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The slow kinetic transients of arylsulphatase A

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A simple model is described to account for the anomalous time course of arylsulphatase A. In the case of the ox liver and human placental enzymes the enzyme–nitrocatechol sulphate complex can, in addition to forming products, slowly break down to form an inactive species which can in turn slowly regenerate active enzyme. When the inactive form binds sulphate the rate of reactivation is enhanced, 218-fold in the case of the ox enzyme. Rat liver arylsulphatase A is refractory to reactivation by sulphate.

The time course of the reaction catalysed by arylsulphatase A (EC 3.1.6.1) using the chromogenic substrate nitrocatechol sulphate can follow three distinct phases. There is an initial rate (v_0) that decays over a period of minutes to a new steady rate (v_f) and this may then be slowly reactivated by the addition of various anions. Since one of the reaction products, sulphate anion, is capable of 'reactivating' the enzyme all three phases can be observed if the time course is monitored over a period of hours. This behaviour has been described for the ox (Nicholls & Roy, 1971), human (Baum *et al.*, 1958) and rabbit (Waheed & Van Etten, 1980) liver and human kidney (Stinshoff, 1972) enzymes. Roy (1978) has reported similar transients using other sulphate ester substrates. Baum & Dodgson (1958) described a complex model for the human liver enzyme, based on the slow exposure of a second site, which has formed the basis of most subsequent studies. However, little analytical treatment has been presented by these authors although Roy and co-workers have formulated several expressions that attempt to describe the initial period of the reaction (Roy, 1972; Nicholls *et al.*, 1974; Roy, 1978). We were initially interested that the time course of the reaction catalysed by rat liver arylsulphatase A had received little attention, and during preliminary studies we could find no evidence for any anion-mediated reactivation. The time course for rat liver arylsulphatase A is adequately described by Frieden's (1970) equation for slow enzyme transients (C. O'Fagain, U. Bond, B. A. Orsi and T. J. Mantle, unpublished work). We have re-examined the slow transients of ox liver and human placenta arylsulphatase A and present a simple model that accounts for the observed phenomenon.

Materials and methods

Materials

Nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) was obtained from Sigma. DEAE-cellulose and CM-cellulose were purchased from Whatman. Sephadex and Sepharose gels were supplied by Pharmacia. All other reagents used were of the highest purity available. Deionized distilled water was used throughout.

Partial purification of rat and ox liver arylsulphatase A

Fresh liver was homogenized in 10 vol. of 10 mM-Tris/acetate/2 mM-EDTA, pH 7.2, (buffer A) using a Kenwood blender at the maximum setting for 40 s. The homogenate was centrifuged at 43 500 ***g*** for 60 min. The resulting supernatant was dialysed or gel-filtered into 10 mM-Tris/acetate, pH 7.2 (buffer B). The desalted supernatant was then applied to a column (2.4 cm × 25 cm) of DEAE-cellulose equilibrated with buffer B. The column was developed using a 0–0.3 M-KCl gradient (2 × 500 ml) in buffer B. This procedure separates the more acidic arylsulphatase A from arylsulphatase B (Worwood *et al.*, 1973; Allen & Roy, 1968) and results in a 25-fold purification over the crude homogenate.

On some occasions both rat and ox liver arylsulphatase A were obtained by a different procedure. A lysosomal pellet was prepared by the method of Worwood *et al.* (1973) and stored frozen at −20°C. Upon thawing, the lysosomal pellet was diluted 7-fold with ice-cold water and centrifuged at 43 500 ***g*** for 1 h. The supernatant was gel-filtered into buffer B and chromatographed on DEAE-cellulose as outlined above.

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Partial purification of human placental arylsulphatase A

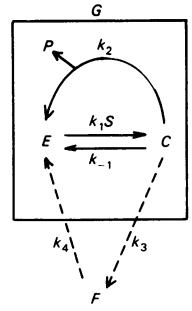
Fresh whole placentas were kindly supplied by the National Maternity Hospital, Holles Street, Dublin 2, Ireland. Following removal of the cord and blood clots, the placenta was minced and homogenized in a Kenwood blender in 7 vol. of ice-cold 10mM-2-mercaptoethanol containing 2mM-EDTA titrated to pH7 with 5M-NaOH. The homogenate was centrifuged at 16300*g* for 10min. A one-fifth volume of 0.5M-sodium phosphate, pH7.2, was added to the resulting supernatant which was made 65% satd. with respect to $(NH_4)_2SO_4$. After stirring for 30min the suspension was centrifuged at 16300*g* for 10min. The pellets were resuspended in a minimum volume of 10mM-Tris/acetate/2mM-EDTA/10mM-mercaptoethanol, pH7.2, (buffer C) and were dialysed against the same buffer (30 vol.) overnight. Non-diffusible material was centrifuged at 43500*g* for 20min. The resulting supernatant was gel-filtered on Sephadex G-25 (column 80cm × 6cm diameter) equilibrated in 10mM-Tris/acetate/10mM-mercaptoethanol, pH7.2, (buffer D) and then applied to a column of DEAE-cellulose (16cm × 7cm diameter) equilibrated with the same buffer. The column was developed with a linear 0–0.3M-KCl gradient. This procedure separates arylsulphatase A from the B enzyme (which does not bind to DEAE-cellulose under these conditions; see Fluharty *et al.*, 1975). Fractions containing arylsulphatase A activity were pooled and dialysed against buffer D.

Assay of arylsulphatase activity

Arylsulphatase A activity was measured by following the production of 4-nitrocatechol spectrophotometrically. Assays were conducted at 37°C (unless stated otherwise) in a final volume of 0.5ml containing 0.33M-sodium acetate/acetic acid buffer, pH5.9, and nitrocatechol sulphate dissolved in the same buffer and titrated to pH5.9 with 5M-acetic acid. The reaction was normally started by the addition of 10–50μl of enzyme solution. When Na_2SO_4 was added during an incubation 10μl was dispensed from a stock solution made up in the assay buffer. The reaction was terminated by the addition of 0.5ml of 1M-NaOH and the nitrocatechol produced was measured by the A_{515} (ε 12600 litre·mol^{-1}·cm^{-1}; Waheed & Van Etten, 1979*b*). Dilutions were performed if A_{515} exceeded 1.2. Time points were determined in triplicate up to 1h and in duplicate thereafter.

Preparation of substrate-modified ox liver arylsulphatase A

Modified ox arylsulphatase A was prepared by incubating the enzyme with 10mM-nitrocatechol sulphate in 0.33M-acetic acid/sodium acetate buffer, pH5.9, at 37°C for 90min. The mixture was applied to a column of Sephadex G-25 (23cm × 2cm) and eluted with buffer B. Fractions corresponding to the void volume were collected and those showing significant A_{280} values were pooled and used immediately to study the kinetics of reactivation.



Scheme 1. For the sake of simplicity the products 4-nitrocatechole and sulphate are referred to collectively as *P*. In addition nitrocatechol release on going from *C* to *F* and sulphate release on going from *F* to *E* have been ignored since flux through this pathway represents a negligible proportion of the flux through the normal catalytic cycle (0.001% in the case of ox liver enzyme). Similar considerations apply to Scheme 2.

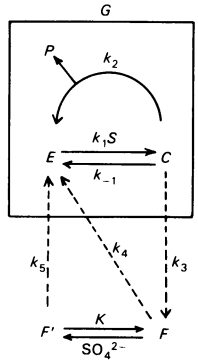
Theory

Slow transient in the absence of anion. The model (shown in Scheme 1) assumes that the formation of the substrate-modified enzyme form (*F*) is derived from the enzyme–nitrocatechol sulphate complex (*C*) and that this process is characterized by the first order rate constant k_3. It is a further feature of the model that the modified form *F* (which is catalytically inactive) can slowly revert to *E* and that this is described by k_4. Since k_3 and k_4 are slow processes we assume they do not affect the steady state within the box and we refer to the sum of these enzyme forms as *G*. The variation of *G* with time (*t*) is then described by:

$$G = \frac{k_4}{k_3+k_4} G_0 + \frac{k_3}{k_3+k_4} G_0 \, e^{-(k_3+k_4)t} \qquad (1)$$

where G_0 is the concentration of *G* at zero time (i.e. total enzyme, E_t). Since $G = C\,(1 + K_m/S)$, eqn. (1) may be written as:

$$C = \frac{k_4}{k_3+k_4} C_0 + \frac{k_3}{k_3+k_4} C_0 \, e^{-(k_3+k_4)t} \qquad (2)$$



Scheme 2

Since $dP/dt = k_2[C]$, eqn. (2) may be recast, after integration, as:

$$P = \frac{k_2k_4}{k_3+k_4}C_0t + \frac{k_2k_3}{(k_2+k_4)^2}C_0\,(1-e^{-(k_3+k_4)t}) \quad (3)$$

Note that this equation is of the same form as the expression described by Frieden (1970). Since the ratio C_0/E_t is $S/(K_m+S)$ eqn. (3) may be written in the substrate concentration dependent form as:

$$P = \frac{k_2E_tS}{K_m+S}\left[\frac{k_4}{k_3+k_4}t + \frac{k_3}{(k_3+k_4)^2}(1-e^{-(k_3+k_4)t})\right] \quad (4)$$

This equation is of the form:

$$P = at + b(1-e^{-ct}) \quad (5)$$

and progress curves were fitted to eqn. (5) by non-linear regression. The apparent first order rate constant c is then k_3+k_4. The ratio of the initial slope $(dP/dt)_0$ and the final steady-state slope $(dP/dt)_f$ is:

$$\left(\frac{k_4}{k_3+k_4}\right)^{-1} = \frac{v_0}{v_f}$$

Knowing c and the ratio of the initial and final slopes the first-order rate constants k_3 and k_4 are easily calculated.

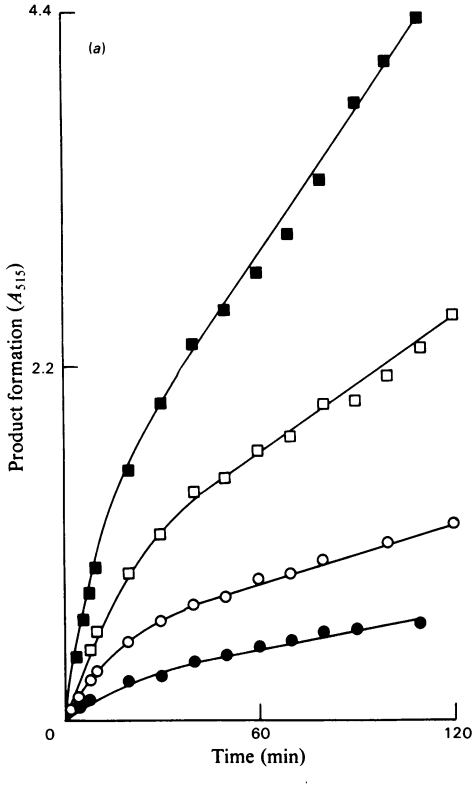
Effect of sulphate on the slow transient. To take account of the reactivating effect of sulphate (in the case of the ox and human placental enzymes) Scheme 1 is extended to Scheme 2. We assume that in the presence of sulphate the modified form F is in equilibrium with its sulphate complex, which we denote F^1, and that this equilibrium is defined by K. We further assume that F^1 can slowly regenerate E in the first-order process characterized by k_5. Product formation with time is then described by:

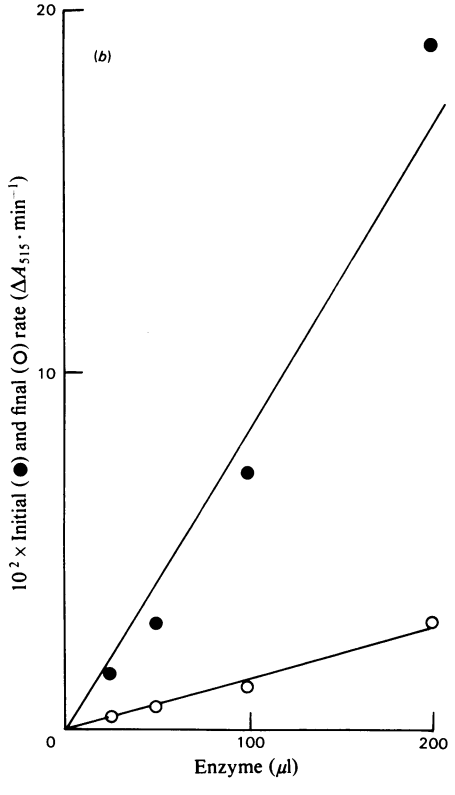
$$P = \frac{k_2E_tS}{K_m+S}\left\{\frac{\left(k_4+k_5\frac{SO_4^{2-}}{K}\right)\cdot t}{k_4+k_5\frac{SO_4^{2-}}{K}+k_3\left(1+\frac{SO_4^{2-}}{K}\right)} + \frac{k_3\left(1+\frac{SO_4^{2-}}{K}\right)^2}{\left[k_4+k_5\frac{SO_4^{2-}}{K}+k_3\left(1+\frac{SO_4^{2-}}{K}\right)\right]^2}\right.$$
$$\left.\times\left[1-\exp-\left(\frac{k_4+k_5\frac{SO_4^{2-}}{K}+k_3\left(1+\frac{SO_4^{2-}}{K}\right)}{1+\frac{SO_4^{2-}}{K}}\cdot t\right)\right]\right\} \quad (6)$$

when the boundary conditions are $t=0$; $G_0 = E_t$ and by:

$$P = \frac{k_2E_tS}{K_m+S}\left\{\frac{\left(k_5\frac{SO_4^{2-}}{K}+k_4\right)\cdot t}{k_3\left(1+\frac{SO_4^{2-}}{K}\right)+k_4+k_5\frac{SO_4^{2-}}{K}} + \frac{\left(k_5\frac{SO_4^{2-}}{K}+k_4\right)\left(1+\frac{SO_4^{2-}}{K}\right)}{\left[k_3\left(1+\frac{SO_4^{2-}}{K}\right)+k_4+k_5\frac{SO_4^{2-}}{K}\right]^2}\right.$$
$$\left.\times\left[\exp-\left(\frac{k_5\frac{SO_4^{2-}}{K}+k_4+k_3\left(1+\frac{SO_4^{2-}}{K}\right)}{\frac{1+SO_4^{2-}}{K}}\cdot t\right)-1\right]\right\} \quad (7)$$

when the boundary conditions are $t=0$; $G_0 = 0$.





Results

Slow transients of rat liver arylsulphatase A

The product–time course for rat liver arylsulphatase A hydrolysis of nitrocatechol sulphate deviates significantly from linearity after a few minutes at 37°C. In this behaviour it resembles arylsulphatase A from other mammalian sources (Nicholls & Roy, 1971), although the ratio v_f/v_0 is considerably higher for the rat liver enzyme. We routinely store the enzyme at pH 7.2 and 4°C. To establish that the slow modification transient involves the enzyme–substrate complex we have started the reaction with enzyme pre-equilibrated at 37°C and pH 5.9 for up to 2 h when we obtain identical product–time curves. We obtain the same result regardless of the order of addition of components including preincubating the substrate for 1 h at 37°C and pH 5.9. The presence of dithiothreitol and the use of alternative buffers are also without effect.

Fig. 1(*a*) shows the effect of enzyme concentration on the product–time curve for rat liver arylsulphatase A. The initial and final slopes show a linear relationship with enzyme concentration as shown in Fig. 1(*b*). The apparent modification constant c is independent of enzyme concentration with a value of $0.0765 \pm 0.0122\,min^{-1}$. The rate constants k_3 and k_4 are calculated to be $0.0495 \pm 0.018\,min^{-1}$ and $0.0146 \pm 0.00338\,min^{-1}$ respectively. When the results in Fig. 1 were analysed graphically using the procedure described by Frieden (1979) a value for c of $0.067 \pm 0.0147\,min^{-1}$ was obtained. The final steady-state rate v_f is linear for 5 h and does not exhibit the 'reactivation' phenomenon apparent with the human placental or ox liver enzymes (see below). The lack of reactivation of the modified form of the rat liver enzyme by sulphate can also be shown by incorporating Na_2SO_4 in the assay mix or by challenging with Na_2SO_4 during the reaction (Fig. 2). At relatively low concentrations of Na_2SO_4 (1 mM; $1/7K_i$ against v_0) there is very slight inhibition of the initial slope (v_0) but no effect on k_3 or k_4 (Fig. 2*a*). At higher concentrations of Na_2SO_4 (20 mM) there is no reactivation during a sulphate-challenge experiment although considerable inhibition of v_0 occurs. The constants k_3 and k_4 are independent of the

Fig. 1. *Effect of enzyme concentration on product–time curves for rat liver arylsulphatase A*

(*a*) Time courses were conducted using: ●, 25 μl; ○, 50 μl; □, 100 μl; and ■, 200 μl of enzyme. (*b*) The data from Fig. 1(*a*) were fitted to eqn. 5 by using the computer program DEMGRID. The initial (v_0) and final (v_f) slopes are plotted against enzyme concentration.

substrate concentration over the range 0.2–48 mM-nitrocatechol sulphate, as predicted by eqn. (4).

Sulphate-independent transients of human placental and ox liver arylsulphatase A

Time-course experiments were conducted with human placental or ox liver enzymes over a limited period (approx. 1–2 h depending on the enzyme concentration) so that the production of sulphate was below 0.1 mM. The resulting progress curves gave excellent fits to eqn. (5). The values for the rate constants k_3 and k_4 are shown in Table 1.

To obtain an independent estimate of k_4 for ox liver arylsulphatase A the enzyme was incubated for 90 min at 37°C with 4 mM-nitrocatechol sulphate and the 'modified form' isolated rapidly by gel filtration. By monitoring the return of enzyme activity over several hours the half-life for recovery was estimated to be 300 min, which is in reasonable agreement with k_4 in Table 1.

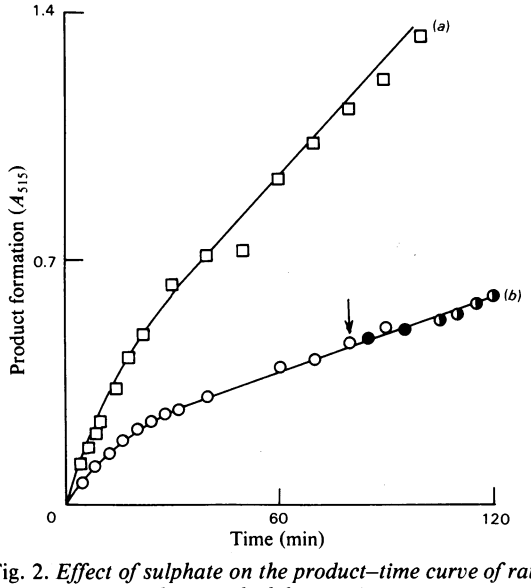


Fig. 2. *Effect of sulphate on the product–time curve of rat liver arylsulphatase A*

(*a*) The solid line is a least squares fit to the control experiment and the data points (□) are from an experiment with 1 mM-Na_2SO_4 included from time zero. The nitrocatechol sulphate concentration was 1.5 mM. (*b*) In a separate challenge experiment 1 mM-Na_2SO_4 was added at the time indicated to the test (●) series of tubes while the controls (○) received buffer. The final concentration of nitrocatechol sulphate was 2 mM.

Sulphate-dependent transients

When extended time courses are studied both ox liver and human placental arylsulphatase A show the 'reactivation' phenomenon (Figs. 3 and 4). Addition of 1 mM-Na_2SO_4 after 1 h incubation stimulates the 'reactivation' process which exhibits a significant lag (Figs. 3 and 4). It is not feasible to analyse the reactivation transient in these experiments by using eqn. (7) because of the experimental error in the relatively high A_{515} values at $t = 0$ (60 min) for the reactivation phase. We tried to overcome this problem by preparing the substrate-modified form of the ox liver enzyme and then initiating the reaction with fresh nitrocatechol sulphate. Although a small proportion of the substrate-modified enzyme reverts to the native form during the gel filtration step, the A_{515} values are much lower, as seen in Fig. 5, and can be used to fit to eqn. 7 in its simplified form:

$$P_t = a \cdot t + b(e^{-ct} - 1) \qquad (8)$$

The apparent half lives for reactivation (c) for the ox enzyme in the presence of 1 mM- and 5 mM-sulphate are 128 min and 65 min respectively. Using the data shown in Fig. 3 a graphical estimate of 0.86 min was obtained for the apparent half-life of reactivation by 1 mM-sulphate when added directly to substrate-modified enzyme. This suggests that some further modification occurs during the isolation of the substrate-modified enzyme.

An alternative approach adopted was to examine the initial transient of ox liver sulphatase A in the

Table 1. *Values of kinetic constants*

Values for the apparent first-order rate constant c, and the first-order rate constants k_3 and k_4 were derived as described in the text. The first order rate constant k_5 and the equilibrium constant K for sulphate binding to F' were obtained by simulation using eqn. 6. All the experiments were conducted at 37°C.

Enzyme source	c (min^{-1})	k_3 (min^{-1})	k_4 (min^{-1})	k_5 (min^{-1})	K (mM)
Rat liver	0.0929	0.0795	0.0134	—	—
Ox liver	0.2370	0.2337	0.0033	0.720	60
Human placenta	0.5220	0.5187	0.00335	—	—

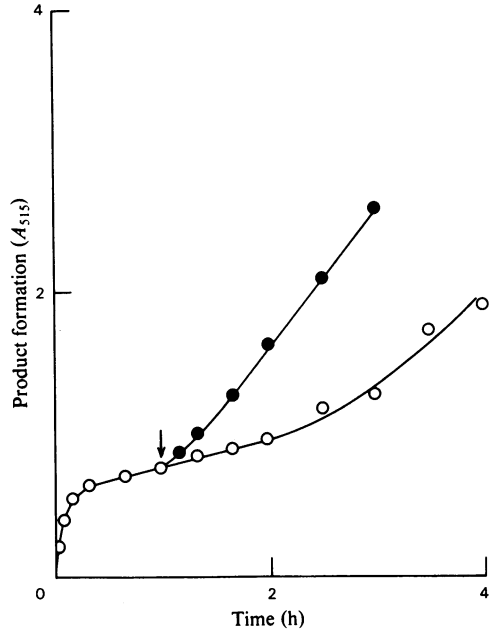


Fig. 3. *Product–time curve for ox liver arylsulphatase A*
Results are shown for a control (○) and 1mM-Na_2SO_4 challenge (●) experiment.

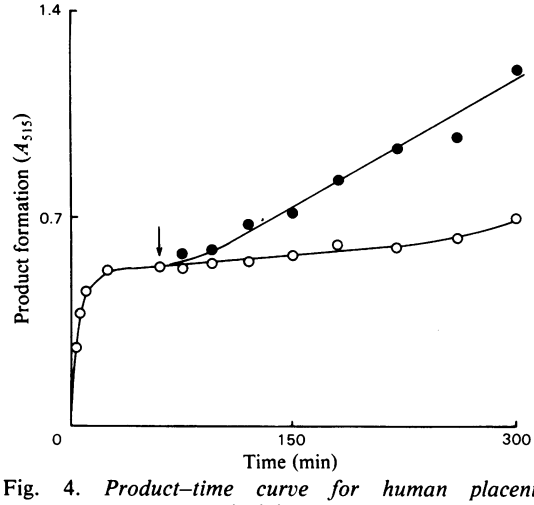


Fig. 4. *Product–time curve for human placenta arylsulphatase A*
Results are shown for a control (○) and 1mM-Na_2SO_4 challenge (●) experiment.

presence of varying amounts of Na_2SO_4. The results of such an experiment are shown in Fig. 6. Also shown are theoretical lines based on eqn. 6.

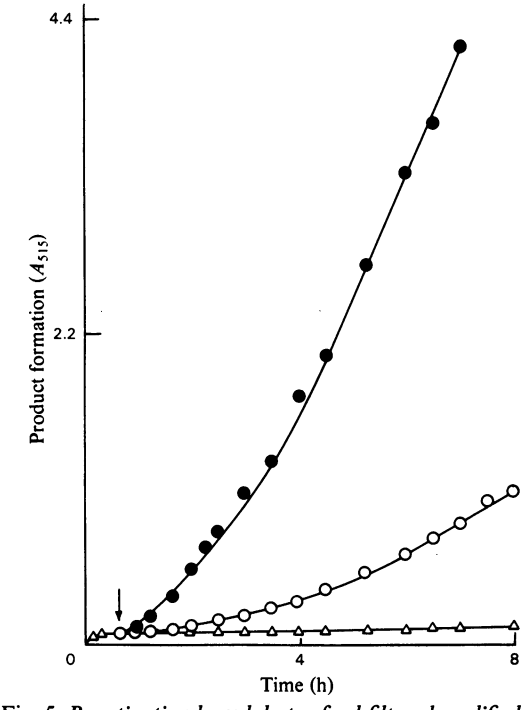


Fig. 5. *Reactivation by sulphate of gel-filtered modified ox liver arylsulphatase A*
Substrate-modified enzyme was prepared as described in the text. At the time indicated the following additions were made; buffer (△), 1mM-Na_2SO_4 (○) or 5mM-Na_2SO_4 (●).

Discussion

Slow substrate-dependent inactivation of enzymes has received little theoretical treatment. Recently Waley (1980) has presented a series of equations describing the substrate-dependent decline in enzyme activity with time. His approach is analogous to that used by Kitz & Wilson (1962) in their treatment of those irreversible inhibitors that form a kinetically significant reversible complex prior to covalent bond formation. Technically these experiments involve rapidly removing the substrate (e.g. by dilution) at various times and measuring initial rates, with fresh substrate (see e.g. Wang & Walsh, 1978). By the nature of the experiment one is measuring the decline of free enzyme and hence the apparent pseudo first order rate constant for inactivation will be a complex function that will vary hyperbolically with the amount of substrate added (Waley, 1980). Our experimental approach has been different since significant product formation occurs during the first

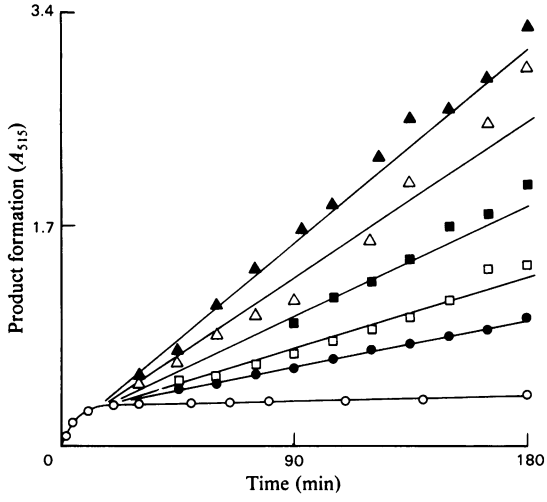


Fig. 6. *Effect of Na_2SO_4 concentration on the product–time curve of ox liver arylsulphatase A*

Time courses were conducted in the presence of zero (○), 1 mM- (●), 2 mM- (□), 4 mM- (■), 8 mM- (△) and 16 mM- (▲) Na_2SO_4. The solid lines are theoretical and based on eqn. 6. Values for k_3 and k_4 were derived as described in the text and the values for k_5 and K were obtained by simulation using eqn. (6) and are shown in Table 1. Data points and theoretical lines below 30 min have been omitted for the sake of clarity.

incubation and cannot be ignored by diluting into fresh substrate (i.e. radiolabelled substrate or an alternative substrate). In addition, since the first substrate-modified form (F) slowly reverts to free enzyme and since this process is further affected by the presence of various anions Waley's (1980) approach would result in an extremely complex rate equation not readily amenable to further analysis. We have, therefore, chosen to examine the product–time course since the first-order rate constants for the formation of the substrate-modified form F and its reversion to form E can be derived directly from this curve when the experiment is conducted in the absence of added anion. In addition we have found that fitting the data to eqn. 5 yields very accurate estimates of the coefficients a, b and c and hence of k_3 and k_4. Since the experiment monitors the decline of free enzyme and enzyme substrate complex, the apparent first order rate constant for inactivation (c) is predicted to be independent of substrate concentration (eqns. 4 and 5) and we have verified this experimentally over the range 0.2–48 mM-nitrocatechol sulphate for the rat enzyme (K_m using true initial rates is 0.7 mM). It is, therefore, surprising that Soper & Manning (1978) observed a hyperbolic dependence of the apparent inactivation constant with substrate concentration. This may have resulted from attempts to analyse the product time curve graphically rather than using a least squares procedure as adopted in the present work.

All of our data from experiments conducted in the absence of added anion (and over a limited period in the case of the ox and human enzymes) fit to eqn. 5 and we have interpreted the data in terms of Scheme 1. According to this model the final steady state rate (v_f) is due to the equilibrium between the enzyme forms in G and the inactive form F. However, it is difficult to eliminate the possibility that F simply has a lower activity. We favour Scheme 1 since we obtain two independent estimates of the rate constant k_4 which are in good agreement.

The nature of the inactivation of the enzyme appears to result from the sulphation of arylsulphatase A. Two groups (Waheed & Van Etten, 1979*b*; Prosser & Roy, 1980) have found stoichiometric amounts of ^{35}S covalently bound to the modified form of the enzyme when nitrocatechol [^{35}S]sulphate is used as substrate. The hydrolysis of nitrocatechol sulphate catalysed by arylsulphatase A is known to take place by O–S bond fission (Spencer, 1958) and Waheed & Van Etten (1980) have suggested that SO_3 is a primary reaction product. Subsequent reaction of SO_3 with nucleophilic groups on the protein rather than with water could then result in the observed modification. The sulphated residue, which has not been identified, is evidently unstable with a half life, at 37°C, of 52, 207 and 211 min for the rat, human and ox enzymes respectively. Direct experimental evidence for the regeneration of active enzyme concomitant with the loss of $^{35}SO_4^{2-}$ from nitrocatechol [^{35}S]sulphate-modified enzyme has been provided by Prosser & Roy (1980).

We confirm the results of many previous workers that the substrate-modified forms of ox liver and human placental arylsulphatase A are reactivated by inorganic sulphate. Our data suggest that, in the case of the ox enzyme, the binding of sulphate to the modified enzyme enhances the regeneration of active enzyme 218-fold. By contrast rat liver arylsulphatase A appears unique in showing no detectable reactivation. The rat liver enzyme undergoes a polymerization similar to that of the ox liver enzyme (Worwood *et al.*, 1973; Jerfy *et al.*, 1976) so that the kinetic difference is unlikely to be explained by different polymeric states. Further work is necessary to determine why the rat liver enzyme is so refractory to reactivation by sulphate.

Our model differs from the earlier scheme proposed by Baum & Dodgson (1958) in that no time-dependent exposure of a second site is postu-

lated. It is a simple scheme that can be analysed as described and has the merit that k_3 and k_4 are obtainable from a single product time curve by using a straightforward least squares procedure.

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