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THE SULPHATASE OF OX LIVER

XIX. ON THE NATURE OF THE POLYMERIC FORMS OF SULPHATASE A PRESENT IN DILUTE SOLUTIONS

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Summary

Weight-average elution volumes of sulphatase A (an arylsulphate sulphohydrolase, EC 3 1 6 1) from Sephadex G-200 have been determined as functions of protein concentration, pH, ionic strength and temperature. The results are used to calculate the apparent association equilibrium constants for tetramer formation and the associated standard-state thermodynamic parameters. While the apparent association constant decreased from 10^{28} to 10^{21} M^{-3} on increasing the pH from 4 5 to 5.6 at ionic strength 0 1, at any particular pH value studied it was relatively insensitive to temperature variation so that ΔH° is close to zero and tetramer formation in solution is associated with a positive entropy change. At pH 5 0, increasing the ionic strength from 0.1 to 2 decreased the association constant by a factor of 100 Methylumbelliferone sulphate has no effect on the association of sulphatase A

The equilibrium results are used to define the degree of association of sulphatase A likely to be encountered in experiments designed to elucidate its kinetic properties. In the liver lysosome, the tetramer is probably the dominant species.

The monomer and tetramer of sulphatase A have similar, or identical, specific activities with nitrocatechol sulphate and 4-methylumbelliferone sulphate as substrates. With nitrocatechol sulphate, sulphatase A shows Michaelis kinetics under conditions where the monomer is the dominant species and non-Michaelis kinetics where the tetramer is dominant There is apparently a negative cooperativity between the monomer units in the tetramer

In 2 mM sodium taurodeoxycholate and 0 035 M $MnCl_2$, but not in 0 1 M NaCl, the tetramer shows Michaelis kinetics This is not due to dissociation of the tetramer The critical micellar concentration of sodium taurodeoxycholate is about 0 8 mM in both 0 1 M NaCl and 0 035 M $MnCl_2$ but the aggregation number is greater in the latter

Introduction

Sulphatase A was first recognised [1] as an arylsulphatase (EC 3 1 6 1) but more recent investigations show that its physiological function is that of a cerebroside sulphatase [2] The latter activity is frequently investigated in vitro at lower pH values and higher enzyme concentrations than is the corresponding arylsulphatase activity [3] This complicates the interpretation of the relationship between the two activities because sulphatase A exists as a pH-dependent polymerising system [4] at protein concentrations above about 0 1 mg/ml the monomer (mol. wt 107 000) is stable above pH 6 5, a tetramer below pH 5 5 and a mixture of polymeric forms between pH 5 5 and 6 5 Studies of the elution of sulphatase A from Sephadex at pH 5 0 suggested that at concentrations of about 1 μg/ml the tetramer became unstable and that lower polymers appeared [4] A detailed interpretation of the results was not possible because the enzyme was detected by a relatively insensitive method which required long incubation times and so was not suited for quantitative assays [1]

Further complications are introduced into many assays of cerebroside sulphatase activity by the presence of Mn^{2+} and taurodeoxycholate in the reaction mixtures [3,5]

The present communication describes a detailed investigation of the polymerisation of sulphatase A over a wide range of protein concentrations This has allowed the estimation of $K^{\star}_{ass}$, the apparent association constant for tetramerisation, under a variety of conditions so that the enzyme species present in any given reaction mixture can be clearly defined Studies have also been made of the kinetics of the arylsulphatase reaction under conditions where the enzyme exists predominantly as a single species

Experimental

Preparation and assay of sulphatase A

Sulphatase A was prepared from ox liver as previously described [6] and the substrate-modified form of the enzyme was obtained as before [7]

Enzyme activity was routinely assayed in a pH-stat at 37°C, pH 5 6, with 3 mM nitrocatechol sulphate as substrate In kinetic studies the reaction mixture contained 0 1 M NaCl and 0 5 mM sodium acetate. The reaction velocity 1 min after starting the reaction, v_1, was usually computed by fitting the data from the pH-stat recording to a rectangular hyperbola [8] In studies of substrate concentration effects this method was invalid because the change in substrate concentration was not negligible [8] and an apparent v_1, $v_1^{\star}$, was computed by fitting the data to the cubic (Eqn 1)

$$u = A + Bt' + Ct'^2 + Dt'^3 \qquad (1)$$

where $t' = t - 1$ and u is the amount of product at time t Obviously

$$\left(\frac{du}{dt}\right)_{t=1} = \left(\frac{du}{dt'}\right)_{t'=0} = B = v_1^{\star}$$

In these circumstances it was appropriate to use $s^{\star}$, the substrate concentration

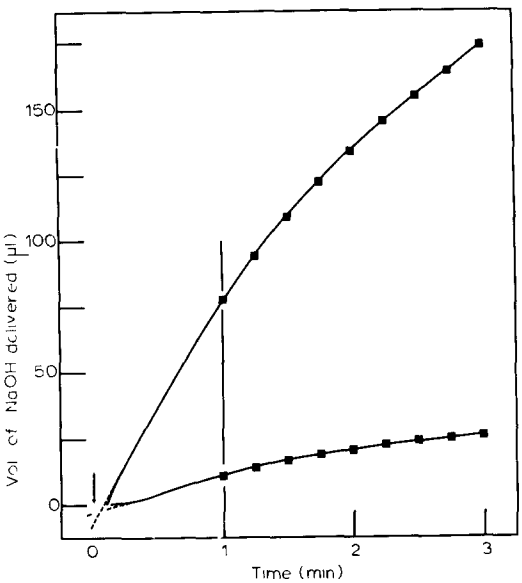

Fig 1 Drawings of pH-stat recordings of the hydrolysis of nitrocatechol sulphate by sulphatase A at pH 4 5 in 0 1 M NaCl Concentration of NaOH in burette 0 0139 M Readings from the curves, taken at 0 25-min intervals from 1 to 3 min after adding the enzyme, were used to compute the coefficients of Eqn 1 the points show values then computed using these coefficients The dotted lines show the extrapolation to zero time required to calculate the amount of NaOH used in the first minute and hence s^* For the upper and lower curves the values of s_0 were 0 628 and 0 0418 mM, respectively the corresponding values of s^* were 0 510 and 0 0241 mM

1 min after the start of the reaction, instead of the initial substrate concentration, s_0, in subsequent computations. Values of s^* were calculated from s_0 and the amount of NaOH delivered by the pH-stat during the first minute of the reaction Typical experimental results are shown in Fig. 1 (the lower curve corresponds to the lowest substrate concentrations shown in Fig 6).

With 4-methylumbelliferone sulphate as substrate, the reaction mixture had a volume of 5 ml and contained buffer of ionic strength 0.1 The temperature was 37° C At suitable intervals, up to a maximum of 15 min, 0 25-ml samples were withdrawn and pipetted into 3 ml of carbonate buffer, pH 10.3 (0 1 M $NaHCO_3$, 0 05 M NaOH). The amount of 4-methylumbelliferone liberated was determined spectrophotometrically ($\epsilon_{362\ nm}$ = 18 400) or fluorimetrically in an Aminco-Bowman spectrofluorimeter. The initial velocity, v_0, was computed in the same way as v_1 with nitrocatechol sulphate

For assays of sulphatase A in column eluates, the reaction mixture had a volume of 0 5 ml and contained 0 01 M 4-methylumbelliferone sulphate at pH 5 6 in 0 5 M sodium acetate/acetic acid buffer After incubation for 15 min at 37° C the reaction was stopped by the addition of 5 ml of carbonate buffer (containing 5 mM EDTA if Mn^{2+} were present see below) and the amount of 4-methylumbelliferone determined as above Such assays gave values of v about 20% lower than v_0 determined by the method used in kinetic studies.

Frontal analysis on Sephadex

The technique was that of Winzor and Scheraga [9] A column (27 cm ×

1 5 cm) of Sephadex G-200 was jacketed at the required temperature and eluted, by upward flow, at a rate of 0 2 ml/min Fractions of approx 0 5 ml were collected and the exact volume of each was determined by weighing, assuming the density of the eluate to be constant at 1 g/ml

A stock solution of sulphatase A (5 mg/ml in 0 1 M Tris HCl, pH 7 4) was diluted into the appropriate buffer and, after keeping at the required temperature for 1 h, a 20-ml sample was applied to the column Elution was continued with the same buffer until the enzyme activity, with 4-methylumbelliferone sulphate as substrate, in the eluate had fallen to zero

The protein concentration in the plateau region was calculated from its enzymic activity and the specific activity of sulphatase A determined under the appropriate conditions The elution volume of the leading boundary, a weight-average elution volume [10], was taken as the volume at which the protein concentration in the boundary was half of that in the plateau region In a few cases the elution volume of the trailing boundary, also a weight-average function [10], was measured but the particular type of column used made it inconvenient to apply precisely 20 ml of enzyme solution in each experiment

Computations

The weight-average elution volume, $\overline{V}$, of a monomer-single higher polymer system is given by

$$\overline{V} = \frac{V_m c_m + V_p(c - c_m)}{c}$$

where V_m and V_p are the elution volumes of the monomer and polymer, respectively, and c and c_m the total protein and monomer concentration, respectively Further, K_{ass}, the association constant of the system, is given by

$$K_{ass} = \frac{c - c_m}{(c_m)^n}$$

where n is the degree of polymerisation Combination of these two equations leads to the relationship [10]

$$\log\left[c\left(1 - \frac{\overline{V} - V_p}{V_m - V_p}\right)\right] = n \log\left(\frac{c(\overline{V} - V_p)}{V_m - V_p}\right) + \log K_{ass} \qquad (2)$$

As V_m and V_p are known, and c and $\overline{V}$ experimentally determined, a plot of Eqn 2 gives a straight line of slope n and intercept on the ordinate of $\log K_{ass}$

When n is known, K_{ass} can be calculated from single experiments using the relationship

$$K_{ass} = c\left(1 - \frac{\overline{V} - V_p}{V_m - V_p}\right) \Big/ \left(\frac{c(\overline{V} - V_p)}{V_m - V_p}\right)^n \qquad (3)$$

In the general case where several polymeric species are present plots of Eqn 2 will not give a straight line A useful paramer is then $c_{0\ 5}$, the protein concentration at which $\overline{V} = (V_m + V_p)/2$ the lower is $c_{0\ 5}$, the greater is the apparent K_{ass} for the system

V_m can be determined directly at pH 7.5 where the monomer is stable [4] or at other pH values by extrapolating plots of $\overline{V}$ against c to $c = 0$. V_p can be determined at high concentrations of protein at pH 4 5 or 5 0, or better by extrapolating plots of $\overline{V}$ against $1/c$ to $1/c = 0$

Light scattering

These measurements were made at room temperature (about 25°C) in a Sofica PGD42000 photogoniodiffusometer (Société Fica, Le Mésnil-Saint-Denis, France) The 90° scattering of a solution of the tetramer of sulphatase A (about 5 mg/ml) at pH 5.0, I = 0 1, was measured and then the pH of the solution rapidly changed to 7 5 by the addition of 0.05 volume of a 2 M buffer, pH 7 5, to form the monomer The 90° scattering was measured as a function of time following the pH change. The change from monomer at pH 7 5 to tetramer at pH 5 0 was similarly studied

Buffers

The following sodium acetate buffers, I = 0 1, were used

pH	NaCl (M)	Sodium acetate (M)	Acetic acid (M)
4.5	0.03	0.07	0 09
5 0	0 03	0 07	0 03
5 6	0 03	0 07	0 075

To give buffers of higher I, the concentration of NaCl was increased appropriately and the pH adjusted as necessary. The manganese acetate buffer contained 0.035 M manganese acetate and 0 09 M acetic acid to give a pH of 4 5 and I approx. 0.1. The Tris buffer, pH 7.5, I = 0 1, had the following composition NaCl, 0 06 M, Tris, 0.05 M and HCl, 0 04 M.

Results

Light scattering

When the pH of a solution of sulphatase A was changed from 5 0 to 7 5 to convert the tetramer to the monomer the decrease in the 90° scattering was complete within about 20 s and was too rapid to allow detailed study The conversion of monomer to tetramer was similarly rapid

Chromatography

A typical elution profile of sulphatase A from a column of Sephadex G-200 is shown in Fig 2 The fact that under all conditions only single elution boundaries were observed showed that any interconversion of polymeric forms is rapid compared to the rate of their separation on the column [10] The elution volumes of the leading boundaries were determined at several protein concentrations under different conditions of pH, temperature and ionic strength At pH 7 5 the elution volumes were independent of protein concentration over the range 0 05—50 μg/ml but at pH 4.5, 5 0 and 5 6 they were highly concentration dependent, although relatively insensitive to temperature The results at 37°C are shown in Fig 3, and the values of $c_{0\ 5}$ under various

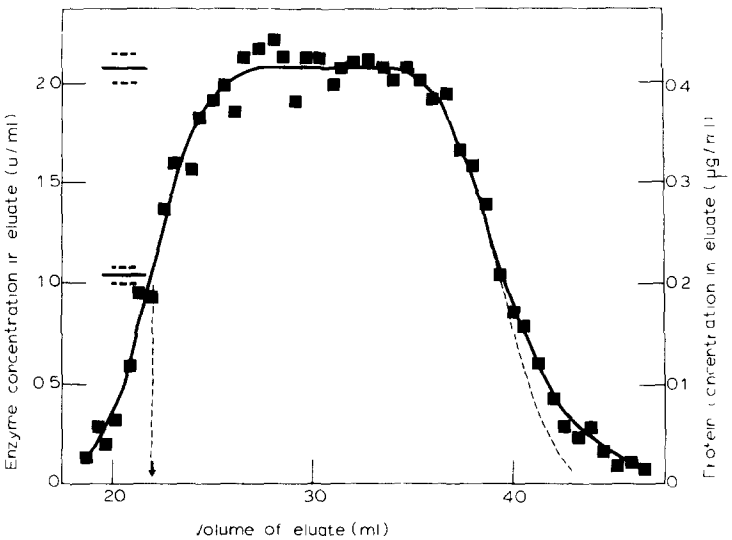

Fig 2 Elution profile of sulphatase A from Sephadex G-200 in sodium acetate buffer, pH 5 0, I = 0 1, at 37°C Applied protein concentration, 0 5 μg/ml The dotted line on the trailing side shows the position of a boundary symmetrical with that on the leading side The upper horizontal line shows the mean plateau level (±1 S D), the lower line shows the half-height and the corresponding elution volume

conditions are shown in Table I The latter also shows that $c_{0\ 5}$ increases, and therefore K_{ass} decreases, with increasing I

In 0 035 M manganese acetate buffer, pH 4 5, the elution pattern of sulphatase A was similar to that in sodium acetate, pH 4 5, I = 0 1 In 0 035 M manganese acetate containing 2 mM sodium taurodeoxycholate the leading boundary was greatly distorted but the trailing boundary was normal, apart

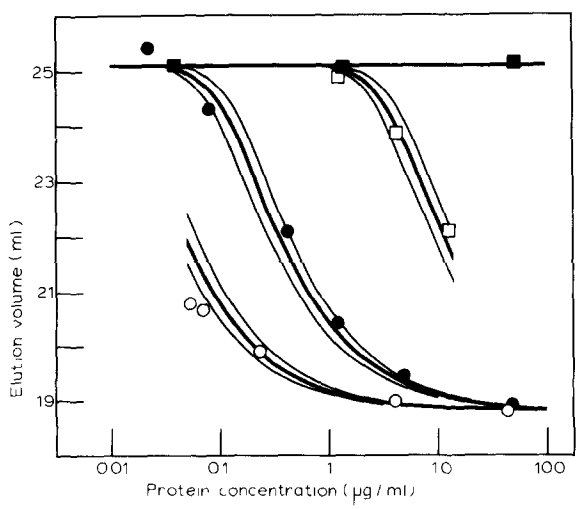

Fig 3 Relationship between elution volume and plateau concentration of sulphatase A at pH 7 5 (■), pH 5 6 (□), pH 5 0 (●) and pH 4 5 (○), I = 0 1, and 37°C The thick lines at the latter three pH values were computed using the values of $K^{\star}_{ass}$ in Table II, and the thin lines using values 0 5 and 2 times those in the table

TABLE I

VALUES OF $c_{0.5}$ FOR THE ELUTION OF SULPHATASE A FROM SEPHADEX G-200 UNDER DIFFERENT CONDITIONS

Under any specified condition, $c_{0.5}$ is the concentration (obtained by interpolation) at which the weight-average elution volume of sulphatase A is the mean of the elution volumes of the monomer and tetramer of the enzyme

T(°C)	I	$c_{0.5}$ (mg/l)		
		pH 4.5	pH 5.0	pH 5.6
5	0.1	—	1.7	—
20	0.1	0.059	0.32	10
20	0.5	—	0.93	—
20	2.0	—	2.1	—
37	0.1	0.042	0.35	13

from some tailing. The elution volumes of the trailing boundary were the same in sodium acetate, manganese acetate and manganese acetate plus taurodeoxycholate, all at pH 4.5, and at protein concentrations of 1.5 and 0.5 μg/ml. Sodium taurodeoxycholate has therefore no effect on the dissociation of the tetramer of sulphatase A at pH 4.5.

At pH 5.0 in the presence of 5 mM 4-methylumbelliferone sulphate the elution volume of sulphatase A (at 0.2 and 1 μg/ml) were not significantly different from those in the absence of substrate. Substrate-modified sulphatase A [7] also had an elution volume indistinguishable from that of the native enzyme. In both these sets of experiments the chromatography was carried out at 5°C and the enzyme was detected by incubating for 1 h with 5 mM nitrocatechol sulphate at pH 5.0 in the presence of 0.25 mM $Na_4P_2O_7$, conditions which minimise difficulties caused by the presence of substrate-modified enzyme [7,11].

Nature of the polymerising system

A detailed study was made only at pH 5.0, I = 0.1, and 20°C. A plot of the data according to Eqn. 2 is shown in Fig. 4A. The points, except for the lowest, could reasonably be fitted by a straight line of slope 3.0—3.5, apparently indicating that sulphatase A existed at a monomer-trimer system under these conditions. This is incompatible with previous findings [4], based on the equilibrium ultracentrifugation of sulphatase A, that at pH 5.0, I = 0.1, the tetrameric form of the enzyme is stable to concentrations of 50 μg/ml or less so that V_p, the elution volume of the polymeric form of sulphatase A is that of the tetramer. The points in Fig. 4A must therefore be fitted with a curve, showing that the system is not that of a monomer and a single higher polymer but of a monomer and several polymeric species, up to a tetramer. This situation has previously been found in studies of the sedimentation of sulphatase A at pH 5.5—6.5 [4].

Nevertheless, if the system is assumed to be that of a monomer-tetramer, the apparent values of K_{ass}, $K^{\star}_{ass}$, calculated by Eqn. 3 are reasonably concentration independent (0.55 10^{11}—2.3 10^{11} l^3 g^{-3} over the concentration range 0.18—4.8 μg/ml), with no obvious trend, and the mean value, 1.4 10^{11}

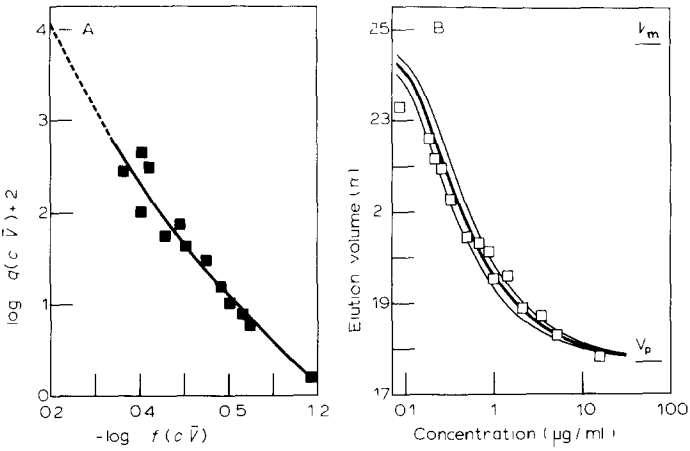

Fig 4 The elution of sulphatase A from Sephadex G-200 at pH 5 0, I = 0 1, and 20°C (A) The results plotted according to Eqn 2 in the text where

$$f(c,\bar{V}) = \frac{c(\bar{V} - V_p)}{V_m - V_p} \quad \text{and} \quad g(c,\bar{V}) = c\left(1 - \frac{\bar{V} - V_p}{V_m - V_p}\right)$$

(B) Elution volumes as a function of protein concentration The lines were computed as described in Fig 3

l^3 g^{-3}, gives a relation between elution volume and protein concentration which agrees well with the experimental values (Fig 4B) It may therefore be concluded that at pH 5.0, I = 0 1, sulphatase A exists as an equilibrium mixture of several polymeric species ranging from monomer to tetramer but that it can adequately be described by a single apparent association constant for tetramerisation

Values of $K^{\star}_{ass}$ were calculated for all series of experiments and are summarised in Table II The appropriate values were used to compute the elution curves shown in Fig 3 again the agreement with the experimental results is satisfactory

TABLE II

APPARENT TETRAMERISATION CONSTANT, $K^{\star}_{ass}$, FOR SULPHATASE A

The values were calculated from Eqn 3 in the text, assuming only monomer and tetramer to be present they are therefore apparent Both weight and molar concentration (italic) constants are given The numbers in parentheses are the numbers of determinations

T(°C)	I	$K^{\star}_{ass}$(l^3 g^{-3} and l^3 mol^{-3})											
		pH 4 5				pH 5 0				pH 5 6			
5	0 1	—		—		3 1	10^9	*9 5*	*10^{23}* (5)	—			
20	0 1	4 1	10^{13}	*1 3*	*10^{28}* (3)	1 4	10^{11}	*4 4*	*10^{25}* (12)	7 4	10^6	*2 3*	*10^{21}* (3)
20		—		—		0 9	10^{11}	*2 8*	*10^{25}* (4)	—		—	
20	0 5	—		—		1 1	10^{10}	*3 3*	*10^{24}* (3)	—		—	
20	2 0	—		—		6 3	10^8	*1 9*	*10^{23}* (3)	—		—	
37	0 1	6 9	10^{13}	*2 1*	*10^{28}* (3)	1 7	10^{11}	*5 1*	*10^{25}* (4)	4 8	10^6	*1 5*	*10^{21}* (2)
37	0 5	—		—		5 8	10^9	*1 8*	*10^{24}* (1)	—		—	

Stability of sulphatase A

During chromatography at pH 7 5 the plateau concentration was identical with that of the applied solution· the mean recovery in eight experiments was 98%. At lower pH values the recovery was considerably less, particularly at low protein concentrations at pH 5 0 and 37°C the recovery was 100% at an applied concentration of 50 μg/ml but only 45% at one of 0 05 μg/ml The loss in activity was due almost entirely to the instability of dilute solutions of sulphatase A at low pH values

At pH 5 0, I = 0 1, the activity of dilute solutions of sulphatase A (<5 μg/ml) slowly decreased The initial velocity of the inactivation, measured over the first hour after dilution, was consistent with first-order inactivation of sulphatase A with a velocity constant of about 10^{-5} s^{-1} at 20°C The rate of inactivation decreased rapidly with time solutions of concentration between 5 and 0.05 μg/ml kept for 48 h at pH 5 and 20°C lost only about 50% of their activity

Some degree of protection was afforded by the presence of bovine serum albumin (1 mg/ml) in the solutions of sulphatase A

Arylsulphatase activity of the monomer and tetramer of sulphatase A

The reaction velocity at pH 5 0, either v_0 with 4-methylumbelliferone sulphate or v_1 with nitrocatechol sulphate, was proportional to the enzyme concentration, over a very wide range, as shown in Fig 5 In these experiments the appropriately diluted enzyme was kept at 37°C for 1 h before starting the reaction by adding either solid 4-methylumbelliferone sulphate or no more than 0 1 volume of nitrocatechol sulphate solution. Similar linear relationships between protein concentration and reaction velocity were found at pH 4.5 and 5 6 In other experiments the reaction was started by adding a small volume of

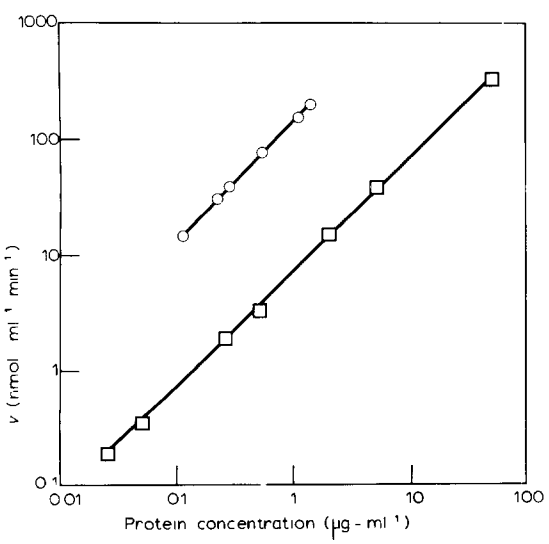

Fig 5 Relationship between arylsulphatase activity and concentration of sulphatase A at pH 5 0, I = 0 1, and 37°C ○, v_1 with nitrocatechol sulphate, □, v_0 with 4-methylumbelliferone sulphate

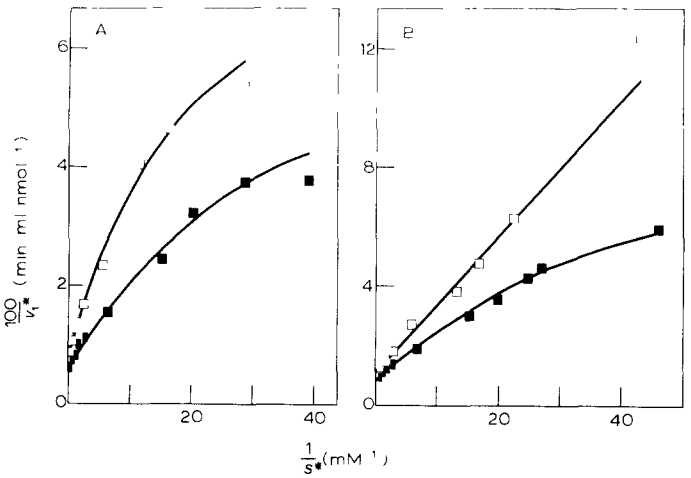

Fig 6 Reciprocal plots for the hydrolysis of nitrocatechol sulphate by sulphatase A (1 4 μg/ml) at pH 4 5 and 37°C (A) In 0 1 M NaCl with (□) and without (■) 2 mM sodium taurodeoxycholate (B) In 0 035 M $MnCl_2$ with (□) and without (■) 2 mM sodium taurodeoxycholate

concentrated enzyme solution at pH 7 5 (i e monomer) to the substrate the results were the same as those in Fig 5

At pH 5 6, I = 0 1, sulphatase A at a concentration of 1 μg/ml (predominantly monomer Fig 3) showed Michaelis kinetics Plots of $1/v_1^*$ against $1/s^*$ were linear and gave a K_m of 0 611 ± 0 026 mM nitrocatechol sulphate by the method of Wilkinson [12] and of 0.609 mM by the method of Cornish-Bowden and Eisenthal [13] The latter method should give a better estimate of K_m where, as in the present situation, there are uncertainties in the substrate concentration At pH 4.5 and a protein concentration of 1 4 μg/ml (predominantly tetramer Fig 3) the reciprocal plots were concave downwards, indicating non-Michaelis kinetics, in either 0 1 M NaCl or 0 035 M $MnCl_2$ (Fig 6)

At pH 5.6 the value of n in the Hill plot and of R_s [14] where 0 93 and 110, respectively, compared with the theoretical values of 1 0 and 81 for an enzyme showing Michaelis kinetics At pH 4 5 the corresponding values were 0 68 and 210, showing that the non-linearity of the reciprocal plots was due to negative cooperativity effects

Effect of taurodeoxycholate on arylsulphatase activity

It was previously reported [3] that the hydrolysis of nitrocatechol sulphate by sulphatase A at pH 4 5 in 0 035 M $MnCl_2$ and 2 mM taurodeoxycholate showed Michaelis kinetics, as did the hydrolysis of cerebroside sulphate This has been confirmed (Fig 6) although there is no evidence to suggest dissociation of the tetramer in this system On the other hand, in 0 1 M NaCl, pH 4 5, the addition of 2 mM taurodeoxycholate did not alter the non-linear reciprocal plot (Fig 6), nor did the addition of 0 4 mM taurodeoxycholate to 0 035 M $MnCl_2$, pH 4 5

The effects of taurodeoxycholate on arylsulphatase activity are complex and apparently related to the physical state of the bile salt because irregularities occur in the region of the critical micellar concentration which is, contrary to

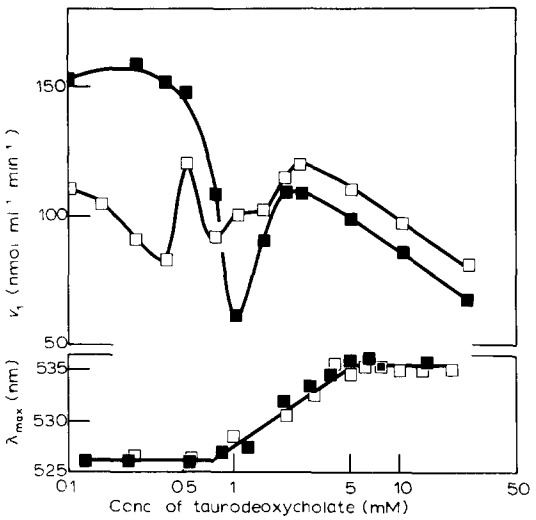

Fig 7 Effect of sodium taurodeoxycholate on the activity of sulphatase A (1 μg/ml) in 0 1 M NaCl (□) and in 0 035 M $MnCl_2$ (■) at pH 4 5 and 37°C The activities in the absence of taurodeoxycholate were 133 and 161 nmol ml^{-1} min^{-1}, respectively The lower curve shows λ_{max} for Rhodamine 6G as a function of taurodeoxycholate concentration in the same solvents

previous suggestions [3], about 0.8 mM taurodeoxycholate in both 0.1 M NaCl and 0 035 M $MnCl_2$ (Fig. 7). The critical micellar concentration was determined by the method of Carey and Small [15] which utilises the different absorption maxima of Rhodamine 6G in water and in detergent micelles.

Micelles of taurodeoxycholate have a higher sedimentation coefficient in 0 035 M $MnCl_2$ ($s^0_{20,w}$ = 1.9 S, $s_{20,w}$ = 1.85 [1 − 0.04c]) than in 0.1 M NaCl ($s^0_{20,w}$ = 1 1 S, $s_{20,w}$ = 1 10 [1 − 0.04c]) where c is the concentration of taurodeoxycholate in g/100 ml. Sedimentation coefficients were measured in conventional double-sector cells at a speed of 68 000 rev /min at 20°C in an An-H rotor in a Beckman Model E ultracentrifuge. The apparent specific volume of the micelles, determined by density measurements (DMA 02D Density Meter, Anton Paar, K G Graz, Austria) of 4 5, 3 0 and 1 0% solutions of the bile salt were 0 737, 0 739 and 0 734 in 0 1 M NaCl and 0 734, 0 737 and 0 734 in 0 035 M $MnCl_2$. These were clearly identical and the mean of 0 735, which agrees well with values previously reported [16] for solution in NaCl, was used for both solvents

As micelles of taurodeoxycholate in 0 1 M NaCl are essentially spherical [16], the greater $s^0_{20,w}$ in $MnCl_2$ must indicate a greater micellar weight (i e aggregation number) in that solvent

Discussion

Light scattering studies confirm the previous claim [4], based on the sedimentation of sulphatase A, that the interconversion of the polymeric forms of the enzyme is rapid At a concentration of about 5 mg/ml it is complete within 30 s. It should nevertheless be noted that $t_{½}$ for a polymerisation is highly dependent on concentration, being proportional to $c^{-(n-1)}$ where c is

the concentration and n the number of monomer units per polymer Therefore, although tetramerisation is rapid at the concentrations accessible by light scattering it could be relatively slow at the concentration of 0 5 μg/ml used in kinetic studies No evidence for this has been found

Frontal analysis on Sephadex G-200 confirmed the rapid interconversion of the polymeric forms of sulphatase A because at all concentrations only single leading and trailing boundaries were observed (for example, Fig. 2) At pH 4.5, 5 0 and 5 6 the elution volumes of sulphatase A were concentration dependent Detailed studies at pH 5 0 (Fig 4) showed that the system contained several polymers but experimental difficulties made it impossible to obtain data of an accuracy sufficient to allow computation of individual association constants The system was therefore treated as one of monomer and tetramer to give values of a single apparent association constant, $K^{\star}_{ass}$ This showed no obvious variation with concentration so that intermediate polymers may be present in only vanishingly small amounts as is apparently the case in some other polymerising systems [17,18] The values of $K^{\star}_{ass}$ have been summarised in Table II and a consideration of the data in Figs 3 and 4 suggest that these will be correct within a factor of about 2 in either direction The values are large, 10^{21}—10^{28} M^{-3}, and whatever their absolute significance it is clear that the tetramer of sulphatase A dissociates only at very low protein concentrations at pH values less than 5 5 The only comparable system appears to be the monomer-dimer of alkaline phosphatase [19]

The corresponding values of ΔG^0 for the tetramerisation range from —120 to —140 kJ mol^{-1} and, at least in the range 20—37°C, ΔH^0 must be close to zero because there is no significant difference in $K^{\star}_{ass}$ in this temperature range It therefore follows that in this temperature range ΔS^0 for tetramerisation must be approximately +60 J $degree^{-1}$ mol^{-1} As polymerisation would, in the absence of any other range, give a decrease in entropy the results suggest

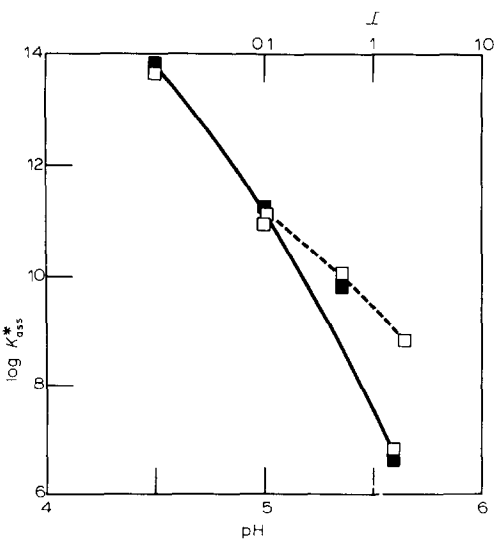

Fig 8 $K^{\star}_{ass}$ (l^3 g^{-3}) for the tetramerisation of sulphatase A as a function of pH and ionic strength at 20°C (□) and 37°C (■) The solid line shows the variation with pH at I = 0 1, and the dotted line shows the variation with I at pH 5 0

that tetramerisation in solution is accompanied by changes in solvent structure This is consistent with the hypothesis that hydrophobic interactions are involved in the formation of the tetramer of sulphatase A [20] Increases in entropy have been noted in other protein-protein interactions for example, in the interaction of tobacco mosaic virus A protein [21] and in the interaction of soybean proteinase inhibitor with chymotrypsin [22].

At pH 5.0, I = 0 1, $K^{\star}_{ass}$ at 5°C is significantly less than that at 20°C so that over this temperature range ΔH^0 is apparently positive At first sight this is inconsistent with hydrophobic interactions being involved in tetramerisation but more detailed studies would be required to allow any interpretation

As shown in Fig 8 the values of $K^{\star}_{ass}$ at different values of pH and of I fall on smooth curves so that interpolations can be made with some confidence Further, $K^{\star}_{ass}$ is not significantly altered by the presence of 0 035 M $MnCl_2$ and 2 mM sodium taurodeoxycholate, conditions commonly used in studies of sulphatase A with lipid substrates [3,5,23,24] so that the values in Table II and Fig 8 allow the prediction of the species of sulphatase A likely to occur in any reaction mixture at pH 4 5—5 6, I = 0 1—0 5 and at 20—37°C In most studies of sulphatase A as an arylsulphatase [3] the enzyme will be present as a monomer (pH 5 6, 0 5—1 μg/ml) although in studies of pH effects (see for example ref 25) it could exist as a tetramer at low pH values, a monomer at high pH values and as an equilibrium mixture of polymers at intermediate values In studies of the cerebroside sulphatase activity of sulphatase A [3] the enzyme will be present as a tetramer (pH 4 5, 10 μg/ml) The form of the enzyme in vivo can also be predicted if it is assumed that the lysosomal volume of ox liver is the same as that of rat liver, 4 μl/g [26] The amount of sulphatase A which can be isolated from ox liver is 1 μg/g [6] therefore the concentration in the lysosomes must be at least 0 25 mg/ml If the intralysosomal pH is about 4—5 [27] then sulphatase A must exist in vivo as the tetramer Even if the pH is in the region of 6 [28,29], the monomer must be a relatively unimportant species

Fig 5 shows that the monomer and tetramer of sulphatase A have the same specific activities because reference to Fig 3 shows essentially complete conversion of these species over the concentration range studied and, as stated, the presence of 5 mM 4-methylumbelliferone sulphate has no obvious effect on the association of sulphatase A There is therefore nothing to suggest that substrate can influence the monomer-tetramer interconversion but proof of this contention is difficult to obtain because 4-methylumbelliferone sulphate is a rather 'poor' substrate (K_m about 12 mM) and practical difficulties preclude the use of 'good' substrates such as nitrocatechol sulphate (K_m about 0 5 mM)

Reciprocal plots for the hydrolysis of nitrocatechol sulphate by sulphatase A are linear at pH 5 6 and non-linear at pH 4 5 at protein concentrations such that the monomer and tetramer, respectively, would be the dominant species A similar phenomenon has recently been noted in the hydrolysis of ascorbic acid 2-sulphate by sulphatase A [30] The effects of sodium taurodeoxycholate are complex and cannot be explained until more detailed information is available on the nature of solutions of the bile salts but it should be recalled that there are also irregular concentration effects in the cholic acid-catalysed hydrolysis of certain nitrophenyl esters [31]

The inactivation of dilute solutions of sulphatase A has not been studied in detail but it is slow and of no significance in normal enzyme assays The initial rate of inactivation has a velocity constant of about 10^{-5} s^{-1} this should be compared with values of 30 s^{-1} for the breakdown of the enzyme-nitrocatechol sulphate complex and of 3 10^{-3} s^{-1} for the transformation of this complex into substrate-modified enzyme [32] The sulphatase A from human urine is apparently less stable than the ox enzyme at a concentration of 100 μg/ml at pH 7 5 it has been described [33] as 'quite unstable' although no quantitative data was given It was also suggested [33] that the inactivation of human sulphatase A was caused by dissociation of the monomer into half-units Unfortunately the inactivation of ox sulphatase A occurs only at concentrations too low to be studied other than by measurements of enzyme activity so that the formation of inactive subunits could not be detected This is particularly unfortunate in view of the previous suggestions [34,35] that the monomer of sulphatase A may be made up of half-units

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