Biochimica et Biophysica Acta, 293 (1973) 178–190

BBA 66801

THE SULPHATASE OF OX LIVER

XVI. A COMPARISON OF THE ARYLSULPHATASE AND CEREBROSIDE SULPHATASE ACTIVITIES OF SULPHATASE A

AGNES JERFY AND A. B. ROY

Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra (Australia)

(Received July 24th, 1972)

SUMMARY

The initial velocities for the hydrolyses of cerebroside sulphate and nitrocatechol sulphate by the sulphatase A of ox liver have been studied under similar conditions in the pH-stat.

Cerebroside sulphatase activity requires the presence of sodium taurodeoxycholate and $MnCl_2$ (or certain other salts) in the reaction mixture. The $MnCl_2$ lowers the critical micellar concentration of the inhibitory ionic form of the bile salt and allows the formation of mixed micelles of taurodeoxycholate and cerebroside sulphate. The K_m varies with taurodeoxycholate concentration and is 0.07 mM cerebroside sulphate at 2 mM bile salt. The reaction is noncompetitively inhibited by K_2SO_4 with a K_i of 0.47 mM. It is 50% inhibited by 25 mM hydroxylamine·HCl.

The kinetics of the reaction are consistent with a uni-ter mechanism in which the substrate is a mixed micelle of taurodeoxycholate and cerebroside sulphate, and the products are released in the order cerebroside, sulphate and taurodeoxycholate.

The arylsulphatase activity is inhibited by ionic taurodeoxycholate. Salts lessen this inhibition by lowering the critical micellar concentration but they also activate the enzyme. At pH 4.5 in the presence of taurodeoxycholate and $MnCl_2$ the properties of the arylsulphatase are not significantly different from those at the optimum pH of 5.6. The arylsulphatase is competitively inhibited by K_2SO_4 with a K_i of 0.15 mM.

Explanations of the apparent differences between the arylsulphatase and cerebroside sulphatase activities of sulphatase A are given and it is concluded that both are due to a single site on the protein.

It has been shown that certain cations form complexes with 4-nitrocatechol: this reaction is accompanied by the liberation of H^+ and by an increased absorption at 430 nm.

INTRODUCTION

Mehl and Jatzekewitz[1] were the first to show the close association of cerebroside sulphatase and sulphatase A activities, a finding confirmed by their subsequent studies[2] and by the work of Austin *et al.*[3] who showed that metachromatic leucodystrophy, a congenital condition in which the catabolism of cerebroside sulphate is disturbed, is characterized by a deficiency of sulphatase A.

Most of the work on the cerebroside sulphatase activity of sulphatase A has been carried out with inadequately characterized preparations of the enzyme, often simply with crude tissue extracts, and the two activities have not been examined under comparable conditions. Most assays of cerebroside sulphatase have been radiochemical methods requiring quite prolonged times of incubation, up to 2 h or more, so that initial velocities cannot be determined. Such methods are not suitable for kinetic studies because of the complex time-dependent kinetics of the arylsulphatase activity of sulphatase A (ref. 4).

The very different chemistry of the two types of substrate made it difficult to accept that sulphatase A and cerebroside sulphatase activities were due to a single enzyme and these doubts were reinforced by the apparently rather different properties of the two activities. The more important of these differences are summarized in Table I: the data for cerebroside sulphatase comes from the work of Mehl and Jatzkewitz[1,2] and of Porter *et al.*[5]. The present investigation was undertaken in an attempt to resolve some of these discrepancies using the well-characterized sulphatase A of ox liver.

TABLE I

DISTINCTIONS BETWEEN CEREBROSIDE SULPHATASE AND ARYLSULPHATASE ACTIVITIES

	Cerebroside sulphatase	*Aryl sulphatase*
Requirement for complementary fraction	Yes	No
Requirement for bile salt	Yes	No
Requirement for Mn^{2+}	Yes	No
Inhibition by K_2SO_4	Noncompetitive	Competitive
Action of hydroxylamine	Activation	Inhibition

EXPERIMENTAL

Enzyme

Sulphatase A was prepared from ox liver as previously described[6]. The preparations had specific activities of between 140 and 160 units/mg and all gave single symmetrical peaks on sedimentation in the ultracentrifuge.

Determination of sulphatase activities

Both arylsulphatase and cerebroside sulphatase activities were determined in a pH-stat (Assembly PHM26-TTT11-SBR2-ABU1; Radiometer Ltd, Copenhagen) by titration with 0.015 M NaOH in an atmosphere of N_2. The volume of the reaction mixture was 10 ml, the temperature 37 °C, the recording speed 4 cm/min, and the reactions were started by adding 10–50 μl of enzyme solution.

The standard conditions for arylsulphatase were 3 mM nitrocatechol sulphate (potassium 2-hydroxy-5-nitrophenyl sulphate) in 0.5 mM sodium acetate, pH 4.5, and an enzyme concentration of about 0.4 μg/ml. Any departure from these is specified below. Because of unavoidable irregularities in the first 30 s of recording, true initial velocities could not be determined and the velocity at 1 min after mixing was computed on a PDP 8 computer by a slight modification of the method of Stinshoff[7] using titres at 0.25-min intervals up to 3 min after the start of the reaction. Stinshoff[7] calculated the initial velocity, v_0, by the following relationship (1) between the time of reaction, t, and the amount of product produced, u.

$$\frac{1}{u} = \frac{1}{v_0} \cdot \frac{1}{t} + \frac{1}{u_{max}} \tag{1}$$

This is better used for computation (although not for graphical evaluation) in the form (2).

$$\frac{t}{u} = \frac{1}{u_{max}} \cdot t + \frac{1}{v_0} \tag{2}$$

Eqn 2 gives more even weighting of the points, as in the analogous forms of the Lineweaver–Burk plots[8], and more precise estimates of v_0. In 10 replicate assays the mean velocity and standard deviation (1 min after the start of the reaction) was 411 $\pm$ 22 μmoles/min computed by Eqn 1 and 414 $\pm$ 16 μmoles/min by Eqn 2.

The standard conditions for cerebroside sulphatase were 0.2 mM cerebroside sulphate (Bovine brain sulphatides: Supelco, Bellefonte, Pennsylvania) in 0.5 mM sodium acetate–acetic acid, pH 4.5, containing 2 mM sodium taurodeoxycholate (A grade; Calbiochem, Los Angeles) and 35 mM $MnCl_2$, at an enzyme concentration of about 10 μg/ml. Any departure from these is specified below. The cerebroside sulphate was usually added to the reaction mixture as a 2 mM solution in 20 mM sodium taurodeoxycholate but in some cases the appropriate volume of a solution of the cerebroside sulphate in chloroform–methanol (3:1, v/v) was taken to dryness in the pH-stat vessel at 37 °C and the residue dissolved in the taurodeoxycholate solution. There was no difference in the results with the two techniques. As the method of Stinshoff was inapplicable here (see results section), the velocity at 1 min after mixing was obtained by drawing the tangent to the progress curve at this point. In kinetic studies at low substrate concentrations the substrate concentration at 1 min after mixing was calculated from the initial value and the amount of hydrolysis which had already occurred. This procedure was necessary because at low substrate concentrations up to 40% of the cerebroside sulphate could be hydrolysed in 1 min.

In all cases kinetic constants were computed by the method of Wilkinson[8] on a PDP 8 computer.

Preparation and assay of substrate-modified enzyme

This was carried out as previously described[4].

Reaction of cations with 4-nitrocatechol

A solution (9 ml) of the required salt (38.9 mM) in 0.5 mM sodium acetate was titrated to pH 5.6 in the pH-stat at 37 °C. To this was added 1 ml of 1.49 mM 4-nitrocatechol in 0.5 mM acetate at pH 5.6, and the amount of alkali required to

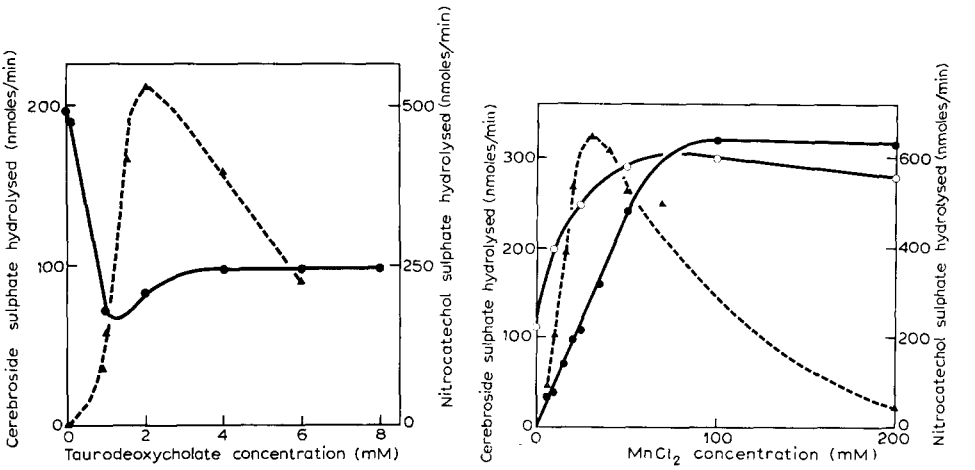


Fig. 1. The effect of varying concentrations of sodium taurodeoxycholate on the cerebroside sulphatase (▲) and arylsulphatase (●) activities of sulphatase A. The substrates were 0.2 mM cerebroside sulphate and 3 mM nitrocatechol sulphate, respectively, and the pH was 4.5 in 0.5 mM sodium acetate–acetic acid containing 35 mM $MnCl_2$.

Fig. 2. The effect of varying concentrations of $MnCl_2$ on the cerebroside sulphatase and arylsulphatase activities of sulphatase A. General conditions as in Fig. 1. For cerebroside sulphatase the taurodeoxycholate concentration was 2 mM (▲) and for arylsulphatase 2 mM (●) or zero (○).

return the pH to 5.6 was measured before taking a sample of the reaction mixture for spectrophotometry.

RESULTS

Cerebroside sulphatase activity

Properties of the enzyme. The optimum pH for cerebroside sulphatase activity under the above conditions was 4.5, in agreement with previous observations[1,5], and the activity fell to 50% of the maximum at pH 4.1 and 4.8, respectively. It was not possible to determine the optimum substrate concentration because of the low solubility of cerebroside sulphate. Cerebroside sulphatase activity was dependent upon the presence of sodium taurodeoxycholate (Fig. 1) and $MnCl_2$ (Fig. 2) or certain other salts (Table II) in the reaction mixture. The optimum concentrations of sodium taurodeoxycholate and $MnCl_2$ were approximately 2 mM and 35 mM, respectively: in both cases the activity fell off sharply on each side of the optimum.

A suspension of 1.9 mg of cerebroside sulphate in 2 ml of water was sonicated for 3 min (power output 75 W) at 0 °C to give an opalescent suspension. Cerebroside sulphate in this form was not hydrolysed by sulphatase A, even in the presence of 35 mM $MnCl_2$. A slow hydrolysis, about 0.02 μmole/min, did occur when 1 ml of diethyl ether was added to the reaction mixture (which remained a single phase because the excess ether rapidly boiled off). Subsequently making the mixture 2 mM taurodeoxycholate allowed the hydrolysis to proceed at the expected rate of about 0.4 μmole/min, showing that the ether had not inactivated the enzyme.

The effect of K_2SO_4 on the hydrolysis of cerebroside sulphate is shown in Fig. 3: the inhibition is noncompetitive. The values of K_m were 75.2 ± 28.8, 71.1 ±

TABLE II

THE EFFECT OF SALTS ON THE CEREBROSIDE SULPHATASE AND ARYLSULPHATASE ACTIVITIES OF SULPHATASE A

pH 4.5 in 0.5 mM sodium acetate; salt concentration 35 mM.

Salt	*Reaction velocity (μmole/mg per min)*		
	2 mM taurodeoxycholate		*No taurodeoxycholate*
	Cerebroside sulphate	*Nitrocatechol sulphate*	*Nitrocatechol sulphate*
None	0	0	72.6
KCl	0.09	25.5	113
KNO_3	—	19.6	103
$MgCl_2$	1.93	139	151
$CaCl_2$	3.82	106	150
$MnCl_2$	5.07	101	170
$CoCl_2$	4.00	106	169
$Co(NO_3)_2$	3.14	104	140
$ZnCl_2$	3.14	44.8	171
$Zn(NO_3)_2$	3.34	69.3	150
$LaCl_3$	7.63	57.9	288
$CeCl_3$	1.18	49.9	223

4.5 and 75.5 ± 8.6 μM cerebroside sulphate at 0, 0.4 and 0.8 mM K_2SO_4, respectively and replots of the intercepts on the ordinate and of the slopes of the lines in Fig. 3 gave values of 0.479 and 0.459 mM K_2SO_4, respectively, for K_i. K_m varies with the concentration of taurodeoxycholate, as shown in Table III. Although there are considerable changes in K_m, V is relatively constant at concentrations of taurodeoxycholate above the optimum (2 mM): at lower concentrations of the latter V falls sharply, presumably because of the high ratio of ionic to micellar bile salt (see Discussion).

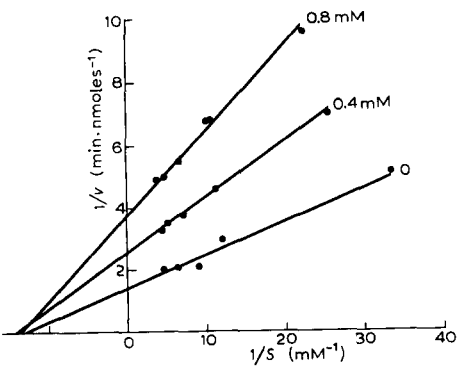


Fig. 3. The effect of varying concentrations of K_2SO_4 on the initial velocity (v) of the hydrolysis of cerebroside sulphate (concentration, S) by sulphatase A at pH 4.5 in 0.5 mM sodium acetate–acetic acid containing 35 mM $MnCl_2$ and 2 mM sodium taurodeoxycholate. The concentrations of K_2SO_4 are indicated on the figure.

TABLE III

K_m AND V FOR THE HYDROLYSIS OF CEREBROSIDE SULPHATE BY SULPHATASE A IN DIFFERENT CONCENTRATIONS OF TAURODEOXYCHOLATE

Taurodeoxycholate concn (mM)	K_m *(μM)*	V *(nmole/mg per min)*
1	32.6 ± 4.5	2.04 ± 0.07
2	61.8 ± 7.0	7.75 ± 0.32
4	201 ± 19.6	10.0 ± 0.44
6	305 ± 23.0	8.62 ± 0.28

The action of the second product, cerebroside, was difficult to investigate because of its insolubility which caused it to precipitate from the standard reaction mixture. By increasing the concentration of taurodeoxycholate to 3.3 mM, 0.1 mM cerebroside (Supelco, Bellefonte, Pa.) could be obtained but this did not inhibit cerebroside sulphatase.

The cerebroside sulphatase activity of sulphatase A was inhibited by hydroxylamine·HCl: 25 mM hydroxylamine·HCl gave a 50% inhibition of the activity.

As only low concentrations of substrate could be used, the hydrolysis rapidly reached completion but the subsequent addition of more cerebroside sulphate caused it to restart, although at a lower rate than initially (Fig. 4). This decrease in rate was due to two factors. First, the addition of the substrate in taurodeoxycholate increased the concentration of the latter to the inhibitory 4 mM (Fig. 1) and second, the inactivation of sulphatase A which occurs when it is kept under the usual reaction conditions but in the absence of substrate. As shown in Fig. 4, the rate of hydrolysis of cerebroside sulphate was similar whether catalysed by sulphatase A which had been kept in the pH-stat for 40 min or by enzyme which had already hydrolysed to completion a quantity of cerebroside sulphate. There is therefore no indication of the production of an inactive form of sulphatase A during its reaction with cerebroside sulphate so that the Stinshoff method of calculating initial velocities is invalid in these conditions.

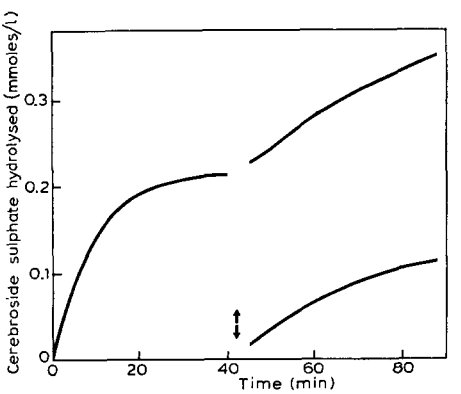


Fig. 4. Progress curves (tracings of pH-stat recordings) for the hydrolysis of 2 μmoles of cerebroside sulphate by sulphatase A: pH 4.5, 35 mM $MnCl_2$ and 2 mM taurodeoxycholate. In the upper curve a further 2 μmoles of cerebroside sulphate was added at 41 min; in the lower curve the enzyme was incubated without substrate until 41 min.

Desialylated sulphatase A produced by the treatment of the native enzyme by neuraminidase (E.R.B. Graham and A. B. Roy, unpublished observations) showed cerebroside sulphatase activity which was indistinguishable from that of the native enzyme.

Effect of taurodeoxycholate on the sedimentation of sulphatase A. Mixtures of sulphatase A and sodium taurodeoxycholate were examined by ultracentrifugation. In contrast to the dissociating effect of sodium dodecyl sulphate[9], sodium taurodeoxycholate had no effect on the sedimentation of the enzyme. The sedimentation coefficients (s_{20}) of the untreated enzyme in acetate buffer, pH 4.5, I = 0.1, in 2 mM sodium taurodeoxycholate in this buffer and in 2 mM taurodeoxycholate and 35 mM $MnCl_2$ in the same buffer were 13.8, 13.8 and 13.9 S, respectively.

Physical nature of the substrate in the reaction mixture. The results of some experiments bearing on the physical state of the taurodeoxycholate and the cerebroside sulphate under the conditions of the assay are shown in Fig. 5. The experimental conditions were not suitable for a detailed investigation of the micelles of bile salt but were chosen to approximate as closely as possible those in the enzyme assays. In all of these experiments the solvent was 0.5 mM sodium acetate–acetic acid, pH 4.5.

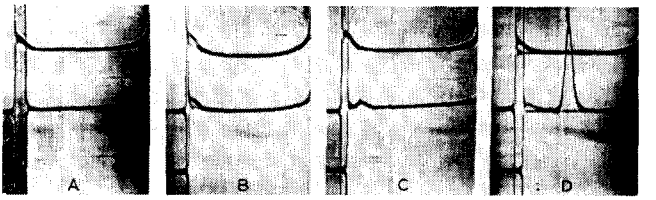


Fig. 5. The sedimentation of 2 mM sodium taurodeoxycholate in 0.5 mM sodium acetate–acetic acid, pH 4.5, under various conditions. Photographs taken at 20 min after reaching speed (52 000 rev./min) in 30 mm cells in an AnE rotor at 20 °C. Bar angle 60°. A, lower curve, no addition; upper curve, 35 mM $MnCl_2$. B, lower curve, 35 mM $MnCl_2$; upper curve, 100 mM $MnCl_2$. C, lower curve, *plus* 0.2 mM cerebroside sulphate; upper curve, *plus* 0.2 mM cerebroside sulphate and 35 mM $MnCl_2$. D, 35 mM $MnCl_2$ with (lower curve) and without (upper curve) sulphatase A (2.5 mg/ml). In B and D double-sector cells were used: in B the reference sectors contained the appropriate concentration of $MnCl_2$ in acetate; in D they contained acetate alone.

A 2 mM solution of taurodeoxycholate in this solvent was below the critical micellar concentration as no sedimenting material was visible in the Schlieren pattern (Fig. 5A, lower pattern): addition of 35 mM $MnCl_2$ caused the formation of micelles of the bile salt (s_{20} about 1 S) which just resolved from the meniscus (Fig. 5A, upper pattern). The effect of $MnCl_2$ is more clearly seen in Fig. 5B where the sedimentation was carried out in double-sector cells, the reference sectors containing the appropriate concentration of $MnCl_2$ in 0.5 mM acetate. As expected, there are more micelles formed in 100 mM (upper pattern) than in 35 mM $MnCl_2$ (lower pattern). This effect is not simply one of ionic strength because there were fewer micelles in 100 mM NaCl than in 35 mM $MnCl_2$ although the ionic strengths were almost identical.

Fig. 5C (lower curve) shows that the addition of 0.2 mM cerebroside sulphate (0.2 mg/ml) to 2 mM taurodeoxycholate was accompanied by the appearance in the Schlieren pattern of a small peak with an s_{20} of 6.5 S: this was not formed when the cerebroside sulphate was added to the bile salt in 35 mM $MnCl_2$ (upper curve).

In the latter case only micelles similar to those of taurodeoxycholate were apparent, indicating the formation of mixed micelles of bile salt and cerebroside sulphate with an s_{20} of about 1 S. The area of the small peak in the lower pattern of Fig. 5C was of the correct order to account for the cerebroside sulphate: the specific refractive increment of the latter is not known but if it is assumed to be 0.001 dl/g, the concentration of the material responsible for the peak would be 0.17 mg/ml, in fair agreement with the concentration of cerebroside sulphate.

In Fig. 5D it is shown that the addition of sulphatase A to taurodeoxycholate in 35 mM $MnCl_2$ (in a molar ratio of sulphatase A, calculated as monomer, to bile salt of 85) had little effect on the number of micelles. Although there were fewer visible in the presence (lower curve) than in the absence (upper curve) of sulphatase A, there was no binding of bile salt comparable to that of sodium dodecyl sulphate[9].

Arylsulphatase activity

Properties of the enzyme. In the presence of 2 mM taurodeoxycholate and 35 mM $MnCl_2$ the pH optimum for the hydrolysis of nitrocatechol sulphate by sulphatase A was pH 5.6. Taurodeoxycholate in the absence of $MnCl_2$ powerfully inhibited the arylsulphatase at pH 4.5 (Fig. 2; Table II): at 1.0 and 0.1 mM taurodeoxycholate the inhibition was 90 and 25%, respectively. The effect of $MnCl_2$ is shown in Fig. 2: it activated the arylsulphatase activity both in the presence and absence of taurodeoxycholate but the effect was more striking in the former case because of the reversal of the inhibition due to the bile salt. Several other salts also caused this activation (Table II). With varying concentrations of sodium taurodeoxycholate at a constant concentration of $MnCl_2$ the inhibition reached a maximum at 1 mM bile salt and thereafter decreased slightly to a constant level of about 50% (Fig. 1).

The effect of K_2SO_4 on the hydrolysis of nitrocatechol sulphate at pH 4.5 in the presence of 2 mM sodium taurodeoxycholate and 35 mM $MnCl_2$ is shown in Fig. 6. The inhibition is clearly competitive. The K_m is 0.516 $\pm$ 0.076 mM nitrocatechol sulphate and the K_i is 0.15 mM K_2SO_4: these values are not significantly different from those determined at pH 5.6 in the absence of taurodeoxycholate and $MnCl_2$.

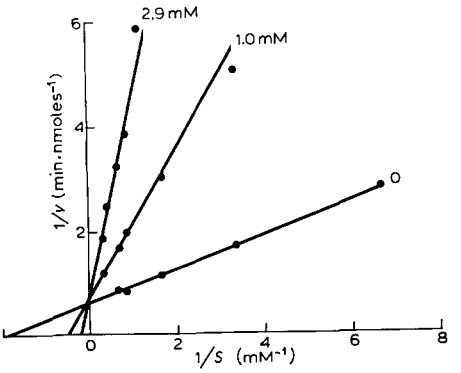


Fig. 6. The effect of varying concentrations of K_2SO_4 on the initial velocity (v) of the hydrolysis of nitrocatechol sulphate by sulphatase A in the presence of taurodeoxycholate and $MnCl_2$ (conditions as in Fig. 3). The concentrations of K_2SO_4 are given on the figure.

In 25 mM hydroxylamine·HCl the arylsulphatase activity of sulphatase A was inhibited by 52%, whether or not taurodeoxycholate and $MnCl_2$ were also present.

Interaction of metal ions and 4-nitrocatechol. The results in Table II show that many salts activate the hydrolysis of nitrocatechol sulphate by sulphatase A at pH 4.5. During attempts to study this activation at pH 5.6, the optimum pH, it became apparent that a reaction occurred between several of these cations and the reaction product, 4-nitrocatechol. This reaction was accompanied by the liberation of up to 2 H^+ per mole of 4-nitrocatechol and by a considerable increase in the absorption in the 430-nm region, as shown in Table IV. These changes presumably reflect complex formation between a cation and the 4-nitrocatechol which results in increased ionisation of the latter with consequent changes in the absorption spectrum.

TABLE IV

COMPLEX FORMATION BETWEEN 4-NITROCATECHOL AND CERTAIN METALLIC SALTS

The concentrations of 4-nitrocatechol and of salt were 0.149 mM and 35 mM, respectively, in 0.5 mM sodium acetate, pH 5.6. The $A_{430\ nm}$ of the 4-nitrocatechol in 0.5 mM sodium acetate was 0.24.

Salt	*Moles of H^+ liberated per mole of 4-nitrocatechol*	*Spectrum of complex*	
		λ_{max}	A_{max}
$MgCl_2$	0.04	—	—
$CaCl_2$	0.04	—	—
$MnCl_2$	0.21	430	0.295
$CoCl_2$	0.97	450	0.684
$ZnCl_2$	0.95	440	1.24
$LaCl_3$	1.62	418	1.07
$CeCl_3$	1.95	430	1.12

The reaction was sensitive to changes in ionic strength and pH. In 0.14 M NaCl the reaction of 4-nitrocatechol with 0.035 M $CoCl_2$ (total ionic strength 0.25) proceeded to only about 70% of that in the absence of NaCl (I = 0.11) as judged either by the liberation of H^+ or by the change in absorbance at 430 nm. At pH 4.5 the reaction between 4-nitrocatechol and cations was much less important. Under the conditions of Table IV, but at pH 4.5, only La^{3+} and Ce^{3+} caused a measurable release of H^+, to about 7 and 8%, respectively of the extent at pH 5.6.

Substrate-modified forms of sulphatase A

The ratio of the rates of hydrolysis of cerebroside sulphate (under standard conditions) and nitrocatechol sulphate (1 mM at pH 4.5) by a preparation containing about 85% of a nitrocatechol sulphate-modified sulphatase A (ref. 4) was 0.054 while the ratio for native sulphatase A was 0.067. It may therefore be concluded that the substrate-modified enzyme, which has no arylsulphatase activity[4], is likewise devoid of cerebroside sulphatase activity. It should be noted that the arylsulphatase activity of the modified enzyme at pH 4.5 is not increased by SO_4^{2-}, contrary to the situation at pH 5.6 (ref. 4).

Attempts were made to isolate an analogous substrate-modified form of sulphatase A from a reaction mixture containing cerebroside sulphate. No such form

could be isolated by the general technique described previously[4] at pH 4.5, pH 6 or pH 7 from reaction mixtures containing 0.2 mM cerebroside sulphate (and 2 mM taurodeoxycholate and 20 mM $MnCl_2$) which had been incubated for times ranging from 0.5 to 6 h. The enzyme concentration in these experiments was 1 μg/ml so that the reaction velocity was about half of that in Fig. 4.

DISCUSSION

The general behaviour of sulphatase A as an arylsulphatase at pH 4.5 in 2 mM sodium taurodeoxycholate and 35 mM $MnCl_2$ is not significantly different from its behaviour at pH 5.6 in the absence of these possible modifiers. In particular, the hydrolysis of nitrocatechol sulphate is competitively inhibited by SO_4^{2-}, behaviour consistent with sulphatase A catalysing a uni-bi[10] reaction with SO_4^{2-} being the last-released product[11].

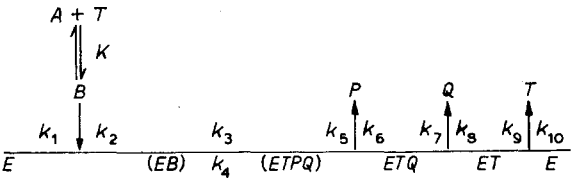
The inhibition of arylsulphatase activity by taurodeoxycholate and its reversal by $MnCl_2$ (Fig. 2) or by other salts (Table II) suggests that this inhibition is due to the ionic form of the bile salt which predominates in a 2 mM solution of sodium taurodeoxycholate in 0.5 mM acetate (Fig. 5A and ref. 12). Addition of $MnCl_2$ or other salt lowers the critical micellar concentration of the bile salt and therefore the concentration of its ionic form. This interpretation is supported by Fig. 1 which indicates that the inhibition by taurodeoxycholate is independent of its concentration once the critical micellar concentration is exceeded. Superimposed upon this is an independent effect of $MnCl_2$ and other salts, an activation of the arylsulphatase activity itself, primarily by the cations (Table II). This activation is rather non-specific and the cations cannot play any direct part in the arylsulphatase reaction.

The situation in the hydrolysis of cerebroside sulphate is more complicated because a micellar bile salt is necessary to solubilise this substrate which otherwise forms large aggregates (Fig. 5C), presumably with particle weights of about 10^5. The formation of micelles of taurodeoxycholate depends upon the presence of a suitable salt in the reaction mixture. The role of $MnCl_2$ in the hydrolysis of cerebroside sulphate is therefore 3-fold. Firstly by lowering the critical micellar concentration it reduces the concentration of ionic taurodeoxycholate to non-inhibitory levels and secondly it directly activates the sulphatase: both of these effects also occur in the hydrolysis of nitrocatechol sulphate. Thirdly, again through lowering the critical micellar concentration, it allows the incorporation of cerebroside sulphate into small micelles of bile salt (Fig. 5C): this obviously has no counterpart in arylsulphatase activity. The apparent absolute dependence of cerebroside sulphatase activity on taurodeoxycholate and $MnCl_2$ is therefore explicable and the differences from arylsulphatase arise solely from the fact that the substrates for the latter do not require solubilisation by bile salts. These effects will be less obvious in assays where a buffer contributes significantly to the salt concentration (*e.g.* ref. 5) and so lowers the critical micellar concentration of taurodeoxycholate[12].

This argument implies that the taurodeoxycholate serves only as a detergent which should be replaceable by other such compounds although a certain specificity might be expected in this, as in many[13], micellar reactions. Certainly in unpurified

systems taurodeoxycholate can be replaced by cholate[14] and, as reported above, it can be replaced, albeit inefficiently, by ether.

The noncompetitive inhibition of cerebroside sulphatase by SO_4^{2-} (Fig. 3 and ref. 5) is strikingly different from the competitive inhibition of arylsulphatase activity under similar conditions (Fig. 6). Noncompetitive inhibition by a product requires that this combines with a different form of the enzyme than does the substrate. This is possible where the substrate is a mixed micelle of taurodeoxycholate and cerebroside sulphate: noncompetitive inhibition by SO_4^{2-} would occur if this were released from the enzyme after the cerebroside but before the bile salt, as in the following scheme (E, A, T, B, P and Q are sulphatase A, cerebroside sulphate, micellar taurodeoxycholate, mixed micelles of cerebroside sulphate and taurodeoxycholate, cerebroside and SO_4^{2-}, respectively).



The assignment of P and Q to cerebroside and SO_4^{2-}, respectively is made only on the analogy with the arylsulphatase activity of sulphatase A. This scheme represents a uni-ter reaction and the velocity equations can be derived in the usual way[10] to give Eqns 3 and 4 for the initial velocities in the absence and presence of SO_4^{2-}, respectively. The kinetic constants are defined according to Cleland[10].

$$v = \frac{V_a}{K_b\left\{1 + \frac{t}{K_{it}}\right\}\left\{1 + \frac{K}{t}\right\} + a} \tag{3}$$

$$v = \frac{V_a}{K_b\left\{1 + \frac{t}{K_{it}}\left[1 + \frac{K_p q}{K_{ip}K_q}\right]\right\}\left\{1 + \frac{K}{t}\right\} + a\left\{1 + \frac{q}{K_{iq}}\right\}} \tag{4}$$

It follows from Eqn 3 that there will be no cerebroside sulphatase activity in the absence of micellar taurodeoxycholate and that the experimental K_m (which must be greater than K_b) will vary with t, the concentration of these micelles, decreasing with increasing t to a minimum at $t = \sqrt{KK_{it}}$ and then increasing, as shown in Table III. Complementary changes will occur in v but the situation will be more complex than predicted by Eqn 3 because of the inhibition by ionic taurodeoxycholate. The effect of metal ions is not considered in these expressions but increasing concentrations of salt must increase the concentration of micellar taurodeoxycholate and so effectively increase t: superimposed upon this, however, is the direct activation by metallic ions. Nevertheless, Eqn 3 is consistent with the behaviour of cerebroside sulphatase shown in Figs 1 and 2. Eqn 4 shows that inhibition by SO_4^{2-} will be noncompetitive with, in general, $K_{i\ \text{slope}}$ and $K_{i\ \text{intercept}}$ being different, and the latter will vary with the concentration of micellar taurodeoxycholate except when $t \gg K_{it}$. The fact that the two experimental K_i values are identical must be due to chance and can occur only when $K_q = K_{iq}$, $K_p = K_{ip}$ and $t \gg K_{it}$.

Eqns 3 and 4 indicate that V should be independent of t whereas the data in Table III show V to vary with t. The reason for this discrepancy is that the model is oversimplified and cannot account for the different structures of mixed micelles formed at different ratios of taurodeoxycholate to cerebroside sulphate. This does not, however, influence the discussion above.

It was expected that the substrate-modified form of sulphatase A produced by its reaction with nitrocatechol sulphate[4] would be devoid of cerebroside sulphatase activity but it was surprising that a corresponding cerebroside sulphate-modified form could not be isolated. This does not mean that such a modified enzyme cannot be formed: the conditions might have been such that it could not accumulate. The rate of formation of modified sulphatase A is highly dependent upon the substrate concentration[7] and in the preparation of nitrocatechol sulphate-modified enzyme[4] the concentration of substrate was about 100 K_m: a corresponding concentration of cerebroside sulphate cannot be used because of its low solubility. Therefore, whether or not a modified form of sulphatase A can be produced during its reaction with cerebroside sulphate must be left undecided at present. If the modification depends upon the formation of complexes of the type FS_2 (4) then the large size of the cerebroside sulphate molecule, and the even greater size of the micelle in which it is incorporated, may prevent this by simple steric reasons.

The removal of the sialyl residues from sulphatase A does not alter its cerebroside sulphatase activity. This disproves the suggestion[15] that metachromatic leucodystrophy, which is characterised by a deficiency of sulphatase A, is due to a deficiency of the sialyl transferase which must be involved in its formation.

The results described above therefore support the view[1,2] that the arylsulphatase and cerebroside sulphatase activities of sulphatase A are the functions of a single active site. Nevertheless, at the concentrations used in arylsulphatase determinations, about 0.4 μg/ml, sulphatase A exists predominantly as a monomer while at the concentrations used for cerebroside sulphatase determinations, 10 μg/ml, it exists mainly as a tetramer[16]: further investigation of this point may be required. The maximum velocities of the two reactions are rather different: 160 μmole/mg per min for nitrocatechol sulphate and 9 μmoles/mg per min for cerebroside sulphate. However, the latter rate is similar to that for phenyl sulphate (about 16 μmole/mg per min) and much greater than that for a potential 'physiological' substrate, oestrone sulphate (about 1 μmole/mg per min).

The most important factor still to be resolved is the nature of the complementary fraction of Mehl and Jatzkewitz[1]. This can no longer be regarded as an absolute requirement for cerebroside sulphatase activity and it may be simply a protein, perhaps a lipoprotein, which solubilises the cerebroside sulphate and so replaces the taurodeoxycholate in the system described above, and presumably also *in vivo*.

There is a minor discrepancy between the present finding that hydroxylamine·HCl inhibits both the cerebroside sulphatase and arylsulphatase activities of sulphatase A at pH 4.5, as it does the latter at pH 5.6 (ref. 17), and the previous observation[1] that hydroxylamine activates cerebroside sulphatase. No explanation can be offered, but the conditions were very different in the two investigations.

Now that cerebroside sulphatase activity can definitely be assigned to sulphatase A, the latter need no longer be regarded as an arylsulphatase and previous

doubts[18] about its physiological role become meaningless. Doubts about the physiological function of other 'aryl' sulphatases still remain and these enzymes must be reinvestigated. In particular, a possible chondrosulphatase activity for sulphatase B should be sought in view of the changes which occur in the activity of this enzyme in Hurler's syndrome[19].

ACKNOWLEDGMENTS

The authors are deeply indebted to Dr H. Kihara, Pacific State Hospital, Pomona, California, and to Dr K. Stinshoff, Max-Planck-Institut für Psychiatrie, Munich, for copies of their works prior to publication.

REFERENCES

1 E. Mehl and H. Jatzkewitz, *Hoppe-Seyler's Z. Physiol. Chem.*, 339 (1964) 260.
2 E. Mehl and H. Jatzkewitz, *Biochim. Biophys. Acta*, 151 (1968) 619.
3 J. Austin, D. Armstrong and L. Shearer, *Arch. Neurol.*, 13 (1965) 593.
4 R. G. Nicholls and A. B. Roy, *Biochim. Biophys. Acta*, 242 (1971) 141.
5 M. T. Porter, A. L. Fluharty, S. D. de la Flor and H. Kihara, *Biochim. Biophys. Acta*, 258 (1972) 769.
6 L. W. Nichol and A. B. Roy, *J. Biochem. Tokyo*, 55 (1964) 643.
7 K. Stinshoff, *Biochim. Biophys. Acta*, 276 (1972) 475.
8 G. N. Wilkinson, *Biochem. J.*, 80 (1961) 324.
9 L. W. Nichol and A. B. Roy, *Biochemistry*, 5 (1966) 1379.
10 W. W. Cleland, *Biochim. Biophys. Acta*, 67 (1963) 104, 173.
11 A. B. Roy, *Biochim. Biophys. Acta*, 198 (1970) 365.
12 D. M. Small, in P. P. Nair and D. Kritchevsky, *The Bile Acids*, Vol. 1, Plenum Press, New York, 1971, p. 249.
13 E. J. Fendler and J. H. Fendler, *Adv. Phys. Org. Chem.*, 8 (1970) 271.
14 A. K. Percy, D. F. Farrell and M. M. Kaback, *J. Neurochem.*, 19 (1972) 233.
15 A. Goldstone, P. Konecny and H. Koenig, *FEBS Lett.*, 13 (1971) 68.
16 L. W. Nichol and A. B. Roy, *Biochemistry*, 4 (1965) 386.
17 A. B. Roy, *Biochim. Biophys. Acta*, 198 (1970) 76.
18 A. B. Roy, *Adv. Enzymol.*, 22 (1960) 205.
19 J. Austin, D. McAfee, D. Armstrong, M. O'Rourke, L. Shearer and B. Bachhawat, *Biochem. J.* 93 (1964) 15c.