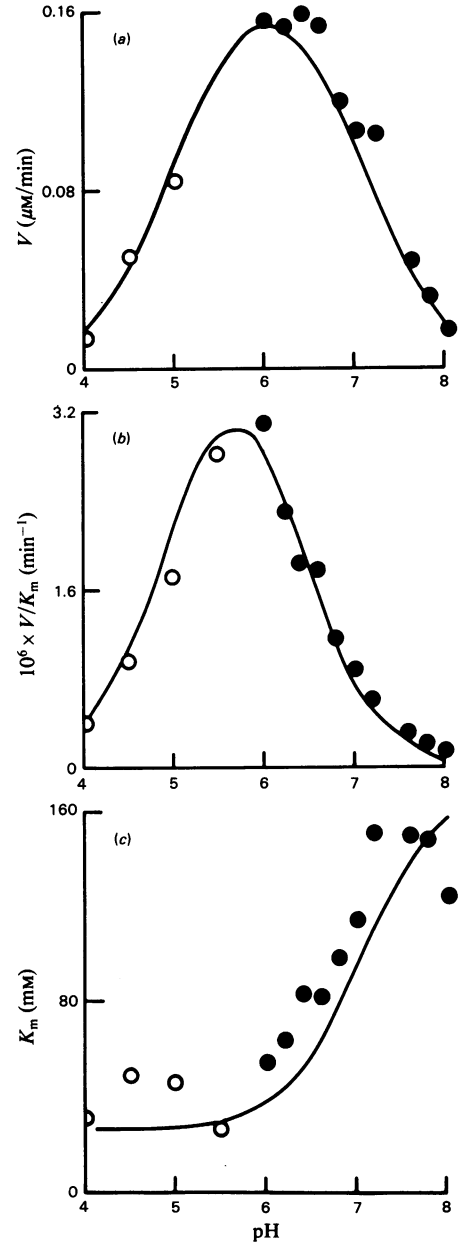


Fig. 3. *pH kinetics of arylsulphatase B with 4-nitrophenyl sulphate as substrate*
The effect of pH on the kinetic parameters V and V/K_m are shown in (*a*) and (*b*) respectively. Data points are shown for acetate (○) and Tris (●) buffers. The continuous lines are based on eqn. (2), where K_1, K_2 and $\bar{f}$ are, for (*a*), 10^{-5}M, 8×10^{-8}M and 0.18, and for (*b*), 10^{-5}M, 5×10^{-7}M and 4.5×10^{-6} respectively. The effect of pH on K_m is shown in (*c*) to point out that K_m is not sensitive to the ionizations characterized by K_1 and K'_1.

Discussion

As both arylsulphatases A and B exhibit a similar pH-dependence of their kinetic parameters and as differences in the observed pK values for the two

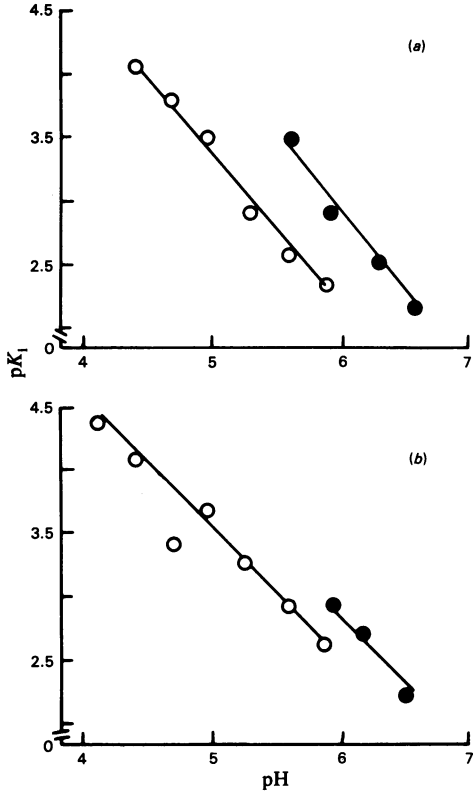


Fig. 4. *Effect of pH on the K_i for sulphate*
The data points are for acetate (○) and Mes (●) buffers with (*a*) arylsulphatase A and (*b*) arylsulphatase B.

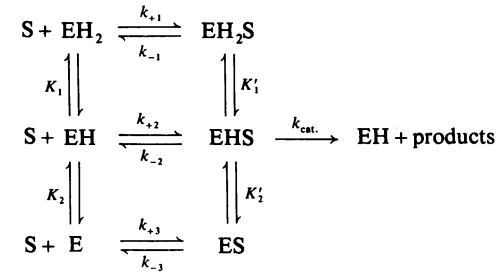
enzymes are small, no distinction is made between them in the following discussion.

Our analysis of the pH effects is based on the model shown in Scheme 1. The initial-rate equation for Scheme 1, with the normal assumption of quasi-equilibrium (see e.g. Cornish-Bowden, 1976; Brocklehurst & Dixon, 1976), is:

$$v = \frac{V[S]}{K_m\left(1+\frac{H}{K_1}+\frac{K_2}{H}\right)+[S]\left(1+\frac{H}{K_1'}+\frac{K_2'}{H}\right)} \quad (3)$$

The two pK's of the enzyme–substrate complex, K_1' and K_2' are apparent in the V data, whereas the two pK's of the free enzyme, K_1 and K_2, are apparent in the V/K_m data. K_2 and K_2' are the two pK's in the K_m data.

The 2-hydroxy group of the substrate 2-hydroxy-5-nitrophenyl sulphate ionizes with a pK of 6.4 (Nicholls & Roy, 1971). However, this is unlikely to represent an experimentally determined pK, since experiments conducted with 4-nitrophenyl sulphate

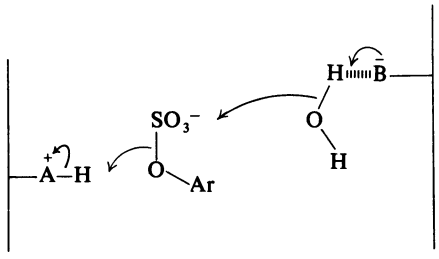


Scheme 1. *Representation of a two-step reaction involving the reversible formation of an intermediate in three protonic states followed by an irreversible step involving only one protonic state*
k_{+1}–k_{+3}, k_{-1}–k_{-3} and $k_{cat.}$ are pH-independent rate constants and K_1, K_1', K_2 and K_2' are molecular acid dissociation constants; 'free' protons are omitted for clarity. S is 2-hydroxy-5-nitrophenyl sulphate.

as substrate showed molecular pK's similar to those observed with 2-hydroxy-5-nitrophenyl sulphate.

The decrease in the K_i for sulphate with decreasing pH suggests that the bisulphate anion HSO_4^- is the true inhibitory species. This interpretation is supported by the independence of the K_i for phosphate over the pH range 4.5–5.9, where it exists predominantly as the monoanion $H_2PO_4^-$. As $H_2PO_4^-$ binding is independent of any residue on the enzyme ionizing over the pH range 4.5–5.9 and assuming that HSO_4^- and $H_2PO_4^-$ bind at the same site (both are competitive inhibitors), then the decrease in K_i observed with sulphate over this pH range must be due to protonation of SO_4^{2-}. The K_i for HSO_4^- (calculated for the acetate buffer series) is 0.39 μM for both arylsulphatases A and B. This very tight binding of HSO_4^- indicates that this species is the true product which normally loses a proton to form SO_4^{2-} only after diffusing from the enzyme surface.

It is known that (i) hydrolysis of 2-hydroxy-5-nitrophenyl sulphate and 4-nitrophenyl sulphate by arylsulphatase A involves O–S bond fission and (ii) there is no evidence for 'sulphate' acceptors, other than water (Spencer, 1958). The latter observation is consistent with a crypto-sequential mechanism where the enzyme, sulphate ester and water form a ternary complex. A consideration of these factors allows us to suggest Scheme 2 as a general model for the mechanism of hydrolysis catalysed by arylsulphatases A and B. Initial binding of the monoanion substrate involves a protonated group (pK 5.7–5.8) and a base with a pK of 4.4–4.5. In the enzyme–substrate complex the pK of the base is unaltered ($pK_1 \simeq pK_1'$), whereas the pK of the acid group is perturbed significantly by substrate binding ($pK_2' > pK_2$). The reaction is shown (Scheme 2) as a



Scheme 2. *Postulated mechanism for the arylsulphatase-catalysed hydrolysis of aryl sulphate esters*

$\overset{+}{A}$–H and $\bar{B}$ are the acidic and basic groups on the enzyme. Ar represents an aryl substituent.

concerted mechanism, resulting in the production of the alcohol and bisulphate anion. In the case of arylsulphatase A we suggest that the enzyme–substrate complex occasionally breaks down, not in a concerted fashion, but by an initial protonation of the bridge oxygen and subsequent breakdown of the cation to form sulphur trioxide and the alcohol product. The sulphur trioxide then reacts with a nucleophilic residue to form an inactive species as described previously (O'Fagain *et al.*, 1982). The model we are proposing (i) suggests reasonable roles for the two ionizing groups identified in the present study, (ii) is consistent with (*a*) the known O–S bond fission, (*b*) the competitive kinetics exhibited by HSO_4^-, (*c*) the apparently crypto-sequential mechanism and (*d*) the substrate-mediated inactivation of sulphatase A which is thought to involve enzyme-bound sulphur trioxide occasionally sulphating a nucleophilic residue on the protein rather than water (Waheed & Van Etten, 1979; O'Fagain *et al.*, 1982).

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