BBA 66555

CEREBROSIDE SULFATASE DETERMINATION IN CULTURED HUMAN FIBROBLASTS

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(Received November 8th, 1971)

SUMMARY

A procedure for the determination of cerebroside sulfatase activity in extracts of cultured human fibroblasts is described. Cerebroside [^{35}S]sulfate prepared from developing rat brain serves as substrate and enzyme activity is estimated by measurement of released inorganic [^{35}S]sulfate. The reaction has a sharp optimum at pH 4.5, shows an absolute requirement for bile salts, and is stimulated by Mn^{2+} and Cl^-. The K_m for cerebroside sulfate is 0.1–0.2 mM. Inorganic sulfate and 4-nitrocatechol sulfate are inhibitory.

INTRODUCTION

The metachromatic leukodystrophies are characterized by accumulation of cerebroside sulfates (sulfatides) in the nervous system and visceral organs[1,2] and by excessive excretion of these lipids in the urine[3]. While a marked deficiency of the lysosomal enzyme arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) had been observed in tissues of patients afflicted with this group of inherited metabolic disorders[4,5], it was not until MEHL AND JATZKEWITZ[6,7] demonstrated that purified pig kidney arylsulfatase A did indeed possess cerebroside sulfatase activity that an enzyme–physiological substrate relation was firmly established. Their assay system required the addition of a heat stable factor from pig kidney for optimal cerebroside sulfatase activity but not for hydrolysis of 4-nitrocatechol sulfate. They observed that normal human tissue was capable of hydrolyzing both sulfatide and 4-nitrocatechol sulfate but tissue from patients with metachromatic leukodystrophies lacked both activities[8,9].

With few exceptions[8–10], all reported estimations of arylsulfatase A activity associated with metachromatic leukodystrophy have been performed with the synthetic substrate 4-nitrocatechol sulfate. One of the unsatisfactory features of this assay is the presence in crude extracts of normal or metachromatic leukodystrophy tissue of other arylsulfatases capable of hydrolyzing 4-nitrocatechol sulfate. Salts

are included in assays for arylsulfatase A to exclude interference by other enzymes, however, the procedure may not be completely selective[11]. Tissues from patients with all forms of metachromatic leukodystrophies have small amounts of arylsulfatase A activity and it has not been possible to determine with certainty if this is due to residual activity of the A type enzyme or whether it is due to incomplete inhibition of other arylsulfatases. Moreover, anomalous kinetics with 4-nitrocatechol sulfate even with purified preparations of arylsulfatase A leads to further equivocation in the interpretation of data[12,13]. Since it has been claimed that the A type enzyme and cerebroside sulfatase are identical[7], a procedure for the determination of the latter in cultured human fibroblast extracts was developed to complement observations with the synthetic substrate. Some findings from the application of this procedure have been reported[14].

MATERIALS AND METHODS

The cerebroside [^{35}S]sulfate was prepared from rat brain by a procedure which will be described in detail elsewhere. Briefly 10- to 14-day-old rats were injected intracerabrally with carrier-free $H_2{}^{35}SO_4$ in saline. After 3 days the animals were sacrificed and the brains homogenized in chloroform–methanol (2:1, v/v). After filtration and mild saponification, the homogenate was partitioned according to the procedure of FOLCH *et al.*[15] The [^{35}S]sulfatide in the lower phase was purified by chromatography on TEAE-cellulose and the potassium acetate used to elute the sulfatide was removed by dialysis against water. The labeled material was taken to dryness under a stream of N_2, redissolved in moist chloroform–methanol (2:1), and stored at $-20°$. Fur use in enzyme assays the [^{35}S]sulfatide was diluted with bovine sulfatide (Supelco, Inc., Bellafonte, Pa.) to achieve a specific activity of approx. 10^6 counts/min per μmole. Sulfatide concentration was extimated colorimetrically according to the procedure described by KEAN[16].

Some preparations of sulfatide when examined by two dimensional thin-layer chromatography showed trace contaminants of other lipid materials. However, all preparations contained significant radioactivity only in zones corresponding to sulfatides. When assayed enzymatically, all preparations were quite similar to rabbit brain sulfatide prepared by a more extended procedure[17]. The latter was essentially pure as determined by two dimensional thin layer chromatography.

Fibroblasts extracts

Human fibroblast cultures were initiated from skin biopsies and maintained as described previously[18]. Cells were harvested by trypsinization, suspended in water, and lysed by 6 cycles of freezing and thawing. The supernatant fraction from a 1-min centrifugation in a Spinco microfuge (approx. 14 000 $\times$ *g*) was dialyzed overnight against at least 100 vol. of 1 mM EDTA in 0.1 M sodium acetate, pH 6.8, followed by a 4-h dialysis against the acetate solution only. The buffering capacity of acetate at this pH is limited, but it does prevent the pH from dropping. Buffers which would normally be employed at this pH such as phosphate or amine hydrochlorides would introduce ions which affect the activity of the enzyme[7]. Protein in the dialyzed extracts was estimated by the method of LOWRY *et al.*[19]. The bulk of enzyme activity was recovered in the supernatant fraction after centrigugation. It was stable for

several months when stored at −20° and retained its activity after repeated freezing and thawing.

Assay of cerebroside sulfatase activity

Assays for cerebroside sulfatase were carried out in 15-ml glass centrifuge tubes with ground glass stoppers. Complete assay mixtures contained the following in a total volume of 0.2 ml: 70 nmoles [^{35}S]sulfatide (10^3 counts/min per nmole); 20 μmoles sodium acetate buffer, pH 4.5; 4 μmoles $MnCl_2$; 250 μg sodium taurodeoxycholate; and 10 to 50 μl of dialyzed fibroblast extract (50 to 200 μg protein). The mixtures were incubated at 37° for 2 h and stopped by addition of 3.5 ml chloroform–methanol (2:1, v/v). Upon the addition of 0.73 ml of 0.12 M NaCl containing 0.4 mM carrier Na_2SO_4 two phases were formed. The lower phase after centrifugation contained the bulk of the unreacted [^{35}S]sulfatide while the upper phase (approx. 2 ml) contained all of the reaction product, inorganic [^{35}S]sulfate, and traces of the labeled sulfatide. An aliquot of the upper phase was then washed with an equal volume of "pure solvent lower phase"[15] which removed the remaining sulfatide. The extent of sulfatide hydrolysis was estimated by determining the radioactivity of a 0.2-ml aliquot of the washed upper phase with a toluene–methylcellosolve scintillant[20] in a liquid scintillation spectrometer. Zero time blanks consisting of the complete reaction mixture including enzyme, were partitioned immediately. The washed upper phase from the blanks always had a small amount of radioactivity but it represented only about 1% of the total present in the assay. Enzyme activities are expressed as nmoles inorganic [^{35}S]sulfate released per h per mg protein.

The procedure described is the final procedure which evolved in the present study. Some of the experiments described below were not performed under these precise conditions and any variations are noted in the legends to the figures and tables. It should also be noted that the specific activities of cerebroside sulfatase in fibroblast extracts determined by the present procedure are consistently higher than those reported earlier[14].

RESULTS AND DISCUSSION

Requirements for cerebroside sulfatase activity

The extent of sulfatide hydrolysis in the complete assay mixture and with each of the components omitted is shown in Table I. There was no activity in the absence of

TABLE I

REQUIREMENTS FOR CEREBROSIDE SULFATASE ACTIVITY

Enzymatic assay was as described under MATERIALS AND METHODS.

Incubation mixture	*Enzyme activity (nmoles/h per mg protein)*
Complete	73.3
Minus fibroblast extract	0
Minus sodium taurodeoxycholate	0
Minus $MnCl_2$	6.6
Minus $MnCl_2$, *plus* 8 μmoles NaCl	20.8

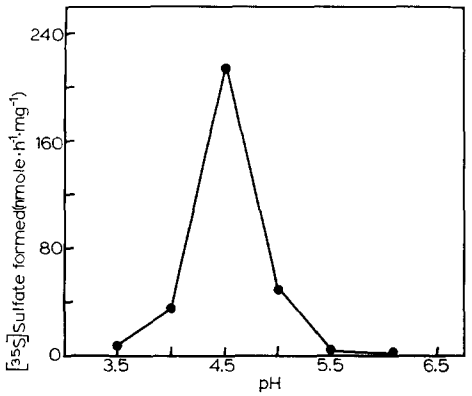


Fig. 1. Effect of pH on cerebroside sulfatase activity. Enzymatic assay was as described under MATERIALS AND METHODS except that reaction mixtures contained 54 nmoles [^{35}S]sulfatide and the sodium acetate buffers were adjusted to the indicated pH.

enzyme or taurodeoxycholate. There was reduced activity without $MnCl_2$ with a partial restoration when NaCl replaced the divalent salt.

pH optimum

A sharp optimum at pH 4.5 was observed in 0.1 M sodium acetate buffer (Fig. 1). When the acetate concentration was increased to 0.4 M, the pH optimum was unchanged but there was a 90% inhibition of the enzyme activity. No increase in activity was observed at acetate concentrations below 0.1 M. Citrate at 0.14 M completely inhibited sulfatide hydrolysis.

Dialysis and proportionality

Dialysis of fibroblast extracts against 0.1 M sodium acetate, pH 6.8, enhanced the sulfatidase activity. Moreover, the proportionality between the rate of hydrolysis and amount of extract was improved (Fig. 2). Some precipitation of protein occurred during dialysis, but the cerebroside sulfatase activity remained soluble. In contrast

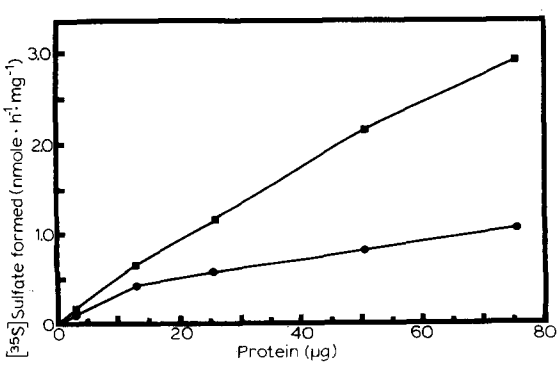


Fig. 2. Effect of dialysis of fibroblast extracts and protein concentration on cerebroside sulfatase activity. Enzymatic assay was as described under MATERIALS AND METHODS except that there were 15.4 nmoles [^{35}S]sulfatide, 8 μmoles NaCl instead of $MnCl_2$, and fibroblast extracts were treated as indicated. ●—●, undialyzed fibroblast extract; ■—■, fibroblast extract dialyzed against 0.1 M sodium acetate, pH 6.85.

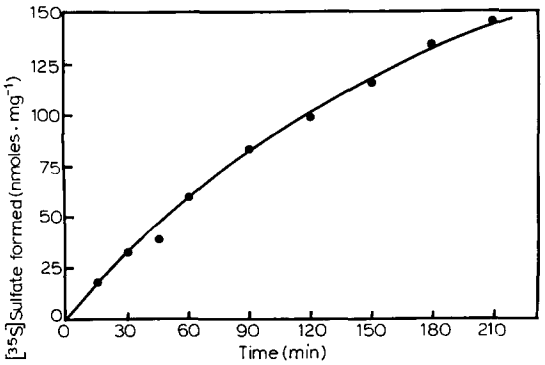


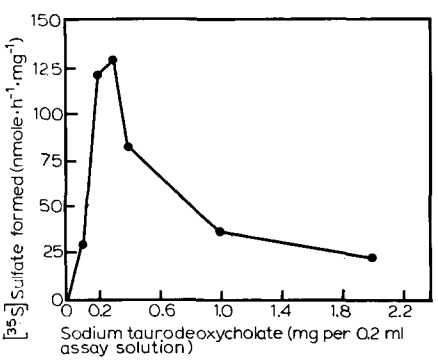
Fig. 3. Time course of cerebroside sulfatase reaction. Enzymatic assay was as described under MATERIALS AND METHODS except that 8 μmoles NaCl was used instead of $MnCl_2$.

when the extracts were dialyzed against water, 75% of the enzyme activity was recovered in the precipitate. Addition of 1 mM EDTA to the dialysis medium enhanced the activity of some cell-free preparations as much as two-fold while other preparations were unaffected.

The time course of the reaction is depicted in Fig. 3. No linearity, even over short time intervals, was observed. The 2-h incubation period was arbitrarily selected for convenience.

Bile salts

In the absence of bile salts no enzyme activity was observed. Detergents including cetyltrimethylammonium bromide, cutscum, Triton X-100, Triton WR-1339, Tween 40, Tween 80, and Brij-35 tested at 250 μg per reaction were inactive. At this concentration, taurodeoxycholate* was the most active, while the two other



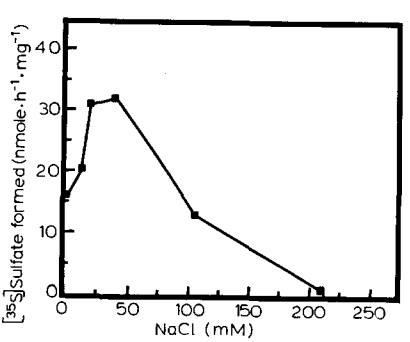


Fig. 4. Effect of sodium taurodeoxycholate on cerebroside sulfatase activity. Enzymatic assay was as described under MATERIALS AND METHODS except that the amount of sodium taurodeoxycholate was varied.

Fig. 5. Effect of Cl^- on cerebroside sulfatase activity. Enzymatic assay was as described under MATERIALS AND METHODS except that $MnCl_2$ was omitted.

* During the early phase of this work we learned that Dr. Howard Sloan and his collaborators at N.I.H. had demonstrated cerebroside sulfatase activity in cultured fibroblast extracts. Dr. Sloan kindly informed us that taurodeoxycholate was essential for enzyme activity. Dr. Sloan has reported some findings with his assay system[10], but at this writing no detailed report of this procedure has appeared.

bile salts tested, cholate and deoxycholate, were about one-half and one-tenth as active, respectively. The concentration of sodium taurodeoxycholate required for maximal activity was rather sharp (Fig. 4) and was independent of sulfatide concentration from 0.02 to 0.4 mM, although it was quite effective in increasing the solubility of sulfatide. Cholate was more effective than taurodeoxycholate but only when its concentration was increased 5- to 10-fold greater than the latter.

Effect of Cl^-

Chloride ion has been reported to be stimulatory for 4-nitrocatechol sulfatase activity[21]. A similar stimulatory effect on cerebroside sulfatase activity was observed (Fig. 5). At high concentrations, enzyme activity was completely inhibited. At 40 mM, the optimum concentration for NaCl, KCl and LiCl stimulated the reaction to about 90 and 70%, respectively, of that by NaCl.

TABLE II

EFFECT OF METAL SALTS ON CEREBROSIDE SULFATASE ACTIVITY

Enzymatic assay was as described under MATERIALS AND METHODS except that $MnCl_2$ was replaced as indicated. The concentration of metal salts was 20 mM unless otherwise noted.

Salt	*Enzyme activity (nmoles/h per mg protein)*	*Relative activity*
None*	69	100
$MgCl_2$	146	212
$MnCl_2$	229	332
$ZnCl_2$**	77	112
$CaCl_2$	149	215
$CdCl_2$	32	46
$HgCl_2$***	26	40
$FeCl_3$	0	0
$SnCl_2$**	0	0
$Cu(NO_3)_2$*	0	0

* Contained 40 mM NaCl.
** Added as suspension.
*** Added as saturated solution.

Effect of metal ions

A number of metal ions, primarily as their chloride salts were examined for their effect on enzyme activity (Table II). Mg^{2+}, Mn^{2+}, and Ca^{2+} were stimulatory at concentrations of 20 mM. Mn^{2+} was the most effective. Zn^{2+} had only a slight stimulatory effect, while other metal ions tested were inhibitory. The extent of stimulation by 20 mM Mn^{2+} varied from 1.5- to 5-fold depending on the [^{35}S]sulfatide and enzyme preparation employed. At concentrations greater than 30 mM, $MnCl_2$ inhibited the enzyme (Fig. 6) as did the other stimulatory metal chloride salts. Acetate and perchlorate salts of Mn^{2+} in the presence of 40 mM NaCl were stimulatory but become inhibitory at 30 and 20 mM, respectively.

Substrate concentration

Some variation of the apparent K_m was observed with different preparations of [^{35}S]sulfatide, but was within the range of 0.1 to 0.2 mM. The reaction appeared to

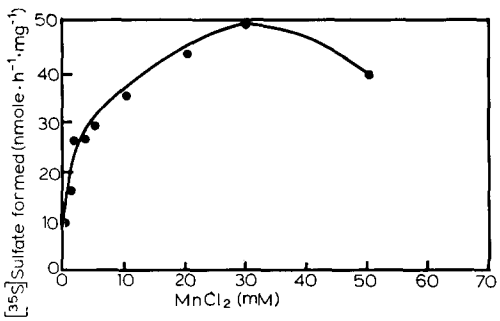


Fig. 6. Effect of Mn^{2+} on cerebroside sulfatase activity. Enzymatic assay was as described under MATERIALS AND METHODS except that $MnCl_2$ concentration was varied and appropriate amounts of NaCl were added to maintain Cl^- concentrations at 40 mM in the test samples containing less than 20 mM $MnCl_2$.

obey Michaelis–Menten kinetics (*cf.* Figs. 7 and 8). Thus under the usual assay conditions the enzyme was saturated with respect to sulfatide.

Effect of inorganic sulfate

Inorganic sulfate has been observed to be either stimulatory or inhibitory in the 4-nitrocatechol sulfate assay for arylsulfatase A depending on factors such as sulfate ion concentration, pH, or substrate concentration. It was also noted to be inhibitory for pig kidney cerebroside sulfatase[7]. In the present procedure inorganic sulfate was inhibitory. The data presented in Fig. 7 indicate non-competitive kinetics with a K_i of 1 mM. NICHOLLS AND ROY[22] observed that while inorganic sulfate was a competitive inhibitor it was also an activator of a modified form of purified arylsulfatase A in the 4-nitrocatechol sulfatase reaction. The non-competitive kinetics of inorganic sulfate in the sulfatidase reaction might be interpreted as evidence of a separate regulatory site independent of the hydrolytic active center. However, too much significance cannot be accorded data on crude systems.

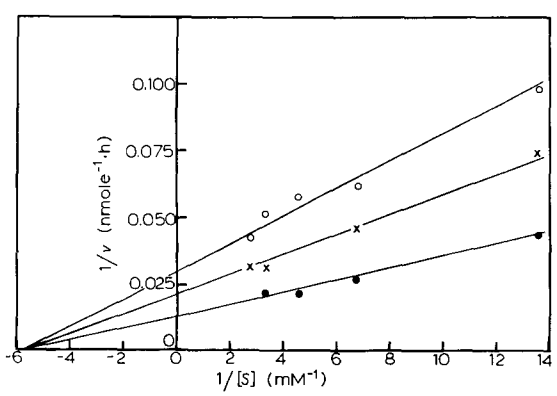


Fig. 7. Lineweaver–Burk plot of cerebroside sulfatase activity in the absence and in the presence of Na_2SO_4. Enzymatic assay was as described under MATERIALS AND METHODS except that the concentration of [^{35}S]sulfatide was varied. ●—●, no additions; ×—×, 0.8 mM Na_2SO_4; ○—○, 1.5 mM Na_2SO_4.

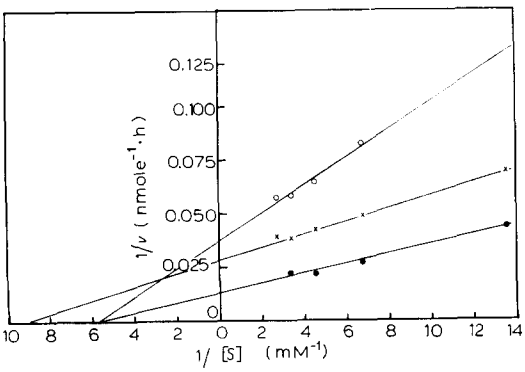


Fig. 8. Lineweaver–Burk plot of cerebroside sulfatase activity in the absence and presence of 4-nitrocatechol sulfate. Enzymatic assay was as described under MATERIALS AND METHODS except that the concentration of [^{35}S]sulfatide was varied. ●—●, no additions; ×—×, 0.26 mM 4-nitrocatechol sulfate; ○—○, 0.51 mM 4-nitrocatechol sulfate.

Effect of other arylsulfatase substrates

If a common reactive site is involved, other arylsulfatase A substrates would be expected to compete with cerebroside sulfate. The effect of 4-nitrocatechol sulfate at two different concentrations on the sulfatidase reaction is shown in Fig. 8. It did not act as a simple competitive inhibitor but rather the kinetics of the inhibition shifted from uncompetitive at low inhibitor concentration to non-competitive at the higher concentration. This behavior, while unusual, is nevertheless consistent with the anomalous kinetics observed when 4-nitrocatechol sulfate serves as the substrate for arylsulfatase A. MEHL AND JATZKEWITZ[7] also reported that it inhibited sulfatidase activity of their pig kidney enzyme. On the other hand 4-methylumbelliferyl sulfate up to an inhibitor to substrate ratio of 3:1 produced no significant inhibition.

General

The goal of the present study was to develop a cerebroside sulfatase assay procedure amenable to fibroblast extracts. We have reported separately that extracts prepared from fibroblasts derived from patients with all forms of metachromatic leukodystrophy were devoid of detectable sulfatidase activity[14]. In contrast, fibroblasts from patients with variant forms of metachromatic leukodystrophy (the so-called juvenile and adult forms) were capable of hydrolyzing cerebroside sulfate added to intact cultures. It would appear that either the mutant enzymes were particularly unstable as cell-free preparations or low levels of enzyme activity were not being detected. The latter possibility cannot be ruled out because the procedure as described earlier[14] was equivocal at low levels of activity. This occurred because during the extraction of inorganic sulfate some cerebroside sulfate partitioned into the aqueous phase. The amount of radioactivity in blanks without enzyme was greater than test samples with cell extracts. In the usual application of the assay this effect was insignificant, but with low enzyme activity preparations it led to equivocation. Washing the upper phase with "pure solvents lower phase" has to a large extent eliminated such ambiguities and has increased the sensitivity and precision of the

procedure, but has not permitted the demonstration of any activity in extracts of fibroblasts from patients with any of the metachromatic leukodystrophy variants.

A number of significant differences in pH optimum and effect of inhibitors and activators between 4-nitrocatechol sulfatase and cerebroside sulfatase activities are apparent. It is tempting to conclude that different enzymes are responsible for the hydrolysis of the two substrates. However, co-purification of both activities (ref. 7 and A. L. FLUHARTY, M. T. PORTER, M. L. SCOTT, B. KLOPFENSTEIN AND H. KIHARA, unpublished results) and their common deficiency in metachromatic leukodystrophy make this possibility unlikely. Moreover, a third substrate for this enzyme, 4-methylumbelliferyl sulfate, has optimal reaction conditions and kinetics distinctly unlike either of the other substrates and its activity also copurifies as found by these latter authors. Some of these differences may be attributable to structural and solubility differences of the substrates themselves which may alter enzyme binding properties. A systematic comparison of the interaction of arylsulfatase A with various substrates should indeed be fruitfyl. However, the significance of such studies would be more meaningfully carried out with purified enzymes rather than with the crude fibroblast extracts described herein.

The present assay procedure has been applied to a highly purified ox liver arylsulfatase A preparation obtained from Dr. A. B. Roy yielding a specific activity of about 50 μmoles of inorganic sulfate released per h per mg protein. No attempts have been made as yet to reevaluate reaction parameters for the refined system. It does appear, nevertheless, to provide significant improvement over the system of MEHL AND JATZKEWITZ[7]. A rate of 8.7 μmole per h per mg in the presence of pig kidney complementary fraction was calculated from their data with a similar enzyme preparation.

ACKNOWLEDGEMENT

We are greatly indebted to Professor A. B. Roy and Miss Agnes Jerfy for their generosity in providing a sample of purified ox liver arylsulfatase A. This work was supported by Grants NS-8839 and HD-4331 from the National Institutes of Health, and the California Department of Mental Hygiene. The opinions and conclusions stated herein are those of the authors and are not to be construed as official or as necessarily reflecting the policy of the Department of Mental Hygiene.

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