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COMPARISON OF THE CEREBROSIDE SULPHATASE AND THE ARYLSULPHATASE ACTIVITY OF HUMAN SULPHATASE A IN THE ABSENCE OF ACTIVATORS

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Summary

A cerebroside sulphatase (cerebroside-3-sulphate 3 sulphohydrolase, EC 3.1.6.8) assay based on radio thin-layer chromatography is described. The substrate was labelled by the catalytic addition of tritium to cerebroside sulphate. Using this assay the cerebroside sulphatase activity of sulphatase A (Aryl-sulphate sulphohydrolase, EC 3.1.6.1) from human liver and kidney in the absence of activators was investigated.

The pH optimum of this reaction depends on the buffer concentration, being pH 4.5 at 50 mM and 5.3 at 10 mM sodium formate. With the latter concentration the apparent K_m for cerebroside sulphate is 0.06 mM; SO_4^{2-} and nitrocatechol sulphate inhibit noncompetitively with a K_i of 4.51 mM for Na_2SO_4 and 0.43 mM for nitrocatechol sulphate.

The cerebroside sulphatase activity of sulphatase A is highly dependent on the ionic strength. The optimum sodium formate concentration is 10 mM, and the cerebroside sulphatase activity decreases rapidly with increasing buffer concentration. The same concentration dependence is observed in the inhibitory effect of cerebroside sulphate on the arylsulphatase reaction. The inhibition decreases at increasing buffer concentrations, becoming an activation at 70 mM sodium formate.

The progress curve of the cerebroside sulphatase reaction shows a deviation from linearity similar to that of the arylsulphatase reaction. Investigation of the effect of preincubation with cerebroside sulphate on the arylsulphatase activity of the enzyme shows that cerebroside sulphatase activity and inactivation of the enzyme by cerebroside sulphate occur simultaneously.

These observations are interpreted as supporting the assumption that cerebroside sulphate and arylsulphates are degraded at an identical active site on the same enzyme. Differences in the properties of the cerebroside sulphatase and the arylsulphatase reaction of the enzyme may be attributed to the differences in the physicochemical state of the two substrates.

Introduction

Sulphatase A (Aryl-sulphate sulphohydrolase, EC 3.1.6.1) of vertebrates, a lysosomal enzyme [1], has long been known as "arylsulphatase A" [2] and references in Ref. 2]. The deficiency of this enzyme in cases of metachromatic leukodystrophy [3], a human sphingolipid storage disease [4,5], gave the first clue to the physiological substrates of the enzyme, which were subsequently identified as cerebroside sulphates [6]. The cerebroside sulphatase (cerebroside-3-sulphate 3 sulphohydrolase, EC 3.1.6.8) activity of sulphatases A from several mammals was confirmed [7,8] and characterized [9,10]. In these investigations the enzyme showed a requirement for activators, such as bile salts [7—10] or a physiological activator [6], for its cerebroside sulphatase activity. In 1971, however, high cerebroside sulphatase activity of purified human sulphatase A in the absence of any activator was reported [11]. This observation seemed important since the arylsulphatase activity of the enzyme, which is primarily measured with nitrocatecholsulphate (2-hydroxy-5-nitrophenyl sulphate) as substrate, also required no activator.

The present investigation was aimed at characterizing the cerebroside sulphatase activity of purified human sulphatase A in the absence of activators, and at gaining insight into the relation between cerebroside sulphatase and arylsulphatase activities of the enzyme.

Experimental

Reagents

If not otherwise stated, reagents of analytical grade were purchased from Merck (Darmstadt, Germany).

Enzyme

Sulphatase A was prepared from human kidney and liver as previously described [12]. The preparation had a specific activity of 30 units per mg, when the arylsulphatase activity was determined according to Baum et al. [13], one unit being defined as the amount of enzyme which degrades one μmole of substrate per min.

Substrate for the determination of the cerebroside sulphatase activity

160 mg of crude bovine cerebroside sulphates [14,15] were dissolved in 5 ml benzene—dimethylformamide (5 :1, by vol.), 150 mg PtO_2 added, and the mixture washed three times with purified N_2. In a closed apparatus 10 Ci carrier-free tritium gas were then added and the reaction mixture gently shaken at 40° C for 24 h. The mixture was subjected to filtration, evaporation, and three times to column chromatography on inactivated Florisil [15]. 59 mg cerebroside sulphate, 25 mg of the hydroxy-fatty acid type and 35 mg of the non-hydroxy fatty acid type, which was later used as substrate, and 17 mg cerebroside were recovered. The specific radioactivity of the substrate preparation was 540 Ci per mole; 93.8% of the tritium gas was added to the fatty acid and 5.3% to the sphingosine moiety of the molecule. Less than 1% of the radioactivity was found in the galactose sulphate moiety. The purity of the cere-

broside sulphate preparation was assessed by densitometry after thin-layer chromatography [16] and by radio thin-layer chromatography. Staining of the thin-layer chromatograms produced no detectable spot besides cerebroside sulphate; radio thin-layer chromatography showed the existing impurities to contain less than 0.1% of the total radioactivity.

Assay of the cerebroside sulphatase activity

(a) Buffer solutions. 0.02, 0.04, 0.08, and 0.16 M sodium formate solutions which had pH values corresponding to the respective incubation pH values were prepared by titrating sodium formate solutions of the respective molarity to the appropriate pH with formic acid.

(b) Additions to the incubation mixture. To investigate the inhibition by nitrocatechol sulphate and by Na_2SO_4, solutions of 2 mM nitrocatechol sulphate (Sigma Chem. Co., St. Louis, Mo.), titrated to pH 5.3 with formic acid, and solutions of 20 mM Na_2SO_4 were prepared. To assess the effect of lipids on the enzyme activity DL-psychosine, L-α-phosphatidylcholine from yolk, sphingomyelin from bovine brain, ceramide from bovine brain (all purchased from Sigma Chem. Co., St. Louis, Mo.), phosphatidylinositol from bovine brain (Koch-Light, Colnbrook, England) and cerebroside from bovine brain were dissolved in chloroform—methanol (2 : 1, by vol.) to give 0.1 mM solutions.

(c) Cerebroside sulphatase assay. 5—75 µl of a 0.185 mM cerebroside sulphate solution in benzene—ethanol (2 : 1, by vol.) were evaporated to dryness within the incubation vessel. In cases in which the effect of lipids was to be investigated, 100 µl of the respective lipid solutions, together with 54 µl of the substrate solution, were evaporated. To each of the dry residues 25 µl of the respective buffer solution and, in some cases 25 µl of the nitrocatechol sulphate or the Na_2SO_4 solution were added. Sufficient water was added to each sample so that after the subsequent addition of the enzyme solution, a final volume of 100 µl was reached. The residues were dispersed by treatment with a sonifier, type LS-75 (Branson Instr., Stamford, Wis.) for 1 min at highest output. After preincubation for 5 min at 37° C the reaction was started by addition of the appropriate volume of a solution of 50 µg of the enzyme preparation in distilled water. After an incubation period of 5 min at 37° C 50 µl were withdrawn, immediately added to 5 µl 1 M NaOH and applied to a thin-layer plate precoated with silica-gel (Merck, Darmstadt, Germany).

Volumes 10 times those given above were used to establish the progress curve of the cerebroside sulphatase reaction. From these mixtures 50 µl were withdrawn after appropriate incubation periods and processed as described above.

The addition of NaOH raised the pH of the incubation mixture to about 11. As shown by control experiments, cerebroside sulphate was hydrolysed neither in the presence nor in the absence of enzyme under these conditions.

(d) Evaluation of the cerebroside sulphatase assay. The thin-layer plates were developed in chloroform—methanol—distilled water (80 : 20 : 3, by vol.) and scanned with a radio thin-layer scanner (Berthold, Wildbad, Germany). Two peaks were detected on the chromatogram. By co-chromatography under various conditions they could be attributed to cerebroside and cerebroside sulphate. The amount of product formation was calculated from the areas under the two peaks.

TABLE I

DETERMINATION OF DEVIATIONS IN THE EVALUATION OF THE CEREBROSIDE SULPHATASE ASSAY

Sample (%), percentage of cerebroside radioactivity within total radioactivity of sample; Eval. (%), percentage of cerebroside radioactivity according to evaluation; Max. dev. (%), percentage of maximum deviation from mean. Number of determinations made at each percentage of cerebroside radioactivity = 7.

Sample (%)	Eval. (%)	Max. dev. (%)	S. D. (%)
50	50.32	7.5	2.5
20	19.88	7.9	2.8
10	10.23	15.0	4.6
5	4.85	21.7	8.3
2	1.79	38.2	11.6
1	0.82	53.6	22.4

To establish the error of this method solutions of labelled cerebroside and cerebroside sulphate in benzene-ethanol (2 : 1, by vol.), containing a constant radioactivity of 11.8 μCi per 50 μl and varying amounts of the two lipids, were prepared.

Labelled cerebroside contributed from 1 to 50% to the total radioactivity of the different solutions. 50 μl samples of these solutions were applied to thin-layer plates which were developed and scanned. Evaluation showed (Table I) that the error increased with decreasing amounts of cerebrosides in the solutions. For this reason the average of 7 incubations was taken for each determination, and the enzyme concentrations were chosen in such a fashion as to give an amount of product formation of about 5% after 5—20 min of incubation.

The method for calculating the reaction parameters of the arylsulphatase reaction [12] requires a high accuracy in determining the product formation at short incubation times and low percentages of substrate degradation. Such an accuracy could not be attained by the method with which the cerebroside sulphatase activity was determined, and it therefore appeared to be unreasonable to apply the calculation to this method. The enzyme activity, instead, was determined by measuring the amount of product formed after an incubation period of 5 min and defining the quotient of product to incubation time was average reaction velocity, $v_{5\,min}$. In those cases in which initial and instantaneous velocities of cerebroside sulphatase reactions are given, they were estimated by drawing the tangent to the respective points of the progress curves.

Assay of the arylsulphatase activity

The assay was performed as described earlier [12]. To determine the arylsulphatase activity in the presence of cerebroside sulphate or other lipids, an appropriate volume of the respective lipid solutions described above was evaporated and dried within the reaction vessel. After dispersion of the lipid in an appropriate buffer solution and 5 min preincubation, the enzyme solution was added. The arylsulphatase reaction was started by the addition of preincubated nitrocatechol sulphate solution. The incubation temperature was 37° C.

The arylsulphatase activity was calculated from the spectrophotometric determination of the amount of product formation [12], each value being the average of 4 measurements (S.D. 1.8%). The U_{max} and V_0 values calculated [12] from 4 progress curves based on 4 series of single measurements showed S.D. values of 4.3% and 4.8%, respectively.

Results

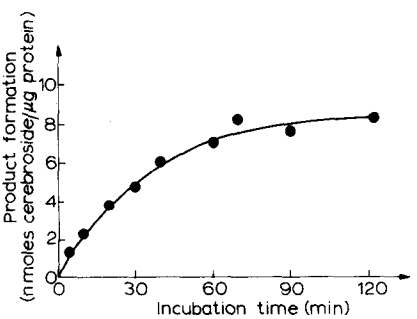
(A) Investigation of the cerebroside sulphatase activity

Progress curve. The progress curve of the catalytic action of sulphatase A on cerebroside sulphate (Fig. 1) shows a similar deviation from linearity to that of the corresponding curve of the arylsulphatase reaction of the enzyme [2]. In contrast to the arylsulphatase reaction, however, the cerebroside sulphatase reaction cannot be reactivated by SO_4^{2-}.

Due to the high error in the cerebroside sulphatase assay it was not possible to apply the method of calculating initial velocities and maximum amounts of product which would be produced at infinite incubation time from the double reciprocal progress curve [12] to this reaction. The method with which the cerebroside sulphatase activity was determined is outlined under Experimental.

pH profile. The pH optimum of the cerebroside sulphatase reaction depends on the concentration of the buffer solution. At 40 mM sodium formate the optimum is pH 4.5, similar to the optima observed for the reactions of sulphatases A from pig kidney [17], human fibroblasts [9], and ox liver [10]. At lower buffer concentrations, however, both the pH optimum and the enzyme activity are increased (Fig. 2). The optimum is pH 5.3 at 0.01 M buffer. Lowering the buffer concentration below 0.01 M reduces the enzyme activity, whereas the optimum pH is not significantly altered.

Effect of substrate concentration on the enzyme activity. In its cerebroside sulphatase reaction, as in its arylsulphatase reaction, the enzyme fol-



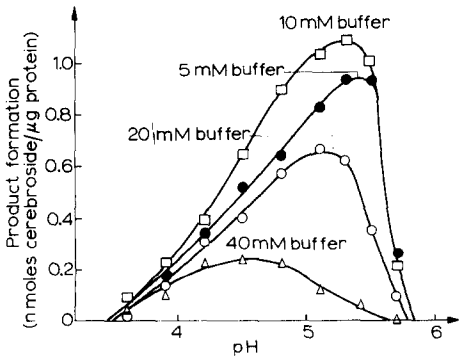


Fig. 1. Progress curve of the cerebroside sulphatase reaction of sulphatase A. Assay conditions: 0.1 mM cerebroside sulphate; 10 mM sodium formate (pH 5.3); 2.8 μg enzyme protein per ml. Product formation after an incubation period of 30 and 120 min corresponding to 14% and 27%, respectively, of the initial substrate concentration.

Fig. 2. pH-dependence of cerebroside sulphatase activity. Assay conditions: 0.1 mM cerebroside sulphate; 32.5 μg enzyme protein per ml; incubation period, 5 min. Buffer: ●———●, 5 mM; □———□, 10 mM; ○———○, 20 mM; △———△, 40 mM sodium formate.

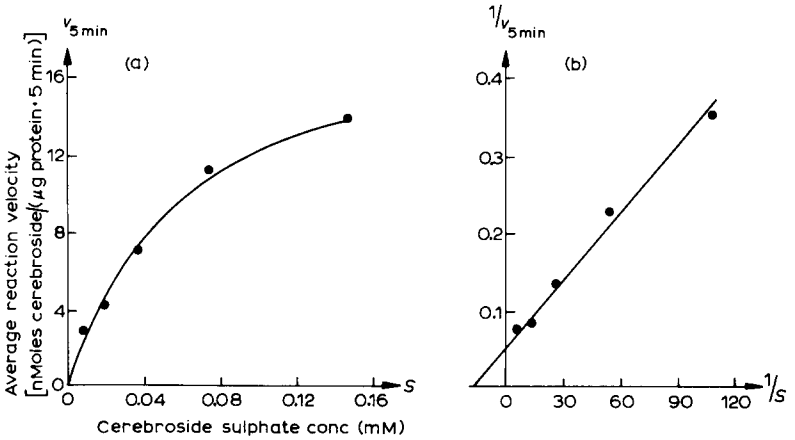


Fig. 3. (a) Michaelis—Menten curve for the cerebroside sulphatase reaction. Assay conditions: 10 mM sodium formate (pH 5.3); 14.2 µg enzyme protein per ml; incubation period, 5 min. (b) Lineweaver—Burk plot corresponding to Fig. 3a.

lows Michaelis—Menten kinetics (Fig. 3). At 0.01 M sodium formate and an incubation period of 5 min the apparent Michaelis constant is 0.06 mM.

Effect of Na_2SO_4, nitrocatechol sulphate and sodium formate on the cerebroside sulphatase activity. Na_2SO_4 and nitrocatechol sulphate inhibit the enzyme reaction noncompetitively; the apparent inhibition constants under the conditions chosen are 4.51 mM for Na_2SO_4 and 0.43 mM for nitrocatechol sulphate (Figs 4a, 4b). Sodium formate displays a complicated mode of inhibition (Fig. 4c). At buffer concentrations above 0.01 M noncompetitive inhibition is observed, whereas the intercept of the lines in the Lineweaver—Burk plot is shifted to the right of the ordinate at buffer concentrations below 0.01 M. A similar observation is made when the effect of buffer concentration on the Lineweaver—Burk plot in the arylsulphatase reaction is investigated [12].

The influence of ionic strength on the progress curve, however, is different in the cerebroside sulphatase and the arylsulphatase reaction. In contrast to the arylsulphatase activity [12], the cerebroside sulphatase activity is not stabilized against the substrate dependent inactivation by increasing buffer concentrations (Fig. 5).

Comparison of the effect of lipids on the arylsulphatase and on the cerebroside sulphatase reaction. The cerebroside sulphatase activity is inhibited to varying degrees by a number of amphiphilic lipids (Table II). Psychosine, which carries a free amino group, is a very potent inhibitor; sphingomyelin and phosphatidylcholine, both exhibiting a zwitterion structure derived from a phosphate and an amino group, do not show as strong an effect. Inhibition is lowest with ceramide and cerebroside, which carry no ionic groups. On the other hand, the arylsulphatase activity is significantly enhanced by these substances.

Phosphatidyl inositol differs from the other lipids in inhibiting not only the cerebroside sulphatase activity but also, though only slightly, the arylsulphatase activity. Phosphatidyl inositol is at the same time the only one among the lipids tested to carry an acid phosphate group not involved in zwitterion structure.

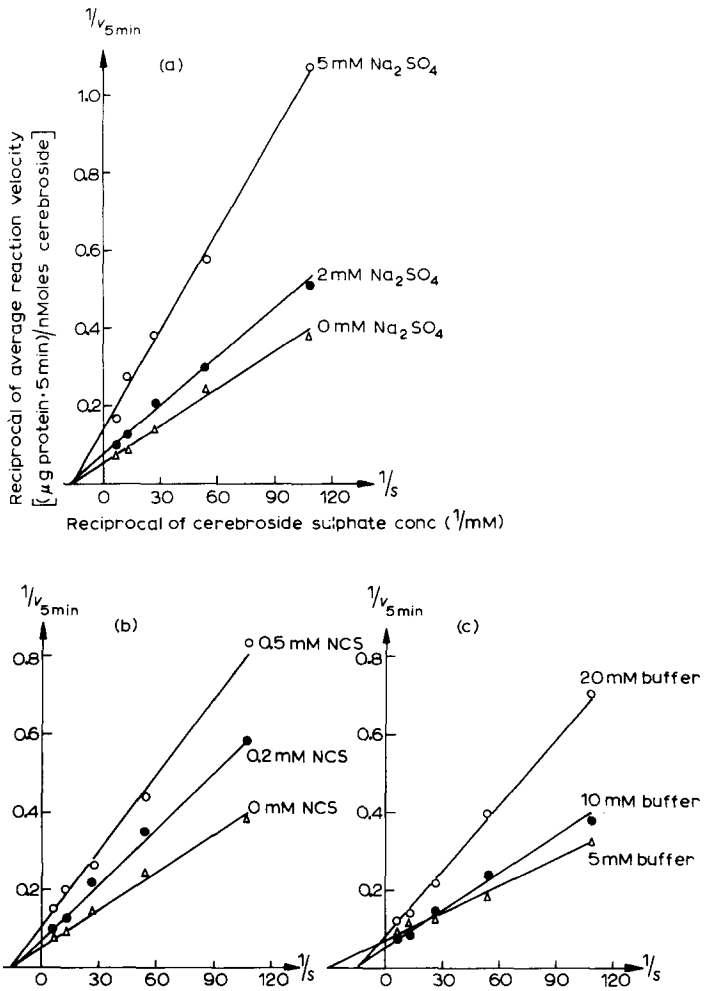


Fig. 4. Influence of Na_2SO_4, nitrocatechol sulphate (NCS), and sodium formate on the Lineweaver—Burk plot of the cerebroside sulphatase reaction. Ordinate, reciprocal of the average reaction velocity; (see fig.3) abscissa, reciprocal of the substrate concentration (see Fig. 3). Assay conditions: 10 mM sodium formate (ph 5.3); 14.2 μg enzyme protein per ml; incubation period, 5 min. (a) Influence of Na_2SO_4. Na_2SO_4 concentration: ○———○, 5 mM; ●———●, 2 mM; △———△, 0 mM. (b) Influence of nitrocatechol sulphate. Nitrocatechol sulphate concentration: ○———○, 0.5 mM; ●———●, 0.2 mM; △———△, 0 mM. (c) Influence of sodium formate. Sodium formate concentration: ○———○, 20 mM; ●———●, 10 mM; △———△, 5 mM.

(B) Effect of cerebroside sulphate on the arylsulphatase reaction

Influence of the buffer concentration on the cerebroside sulphate inhibition. As mentioned above, the cerebroside sulphatase activity of the sulphatase A is highly dependent on the buffer concentration. The inhibition of the arylsulphatase activity of the enzyme also depends on the buffer concentration (Fig. 6), and the arylsulphatase inhibition is high at those sodium formate concentrations at which the cerebroside sulphatase activity is high. The lower the cerebroside sulphatase activity becomes with increasing ionic strength, the

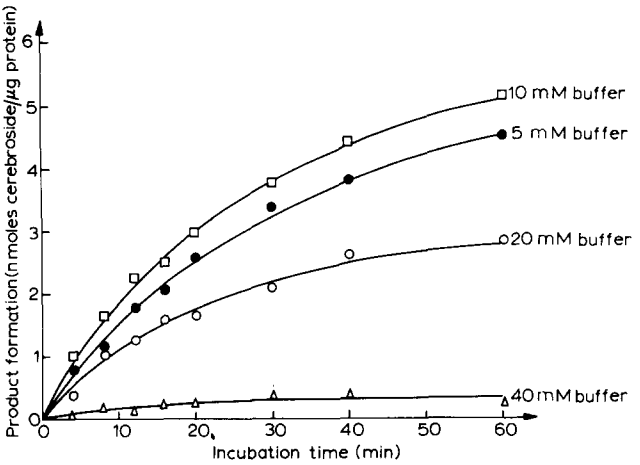


Fig. 5. Effect of buffer concentration on the progress curve of the cerebroside sulphatase reaction. Assay conditions: 0.1 mM cerebroside sulphate; 7.1 μg enzyme protein per ml; incubation pH, 5.3. Buffer: ●———●, 5 mM; □———□, 10 mM; ○———○, 20 mM; △———△, 40 mM sodium formate.

smaller is the inhibitory effect of cerebroside sulphate, and at formate concentrations higher than 0.07 M, where cerebroside sulphatase activity in the absence of activators can no longer be observed, the cerebroside sulphate inhibition of the arylsulphatase reaction changes into an activation.

Effect of cerebroside sulphate on the reaction parameters of the arylsulphatase reaction. From the double reciprocal plot of the progress curve of the arylsulphatase reaction parameters, such as the initial reaction velocity, V_o, and the maximum amount of product formation, U_{max}, can be calculated [12]. To investigate the effect of cerebroside sulphate on the hydrolysis of nitrocatechol sulphate by sulphatase A, the enzyme was (a) incubated with nitrocatechol

TABLE II

EFFECT OF LIPIDS ON CEREBROSIDE SULPHATASE AND ON ARYLSULPHATASE ACTIVITY OF SULPHATASE A

Conditions: Cerebroside sulphatase assay; 0.1 mM Cerebroside sulphate, 10 mM sodium formate (pH 5.3), 14.2 μg enzyme protein per ml, 20 min incubation, substrate and lipid were dispersed together. Arylsulphatase assay: 5 mM nitrocatechol sulphate, 10 mM sodium formate (pH 5.3), 1.25 μg per ml enzyme protein, 5 min.

Lipid	Concentration (mM)	% Activity	
		Cerebroside sulphatase reaction	Arylsulphatase reaction
—	—	100	100
Psychosine	0.1	0	124.7
Phosphatidylcholine	0.1	20.6	154.6
Sphingomyelin	0.1	28.9	143.0
Phosphatidylinositol	0.1	23.3	93.8
Cerebroside	0.1	45.2	136.4
Ceramide	0.1	72.8	147.2

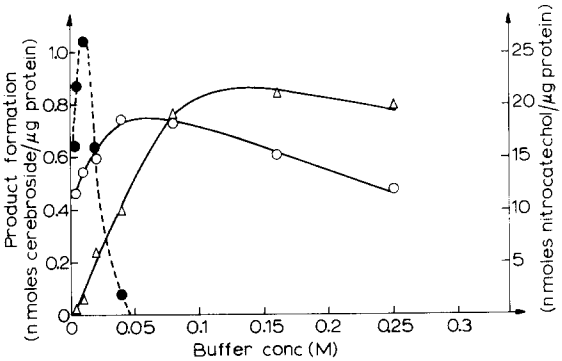


Fig. 6. Effect of buffer concentration on arylsulphatase and cerebroside sulphatase activity and on cerebroside sulphate inhibition of arylsulphatase reaction. Buffer: sodium formate, pH 5.3. Assay conditions: cerebroside sulphatase reaction; 0.1 mM cerebroside sulphate, 32.5 μg enzyme protein per ml, 5 min incubation period. Arylsulphatase reaction, 0.5 mM nitrocatechol sulphate, 3.125 μg enzyme protein per ml, 5 min incubation period. ○———○, arylsulphatase activity in the absence of cerebroside sulphate; △———△, arylsulphatase activity in the presence of 0.1 mM cerebroside sulphate; ●- - - - -●, cerebroside sulphatase activity.

sulphate alone, (b) incubated with cerebroside sulphate and nitrocatechol sulphate together, and (c) preincubated for 30 min with cerebroside sulphate alone, before the arylsulphatase reaction was started by the addition of nitrocatechol sulphate. This set of experiments was performed at two different nitrocatechol sulphate and sodium formate concentrations. The result shows that cerebroside sulphate inhibits the initial velocity of the arylsulphatase reaction, at low buffer concentration, by about 48%, whereas the maximum amount of product formation is not significantly affected (Table III, lines 2

TABLE III

EFFECT OF CEREBROSIDE SULPHATE AND OF NITROCATECHOL SULPHATE ON THE INITIAL VELOCITY AND ON THE MAXIMUM AMOUNT OF PRODUCT FORMATION IN THE ARYLSULPHATASE REACTION

Nitrocatechol sulphate, cerebroside sulphate and buffer concentration (mM) as indicated in the Table. Enzyme concentration: 1.25 μg per ml. Incubation pH: 5.3. Preinc.: 30 min preincubation in the presence of cerebroside sulphate and absence of nitrocatechol sulphate. Determination of initial velocity V_0 (nmole nitrocatechol per μg protein and min) and maximum amount of product formation U_{max} (nmole nitrocatechol per μg protein) as described earlier [12]. Further details see Experimental.

Concentration (mM)			Preincubation	U_{max}	V_0
Buffer	Nitrocatechol sulphate	Cerebroside sulphate			
10	0.5	0	no	27.7	3.9
10	0.5	0.1	no	26.4	2.0
10	0.5	0.1	yes	13.5	1.1
10	5	0	no	28	6.4
10	5	0.1	no	27.8	3.4
10	5	0.1	yes	12.4	2.0
100	0.5	0	no	74	7.5
100	0.5	0.1	no	76	9.1
100	0.5	0.1	yes	72	9.2

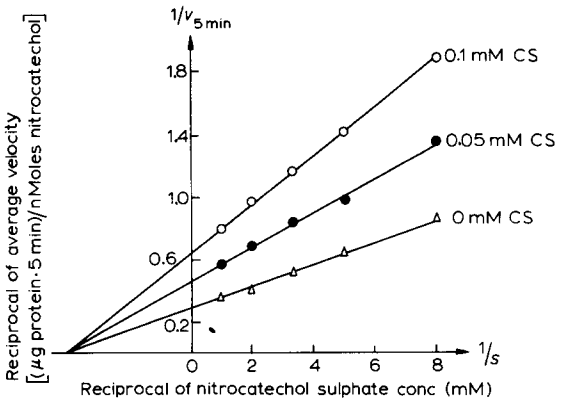


Fig. 7. Influence of cerebroside sulphate on the Lineweaver—Burk plot of the Arylsulphatase Reaction. Assay conditions: 10 mM sodium formate (pH 5.3); 3.125 μg enzyme protein per ml; incubation period, 5 min. Cerebroside sulphate (CS) concentration: ○———○, 0.1 mM; ●———●, 0.05 mM; △———△, 0 mM.

and 5 as compared to lines 1 and 4, respectively). After preincubation with cerebroside sulphate the initial velocity is again decreased, now by 40—45%, but this time the maximum amount of product formation is also significantly diminished (49—59%) (Table III, lines 3 and 6 as compared to lines 2 and 5, respectively). At 0.1 M sodium formate the presence of cerebroside sulphate only slightly altered the maximum amount of product formation of the arylsulphatase reaction, whereas the initial velocity was enhanced by 21%. Preincubation of the enzyme with cerebroside sulphate had no significant effect under these conditions.

Mode of inhibition of the arylsulphatase reaction by cerebroside sulphate. As shown in Fig. 7 cerebroside sulphate inhibits the arylsulphatase reaction noncompetitively at 0.01 M sodium formate concentration. Under the conditions chosen, the apparent Michaelis constant is 0.24 mM, the inhibitor constant for cerebroside sulphate 0.08 mM.

Discussion

Purified human sulphatase A has no absolute requirement for activators when acting on cerebroside sulphate. Provided the optimum conditions are chosen the cerebroside sulphatase activity of the enzyme is relatively high when compared to the activity of other human sulphatases A in the presence of activators [7—9]. This activity, however, requires incubation in buffers of low ionic strength (Figs 2, 5 and 6). At low buffer concentrations cerebroside sulphate, in addition, inhibits the arylsulphatase reaction of the enzyme. The cerebroside sulphatase activity and the inhibition of the arylsulphatase reaction by cerebroside sulphate decrease simultaneously when the buffer concentration is increased. These observations are taken as additional evidence for the identity of cerebroside sulphatase and arylsulphatase A. They, furthermore, confirm the view that the physiological activator of the cerebroside sulphatase activity of the sulphatase A [23], in contrast to earlier suggestions [6,17], is not a complementary fraction of the enzyme.

The inactivation to which the enzyme is subjected in its arylsulphatase reaction, and which leads to the characteristic non-linear progress curve [2], can also be seen in its cerebroside sulphatase reaction (Figs 1 and 5). The reaction velocity, as determined from the slopes of the progress curve at 0 and 30 min incubation time, decreased to about 33% of its initial value within 30 min of incubation. The substrate concentration had decreased to about 86% of the initial concentration during this time, and the SO_4^{2-} concentration had increased to 14 μM.

Comparison of these values with the data from Figs 3 and 4a indicates that neither substrate depletion, product inhibition, nor the sum of these effects can be the sole reason for the decrease in enzyme activity. This inactivation probably follows a mechanism similar to the one observed in the arylsulphatase reaction [12,18,19].

Further evidence for this interpretation of the progress curve of the cerebroside sulphatase reaction can be gained from a comparison of the initial velocity and the maximum amount of product formation of the arylsulphatase reaction in the absence of, in the presence of, and after preincubation with cerebroside sulphate (Table III). The loss of arylsulphatase activity after preincubation with cerebroside sulphate clearly shows that the enzyme is inactivated in its cerebroside sulphatase reaction, and the stability of the enzyme activity towards cerebroside sulphate under conditions in which this substrate cannot be degraded indicates a reaction dependent inactivation and not a denaturation of the enzyme protein due to the detergent character of the substrate [10].

The inactivation of the enzyme by cerebroside sulphate is much slower, however, than with nitrocatechol sulphate. After 30 min incubation under comparable conditions (0.01 M sodium formate, pH 5.3, a substrate conc. 1.7 times the apparent K_m value) 33% of the enzyme activity is left in the cerebroside sulphatase reaction (Fig. 1), compared to 3.7% in the arylsulphatase reaction. The corresponding initial velocities are 2.7 nmole of substrate hydrolysed per μg protein per min in the cerebroside sulphatase reaction and 6.4 nmole per μg per min in the arylsulphatase reaction.

The differences in the reaction and inactivation velocities as well as in other parameters of the arylsulphatase and the cerebroside sulphatase reaction may be attributed to the differences in the physicochemical properties of the respective substrates, but especially to the fact that cerebroside sulphates form micelles in aqueous solutions [10], in contrast to nitrocatechol sulphate which dissolves monomolecularly.

Thus the pH optimum of the cerebroside sulphatase reaction, as determined after an incubation period of 5 min, is significantly lower than that of the arylsulphatase reaction (Stinshoff, in preparation). In both cases, however, the optimum depends on the ionic strength of the incubation mixture, being higher, the lower the ionic strength (Fig. 2).

The cerebroside sulphatase activity of human sulphatase A is noncompetitively, the arylsulphatase activity competitively, inhibited by Na_2SO_4 (Fig. 4a; ref. 12). A similar difference was observed in the mode of inhibition SO_4^{2-} exerts on the detergent-activated cerebroside sulphatase reaction and the arylsulphatase reaction of bovine sulphatase A [10]. Jerfy and Roy have explained their findings by suggesting a uni-ter mechanism with the bile salt micelle being

the reaction participant released last from the enzyme molecule. If not the detergent but a substrate micelle is regarded as the participant which leaves the complex last, a similar explanation may account for the present observation.

Noncompetitive inhibition is also seen, when the influence of nitrocatechol sulphate on the cerebroside sulphatase activity and the effect of cerebroside sulphate on the arylsulphatase activity are investigated.

The difference in the physicochemical state of the two substrates may also account for the different influence the buffer concentration exerts on the arylsulphatase and on the cerebroside sulphatase reaction. As described earlier [12], increasing ionic strength inhibits both the initial reaction velocity and the inactivation velocity of the arylsulphatase reaction. In the cerebroside sulphatase reaction an exact calculation of reaction parameters such as the initial velocity is not possible. According to Fig. 5, however, increasing buffer concentrations also inhibit the initial velocity of this reaction, but no influence on the velocity of the inactivation of the enzyme can be observed. Fig. 6 shows that velocity of the cerebroside sulphate degradation is more strongly effected than that of the nitrocatechol sulphate degradation. The same observation was made by Lipovac et al. [20] who investigated the action of *Vibrio cholerae* sialidase on micellar ganglosides and monomolecular sialyllactose. It led to the conclusion that ionic interaction with the enzyme affects not so much the catalytic activity of the active site as the conformation of the enzyme, thus modifying the steric availability of this active site for large substrate micelles. The effect of ionic strength on the enzyme activities of the sulphatase A may be similarly explained.

The effect of lipid inhibitors on the activity of the sulphatase A also does not seem to be due to any modification of the active center. The inhibition of the cerebroside sulphatase reaction may be caused by the effect of the lipids on the conformation of the enzyme, or by their influence on the substrate micelles. Cerebroside sulphate is known to form mixed micelles with other amphiphilic lipids, the surface charge of which is dependent on the hydrophilic groups of both lipids [21]. Thus, the degree of inhibition of the cerebroside sulphatase reaction appears to be related to the ionic groups of the respective lipid, whereas the arylsulphatase reaction, in which a monomolecular substrate is hydrolysed, is enhanced by most of these lipids. Inhibition of this reaction is only observed with phosphatidyl inositol which carries a free phosphate group. Phosphate is known to be a strong inhibitor of the arylsulphatase reaction [22], whereas the lack of inhibition of the arylsulphatase reaction by phosphatidyl choline and sphingomyelin, which also carry phosphate groups, may be due to the fact that their phosphate groups are bound by zwitterion formation.

Another possibility of interpreting the complex inhibition pattern of the arylsulphatase and the cerebroside sulphatase reaction of sulphatase A is to assume the existence of two different active sites for the two activities. With regard, however, to the similarity of (a) the two hydrolytic reactions, (b) the dependence of the cerebroside sulphatase activity and the arylsulphatase inhibition by cerebroside sulphate on the ionic strength, and (c) the characteristic deviations of the progress curves of the two reactions from linearity, this assumption seems highly improbable.

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