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CEREBROSIDE 3-SULFATE AS A PHYSIOLOGICAL SUBSTRATE OF ARYLSULFATASE A

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SUMMARY

1. A method was developed for isolating a pure enzyme with cerebroside-sulfatase activity from the pig kidney. It was found that arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) was confined to the same isolated enzyme preparation. As disc electrophoresis revealed, this enzyme is strikingly different from the arylsulfatase A from ox liver which was isolated in ROY's laboratory by an essentially different procedure. Nevertheless, ROY's enzyme also exhibited, like ours, a slight degree of cerebroside sulfatase activity which in both preparations was strongly enhanced by addition of the same heat-stable 'complementary fraction' obtained from pig kidney. Analytical disc electrophoresis under various conditions indicated purity of the two arylsulfatases A. The quotient of the sulfatase activities with aryl and cerebroside sulfate as substrate remained constant after further disc-electrophoresis separation.

2. As sulfatase activity with ^{35}S-labelled synthetic galactose 6-sulfate and cerebroside 6-sulfate could not be detected, the enzyme is highly specific for its naturally occurring substrate, cerebroside 3-sulfate (K_m = 0.105 mM) (see ref. 5).

3. Galactose 3-sulfate, galactose 6-sulfate and cerebroside 6-sulfate were found to inhibit the activity with cerebroside 3-sulfate as substrate as well as with arylsulfate (2-hydroxy-5-nitrophenyl sulfate).

4. Cerebroside 3-sulfate was found to inhibit sulfatase activity with 2-hydroxy-5-nitrophenyl sulfate as substrate and *vice versa*; a result which indicates a common enzyme for both substrates.

5. Arylsulfatase B is only slightly active towards cerebroside sulfate, provided the heat-stable complementary fraction is added.

Thus it is very evident that cerebroside 3-sulfate is a naturally occurring substrate of arylsulfatase A.

INTRODUCTION

The arylsulfatases which liberate sulfate from synthetic phenyl sulfuric acids and derivatives thereof, have been found[1] in bacteria, fungi, molluscs, insects, birds and mammals. The widespread distribution of these enzymes in nature suggests that

they have important physiological functions, although the natural substrates of the arylsulfatases are for the most part uncertain. The assumption that cerebroside sulfates would be natural substrates of arylsulfatase A was initially based on the observation of an arylsulfatase deficiency[2] in metachromatic leukodystrophy. This hereditary disease is characterized by an accumulation of cerebroside sulfates (*cf.* ref. 3) and a deficiency of cerebroside-sulfatase activity[4] and arylsulfatase A. Assuming only a single gene defect, it could be supposed that both cerebroside and aryl sulfate would be substrates of the mammalian arylsulfatase A. The results of these experiments obviously confirm this assumption.

MATERIALS AND METHODS

Preparation of ^{35}S-labelled substrates

[^{35}S]Cerebroside 3-sulfate of the cerasine type was isolated[5] from the brain of 4-week-old rabbits after intracerebral injection of [^{35}S]sulfate. The specific activity of the isolated cerebroside 3-sulfate was $5.0 \cdot 10^6$ counts/min per mg. [^{35}S]Cerebroside 6-sulfate, D-[^{35}S]galactose 3-sulfate and D-[^{35}S]galactose 6-sulfate were synthesized as described elsewhere[6].

Enzyme preparations

The arylsulfatase A and the heat-stable 'complementary fraction' required for maximal cerebroside-sulfatase activity were isolated from pig kidney. A 6000-fold purified preparation was finally obtained[5] after carrier-free electrophoresis in 25 mM ammonium acetate (pH 5.1). This preparation was used for the experiments described under the results.

For final isolation of arylsulfatase A, the standard disc electrophoresis on polyacrylamide according to the method of DAVIS[7] was adapted to the preparative scale as follows. The sample gel had a volume of 1 ml and contained 1.7 mg of purified arylsulfatase A preparation. The running gel was prepared to a height of 10 cm and the spacer gel to a height of 2.5 cm in a glass tube of 1.7 cm inside diameter. Usually a current of 15 mA was applied for 5 h at 4°. The gel column was then sectioned into individual blocks 2 mm thick. From each block a small piece was removed for arylsulfatase assay. Arylsulfatase A migrated about half as rapidly as the bromophenol blue marker. The gel block containing the arylsulfatase was extracted by electrophoresis under the same conditions as those used for separation.

Arylsulfatase A from ox liver was isolated in ROY's laboratory[8].

Assay of enzyme activity

Isomers of [^{35}S]cerebroside sulfate as substrate. The reaction mixture[5] contained 100 munits of arylsulfatase A, 80 μg of complementary fraction, and 10 mμmoles of cerebroside sulfate dissolved in 1 ml of a 0.2 M sodium acetate buffer solution (pH 4.5). The samples were incubated for 15 to 30 min at 37°. The liberated sulfate was precipitated as benzidine sulfate after removal of undegraded cerebroside sulfate[5].

Isomers of D-[^{35}S]galactose sulfate as substrate. The reaction mixture contained 100 munits of arylsulfatase A, 80 μg of complementary fraction and 0.2 μmole of D-galactose sulfate dissolved in 0.2 ml of a 0.1 M sodium acetate buffer solution (pH 4.5). After incubation for 3 h at 37°, the samples were subjected to thin-layer chro-

matography on MN-silica gel-N (Macherey, Nagel and Co., Düren, Germany) with 0.2 M sodium acetate (pH 4.5)–ethanol (1:4, v/v) as solvent. Galactose sulfate (R_F 0.85) and free sulfate (R_F 0.08) were localized by means of a thin-layer scanner. The activity of the corresponding silica gel samples was counted in a Packard scintillation counter.

p-Nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) as substrate. The samples contained 20 munits of arylsulfatase A, 4 μmoles of *p*-nitrocatechol sulfate dissolved in 1 ml of 0.2 M sodium acetate (pH 4.5). After incubation for 15 min at 37°, 4 ml of 1 M NaOH were added. The absorbance was read at 515 mμ.

The protein content was determined by the method of LOWRY *et al.*[9].

RESULTS

Enhancement of cerebroside-sulfatase activity of arylsulfatase A by the complementary fraction from pig kidney

The enzyme was extracted from a lysosome-containing mitochondrial fraction of pig kidney in the presence of butanol at pH 5.1, and further purified by precipitating impurities with $CaCl_2$ and precipitating the enzyme with acetone, with subsequent gel filtration on Sephadex[5]. After preparative carrier-free electrophoresis at pH 5.1, two main fractions of arylsulfatase activity were obtained, as shown in Fig. 1. One of these fractions was identified as arylsulfatase A by its activity with *p*-nitrocatechol sulfate as the substrate in an incubation mixture containing pyrophosphate and NaCl (ref. 10). The other fraction was identified as arylsulfatase B upon assay in the presence of barium acetate[10].

The distribution of the cerebroside-sulfatase activity after carrier-free electrophoresis parallels the distribution of arylsulfatase A to some extent, although not exactly, as shown in Fig. 1. This could be explained by the incomplete separation of arylsulfatase A from the neighbouring so-called complementary fraction. As shown in

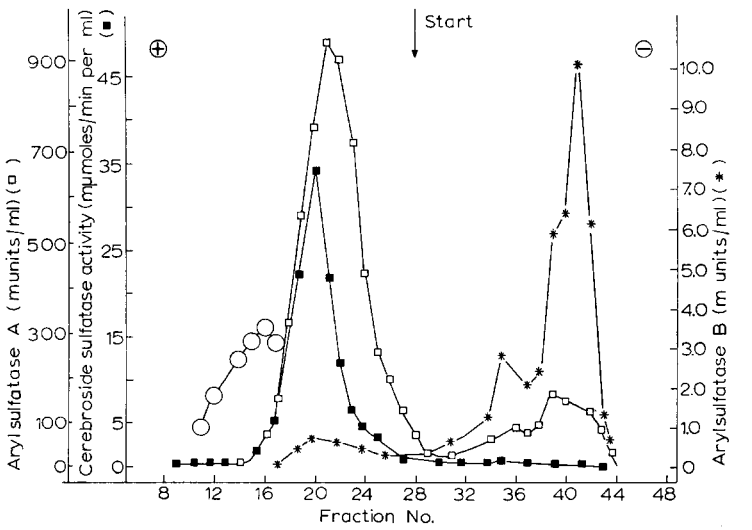

Fig. 1. Sulfatase activities of pig kidney after the fractionation by preparative carrier-free electrophoresis at pH 5.1. □—□, arylsulfatase A; ■—■, cerebroside sulfatase activity; ○, complementary fraction; *—*, arylsulfatase B.

TABLE I

EFFECT OF THE COMPLEMENTARY FRACTION ON RELEASE OF SULFATE FROM CEREBROSIDE 3-SULFATES BY ARYLSULFATASES A AND B FROM PIG KIDNEY

Temperature, 37° and pH 4.5. The details for assay of enzyme activity (Tables I–VI) are described under MATERIALS AND METHODS.

Preparation (units)	*Addition of complementary fraction (μg/ml)*	*Sulfate released from*	
		Cerebroside sulfate (counts/min)	*p-Nitrocatechol sulfate (μmoles)*
Arylsulfatase A (0.68)	—	1 460	10.2
	80	10 700	11.4
Arylsulfatase B (1.53)	—	<10	23.0
	80	420	
	80	90*	
Complementary fraction	80	<10	0.0

* Incubation at pH 6.1 (pH optimum for arylsulfatase B).

Table I, this complementary fraction was free of residual cerebroside-sulfatase and arylsulfatase activity after heating in a boiling-water bath for 20 min. The addition of this complementary fraction to the arylsulfatase fraction increased the cerebroside sulfate hydrolysis from 1460 counts/min to 10 700 counts/min. The *p*-nitrocatechol sulfate hydrolysis, on the contrary, was barely affected by the addition of the complementary fraction.

Arylsulfatase B exhibits no detectable cerebroside-sulfatase activity. After the addition of the complementary fraction, the rate of sulfate release from cerebroside sulfate obtained with arylsulfatase B at pH 6.1 is about 1% of that obtained with arylsulfatase A at pH 4.5. Apparently the cerebroside-sulfatase activity is not a non-specific side effect of sulfatases in general.

The inseparability of cerebroside-sulfatase activity from arylsulfatase A isolated from pig kidney and ox liver

Analytical disc electrophoresis[7] revealed that the arylsulfatase A preparation from the pig kidney contained at least 4 protein components even after completion of the purification step by carrier-free electrophoresis. Therefore, further purification was accomplished by preparative disc electrophoresis on polyacrylamide gel. Regardless of whether the enzyme activity was tested before or after this effective purification step, 100 munits of arylsulfatase A hydrolyzed a constant quantity of cerebroside sulfate per unit time, as will be seen from Table II. The same results were obtained when the pH of the running gel was changed from pH 8.9 to 7.5. The above shows that the cerebroside-sulfatase activity remained confined to the arylsulfatase A.

In order to check whether this arylsulfatase A was obtained in a pure state, analytical disc electrophoresis[7] of 20 μg of enzyme protein was performed under different conditions, with the result that only one zone was stained by amido black. After microdensitometric tracing of this stained protein zone, a fairly symmetrical

TABLE II

INSEPARABILITY AS DETERMINED BY PREPARATIVE DISC-ELECTROPHORESIS OF CEREBROSIDE-SULFATASE ACTIVITY FROM ISOLATED ARYLSULFATASE A

Samples of 100 munits arylsulfatase A from the pig kidney were incubated in the presence of 1 mM barbituric acid and 80 μg heat-inactivated complementary fraction.

Preparative disc-electrophoresis	*pH of running gel*	*Arylsulfatase A (munits)*	*Sulfate released from cerebroside sulfate (mμmoles/30 min) at 37° and pH 4.5*
Not performed	—	100	12.1
In Tris–barbituric acid[11]	7.5	100	12.5
In Tris–glycine[7]	8.9	100	11.7

distribution of density was obtained (Fig. 2), without indicating appreciable amounts of a contaminating protein.

The existence of a species difference in arylsulfatase A made it possible to establish further that arylsulfatase A activity and the cerebroside-sulfatase activity reside in the same enzyme. The arylsulfatase from the ox and arylsulfatase A from the pig migrate at different rates when studied by disc electrophoresis according to the method of DAVIS[7]. Using bromophenol blue as a reference, a relative mobility of 0.5 was found with arylsulfatase from liver and kidney of the pig and a relative mobility of 0.75 was found with arylsulfatase from the liver and kidney of the ox. These different relative mobilities were also observed when crude preparations were compared such as the arylsulfatase of a butanol extract[5] prepared from a lysosome-containing fraction of liver and the kidney of both the ox and the pig. The arylsulfatase zone became visible

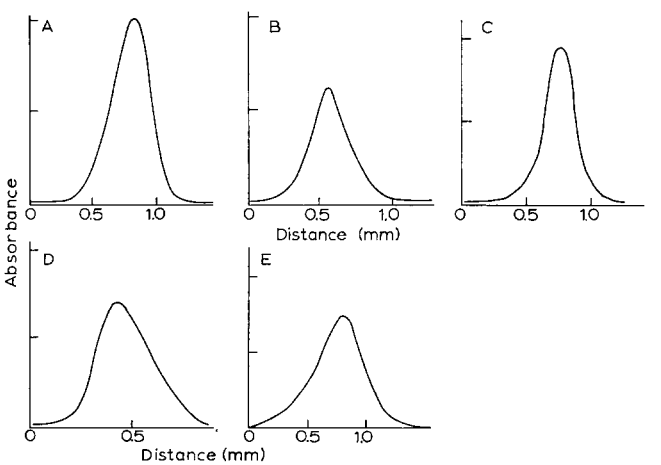

Fig. 2. Density curves of isolated arylsulfatase A after analytical disc electrophoresis. The density curves were obtained with 20 μg of pure arylsulfatase A isolated from pig kidney (A, B, C) and ox liver (D, E). After electrophoresis in a 7% polyacrylamide gel, the samples were stained with amido black. The running gels were prepared under the following conditions: A, pH 8.9 (ref. 7); B, pH 7.5 (ref. 11); C, phenol–acetic acid–water (2:1:1, w/v/v) according to WORK[12]; D, pH 8.9; E, pH 8.9 with 6 M urea (ref. 7).

TABLE III

INSEPARABILITY OF CEREBROSIDE-SULFATASE ACTIVITY FROM ISOLATED ARYLSULFATASE

Cerebroside-sulfatase activity of 100 munits arylsulfatase A was assayed as described under MATERIALS AND METHODS.

Source of enzyme	*Arylsulfatase A (munits)*	*Addition of complementary fraction (μg/ml)*	*Degradation of cerebroside sulfate (mμmoles/30 min) at 37° and pH 4.5*
Pig kidney	100	80	12.1
Ox liver	100	80	8.73
	100	—	1.44
Limpet*	100	80	<0.03

* Partially purified enzyme (Sigma, St. Louis, Mo.).

as a purple band after incubation for 10 min in a medium containing *p*-nitrocatechol sulfate and after final transfer of the gel column into 1 M NaOH.

It should be noted that the arylsulfatase A from the ox and that from pig were isolated by essentially different isolation procedures in different laboratories (*cf.* MATERIALS AND METHODS). Nevertheless, both isolated arylsulfatase A enzymes hydrolyze cerebroside sulfate at comparable rates (Table III). 100 munits of ox liver arylsulfatase A correspond to about 2 μg of protein. In both cases the heat-treated complementary fraction obtained from pig extracts enhances the cerebroside-sulfatase activity of arylsulfatase A. It is highly improbable that these results would be obtained unless the arylsulfatase A and the cerebroside-sulfatase activity resided in the same enzyme protein.

The cerebroside-sulfatase activity does not seem to be a characteristic property of arylsulfatase A of all species, since a partially purified preparation from the limpet was devoid of cerebroside-sulfatase activity even in the presence of the complementary fraction.

Common inhibitors of arylsulfatase A activity with 2-hydroxy-5-nitrophenyl sulfate and cerebroside 3-sulfate as substrate

The naturally occurring cerebroside sulfates are cerebroside 3-sulfates with the sulfate ester group positioned at the third carbon atom of the D-galactose group[6,13,14]. Therefore one would expect a high cerebroside-sulfatase activity with the natural substrate cerebroside 3-sulfate and a low activity with synthetic cerebroside 6-sulfate. It is evident from Table IV that the activity with cerebroside 3-sulfate as substrate is at least 200 times higher than that obtained with cerebroside 6-sulfate. This high specificity of enzyme activity with respect to cerebroside 3-sulfate and cerebroside 6-sulfate apparently is not ascribed to the presence of traces of impurities in the cerebroside 6-sulfate preparation, since a synthetically obtained substance of the same R_F value (thin-layer chromatography) as the natural cerebroside 3-sulfate[6] was attacked at a substantially similar rate as the cerebroside 3-sulfate isolated from rabbit brain. These two preparations of synthetic cerebroside 3- and 6-sulfates were obtained from the same reaction mixture after chromatographic separation.

In conformity with the results obtained with the cerebroside sulfates, a rather

TABLE IV

ACTIVITY OF ARYLSULFATASE A WITH CEREBROSIDE 3-SULFATE, CEREBROSIDE 6-SULFATE AND THE CORRESPONDING DERIVATIVES OF D-GALACTOSE AS SUBSTRATES

100 munits of arylsulfatase A (including the complementary fraction) were used for each incubation.

Type of substrate	*Concn. of substrate (μM)*	*Sulfate released (mμmoles/h) at 37° and pH 4.5*
Cerasine 3-sulfate	10	35.5 319.0*
Phrenosine 3-sulfate (synthetic)	10	22.4 201.6*
Galactose 3-sulfate	500	4.6
Phrenosine 6-sulfate (synthetic)	10	<0.1
Galactose 6-sulfate	500	<0.2

* Calculated for substrate saturation.

TABLE V

INHIBITION OF CEREBROSIDE-3-SULFATASE ACTIVITY BY ADDITION OF CEREBROSIDE 6-SULFATE AND THE CORRESPONDING ISOMERS OF D-GALACTOSE

100 munits of arylsulfatase A were assayed in the presence of 0.05 mM cerebroside 3-sulfate.

Added sulfate ester type	*Concn. (mM)*	*% Inhibition of the control activity*
p-Nitrocatechol sulfate	55	100
Free sulfate + *p*-nitrocatechol*	19	77
Cerebroside 6-sulfate	0.25	74.8
D-Galactose 3-sulfate	2.5	22.1
D-Galactose 6-sulfate	2.5	17.0

* In same amounts as liberated during the incubation with *p*-nitrocatechol sulfate (preceding line).

TABLE VI

INHIBITION OF ARYLSULFATASE A ACTIVITY WITH *p*-NITROCATECHOL SULFATE AS SUBSTRATE BY ISOMERS OF CEREBROSIDE SULFATE AND GALACTOSE SULFATE

Enzyme activities were assayed in a 0.4 mM solution of *p*-nitrocatechol sulfate at pH 4.5.

Added sulfate ester type	*Concn. (mM)*	*% Inhibition of the control activity*
Cerebroside 3-sulfate	0.1	46.5
Cerebroside 6-sulfate	0.25	39.5
D-Galactose 3-sulfate	2.5	33.3
D-Galactose 6-sulfate	2.5	17.0

slight activity was obtained with free galactose 3-sulfate as substrate, whereas no activity was detectable with galactose 6-sulfate.

The sulfate liberation from cerebroside sulfate is reduced in the presence of *p*-nitrocatechol sulfate (Table V), and the sulfate liberation from *p*-nitrocatechol sulfate is reduced in the presence of cerebroside sulfate (Table VI). With reference to Table V, it should be mentioned that the activity of arylsulfatase A with cerebroside sulfate as substrate is inhibited by free sulfate. Therefore a 55 mM solution of *p*-nitrocatechol sulfate was employed to keep the *p*-nitrocatechol sulfate concentration high in comparison to the sulfate released. In addition, a control assay was performed in which the *p*-nitrocatechol sulfate was replaced by the amount of sulfate released in the preceding experiment.

A further indication that the enzyme activities with cerebroside sulfate and *p*-nitrocatechol sulfate as substrate are due to the same enzyme is that the liberation of sulfate from *p*-nitrocatechol sulfate as well as cerebroside 3-sulfate was inhibited by cerebroside 6-sulfate, D-galactose 3-sulfate and D-galactose 6-sulfate (Tables V and VI).

DISCUSSION

The study of the substrate specificity of arylsulfatase A was complicated by two facts: The cerebroside-sulfatase activity is low compared to the activity with *p*-nitrocatechol sulfate as the substrate. The arylsulfatase A released sulfate from *p*-nitrocatechol sulfate about 30 times faster than from the cerebroside sulfate, when a fraction, obtained after the carrier-free electrophoresis step and containing the complementary fraction, was assayed. However, when this fraction was dialyzed and concentrated by ultrafiltration, the cerebroside-sulfatase activity fell to 10% of its original activity, contrary to the unaffected activity with *p*-nitrocatechol sulfate. Therefore, it was more difficult to exclude the possibility that the cerebroside-sulfatase activity resulted from a trace of impurity from the arylsulfatase A preparation. Moreover, the pronounced effect of the complementary fraction on cerebroside-sulfatase activity, while leaving the activity with *p*-nitrocatechol sulfate substantially unaffected, excluded the possibility of evaluating a constant activity quotient throughout the purification procedure.

Since cerebroside 3-sulfate is a substrate of arylsulfatase A in contrast to cerebroside 6-sulfate, galactose 6-sulfate, chondroitin sulfate and steroid sulfate[5], this high specificity largely precludes the possibility that the cerebroside-3-sulfatase activity is an immaterial side effect of arylsulfatase A. In addition, the only known substance which accumulates in patients suffering from metachromatic leukodystrophy is cerebroside sulfate (*cf.* ref. 3) as a consequence of the hereditary lack of arylsulfatase A (ref. 2) and its cerebroside-sulfatase activity[4].

Moreover, the cerebroside-sulfatase activity is inhibited[5] by sulfate, sulfite, phosphate, pyrophosphate and fluoride but not by cyanide, in these respects resembling arylsulfatase A[1].

The chemical nature of the 'activating factor' in the complementary fraction as well as its mechanism of action still remain to be elucidated.

Since the arylsulfatase A of the limpet is devoid of cerebroside-sulfatase activity, cerebroside 3-sulfate does not seem to be the only physiological substrate of all arylsulfatases A. However, it has been shown by the experiments reported above that

at least in mammalian tissues arylsulfatase A is the basic constituent of an enzyme which attacks the physiologically important cerebroside sulfates.

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REFERENCES

1 K. S. DODGSON AND B. SPENCER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. IV, Interscience, New York, 1957, p. 211.
2 J. AUSTIN, D. ARMSTRONG AND L. SHEARER, *Arch. Neurol.*, 13 (1965) 593.
3 H. JATZKEWITZ, *Z. Physiol. Chem.*, 318 (1960) 265.
4 E. MEHL AND H. JATZKEWITZ, *Biochem. Biophys. Res. Commun.*, 19 (1965) 407.
5 E. MEHL AND H. JATZKEWITZ, *Z. Physiol. Chem.*, 339 (1964) 260.
6 H. JATZKEWITZ AND G. NOWOCZEK, *Chem. Ber.*, 100 (1967) 1667.
7 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
8 L. NICHOL AND A. ROY, *J. Biochem.*, 55 (1964) 643.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 H. BAUM, K. DODGSON AND B. SPENCER, *Clin. Chim. Acta*, 4 (1959) 453.
11 D. E. WILLIAMS AND R. A. REISFELD, *Ann. N.Y. Acad. Sci.*, 121 (1964) 373.
12 T. S. WORK, *J. Mol. Biol.*, 10 (1964) 544.
13 T. N. YAMAKAWA, N. KISO, S. HANDA, A. MAKITA AND S. YOKOYAMA, *J. Biochem.*, 52 (1962) 226.
14 P. STOFFYN AND A. STOFFYN, *Biochim. Biophys. Acta*, 70 (1963) 218.