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3′-PHOSPHOADENYLYLSULFATE:GALACTOSYLCERAMIDE 3′-SULFOTRANSFERASE

AN OPTIMIZED ASSAY IN HOMOGENATES OF DEVELOPING BRAIN

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Summary

An optimized in vitro assay of 3′-phosphoadenylylsulfate:galactosylceramide 3′-sulfotransferase (EC 2.8.2.11, galactosylceramide sulfotransferase, formerly known as galactocerebroside sulfotransferase) activity is presented, that can be used in crude homogenate of brain tissue of various developmental stages. The enzyme activity is determined by measuring the [^{35}S]sulfatides formed by the enzymic transfer of [^{35}S]sulfate from 3′-phosphoadenoside 5′-phospho[^{35}S]-sulfate to galactosylceramides.

The sulfatide formation at 30°C is linear up to 30 min and up to a protein concentration of 1 mg per 0.5 ml assay volume. The presence of 0.4% Triton X-100 and 50 μM exogenous bovine cerebrosides are optimal for enzyme activity. The pH optimum of the reaction is at pH 6.5 using 0.1 M imidazole buffer. The enzyme reaction is stimulated by NaCl, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, ATP and inhibited by ADP.

The developmental enzyme activity pattern of mouse brain is the same, if derived from homogenates and microsomes, respectively, under our assay contions.

Introduction

The synthesis and turnover of sulfatide deserves special attention: Sulfatide is enriched in the myelin membrane [1—3] and its rate of synthesis is paralleled by the intensity of myelin formation in rat [4,5] and mouse brain [6,7] during

Abbreviations: APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

development. In brains of myelin-deficient mutant mice, the synthesis of sulfatide is strongly reduced [6,7].

The formation of sulfatide (sulfogalactosylceramide) from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and galactosylceramide (galactocerebroside) is catalyzed by the enzyme 3′-phosphoadenylylsulfate:galactosylceramide 3′-sulfotransferase (galactosylceramide sulfotransferase, EC 2.8.2.11, formerly known as galactocerebroside sulfotransferase) [6,8—11]. The enzyme activity in vitro is estimated by use of [^{35}S]PAPS as the sulfate donor and by measurement of [^{35}S]sulfatide formed [4].

In brain tissue, this enzyme is enriched in the microsomal fraction [4,9], and most of the available data have been elaborated using microsomes or a microsomal extract as enzyme sources [6,9,12—15]. As shown in rat brain, however, galactosylceramide sulfotransferase activity is not only found in microsomes, but considerable amounts of activity are found in other membranous structures, such as in the light myelin fraction [9]. In rat kidney, the bulk of enzyme activity is associated with the Golgi apparatus [16].

Thus the use of microsomes as enzyme source becomes problematic, if the enzyme activity of different organs has to be compared, or if developmental activity patterns would be studied, since enzyme location as well as the physical properties of subcellular constituents may change during cell growth. In addition, the amount of cell material necessary for cellular subfraction may be not available. These difficulties are reduced by the use of homogenate as enzyme source.

In the present paper, an optimized sensitive assay is described, which allows the in vitro determination of galactosylceramide sulfotransferase activity in homogenates from developing mouse brain, and the enzyme activity patterns derived from mouse brain homogenates and microsomes, respectively, are compared over the postnatal development.

Materials and Methods

Chemicals. ATP, ADP, APS and bovine cerebrosides were from Sigma Chemicals, bovine sulfatides from Technosa and [^{35}S]PAPS (specific activities 0.8—4.0 Ci/mmol) from NEN Chemicals. Carrier-free [^{35}S]sulfate was from the Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland. Triton X-100 was purchased from Mann Research Laboratories and Permablend ® II (PPO 98%, POPOP 2%) from Packard Instr. Co.). All the other chemicals used were of analytical grade and originated from Merck.

Animals. Mice of the C57 BL/6J-A^{W-J} strain (Jackson Laboratories, Bar Harbor, Maine, U.S.A.) were used.

Enzyme sources. The animals were decapitated, the brains removed, weighed and immediately homogenized at 0° C in an all glass homogenizer or frozen and stored at —80° C. Storage of whole organs at —80° C and even at —20° C was possible for several months without any loss of enzyme activity. After homogenization, the samples were sonicated at 0° C for 15 s at 50 W (Sonifier B-12, Branson Sonic Power, Danbury, Conn.). Microsomes were isolated essentially as described in a previous paper [17].

Enzyme assay. The incubation mixture (0.5 ml) consisted, except stated

otherwise, of 100 mM imidazole buffer adjusted to pH 6.5 by 37% HCl, 4.0 mM ATP, 200 mM NaCl, 20 mM $CaCl_2$, 50 μM cerebrosides from bovine brain (assumed M_r = 800), 0.4% Triton X-100 and 200—400 mM [^{35}S]PAPS (220 000—440 000 dpm). The reaction was started by adding 0.1 ml of 5% or 10% aqueous brain homogenate (w/v, 300—1000 μg protein) and the samples were incubated in duplicates at 30° C for 20 min in 12-ml polypropylene tubes. The reaction was stopped by the addition of 5.0 ml chloroform/methanol (1 : 1, v/v) and the tubes were centrifuged at 1200 × *g* for 15 min. The precipitate was discarded and 2.5 ml chloroform and 1.5 ml 0.74% KCl solution were added. To allow partitioning, the emulsion was centrifuged (1200 × *g*, 15 min), the upper phase discarded and the lower phase washed three times with 1.2 ml "theoretical upper phase" according to Folch et al. [18]. An aliquot of the lower phase was transferred to a liquid scintillation counting vial and dried. After the addition of 10 ml toluene containing 0.4% Permablend ® II, radioactivity was determined in a Packard Tri-Carb scintillation spectrometer.

If microsomes were used as the enzyme source, the incubation conditions were the same as those described above. The enzyme activities were expressed as pmol [^{35}S]sulfatide formed per h and per mg protein. 1 pmol [^{35}S]sulfatide corresponded to 2200 dpm. Protein determinations were made according to Lowry et al. [19].

Results

Optimal assay conditions

The effect of Triton X-100 on the galactosylceramide sulfotransferase activity was checked in brain homogenates (10%, w/v) of 18-day-old mice. The incubation mixtures contained 0.2, 0.4, 0.6, 0.8 and 1.6% Triton X-100, respectively, but no exogenous galactosylceramides. Maximal enzyme activity was observed in the presence of 0.4% Triton X-100 being about twice of that without detergent.

The enzyme activity was linear with time up to 30 min at 30° C, but not at 37° C. The rate of in vitro sulfatide formation was directly proportional to the protein concentration between 20 and 1000 μg of total protein per assay.

The effect of exogenous substrate (cerebrosides from bovine brain) on the galactosylceramide sulfotransferase activity in homogenates of 6-, 17- and 40-day-old brains is shown in Fig. 1a: Substrate saturation in each age group was obtained by the addition of 20 μg of bovine cerebrosides per assay (50 μM). Similar experiments were carried out using mouse brain cerebrosides and homogenate of 17-day-old mice and the same results were obtained as with bovine cerebrosides. Bovine sulfatides added to the assay mixture up to a final concentration of 100 μM (assumed M_r = 900) did not inhibit galactosylceramide sulfotransferase activity in any one of the three age groups.

The plot of PAPS concentration versus activity (Fig. 1b) demonstrates that under the assay conditions the degree of PAPS saturation is low. The pH optimum of the enzyme reaction in homogenates of 5-, 18- and 31-day-old brains was between pH 6.3 and 6.6. The imidazole concentration of the incubation mixtures was kept close to 100 mM by adjusting the pH to the appropriate

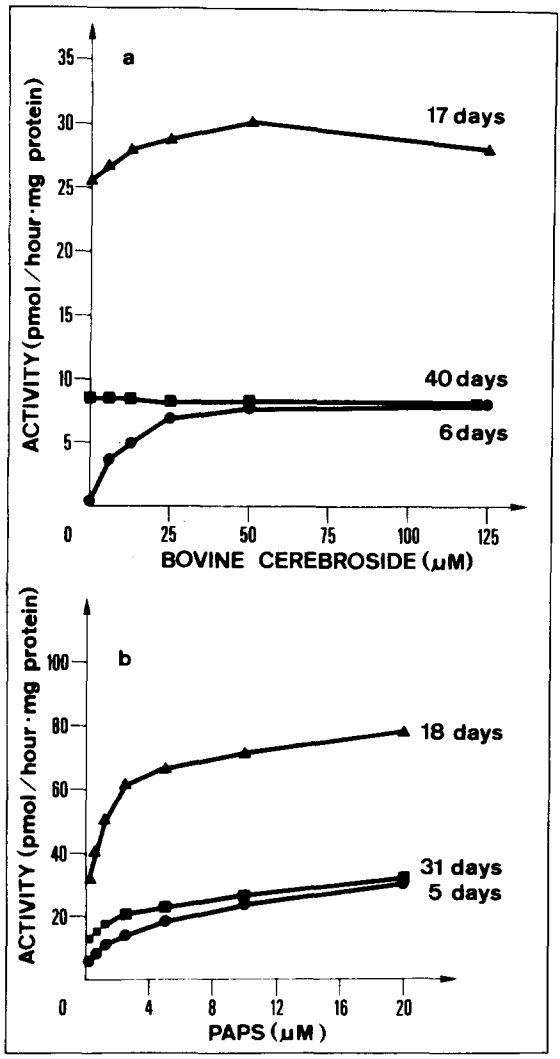


Fig. 1. Effect of various concentrations of exogenous cerebrosides from bovine brain on the in vitro sulfatide formation of brain homogenates of different developmental stages (a) and the dependence on the availability of [^{35}S]PAPS (b). Ordinate: Enzymic formation of [^{35}S]sulfatide from [^{35}S]PAPS and galactosylceramides in vitro. 1 pmol [^{35}S]sulfatide = 2200 dpm.

values (from pH 5.8 up to pH 7.9) with concentrated HCl (37%). For the standard assay pH 6.5 was used.

Monovalent (Fig. 2a) and bivalent cations (Fig. 2b) stimulated the in vitro sulfatide formation in homogenates of 18-day-old brains. A similar relative stimulation was observed in homogenates of 2- and 36-day-old brains. The stimulating effect of calcium (Fig. 2c) could be substituted by Na^+ in the system. Maximal enzyme reaction was observed, if a combination of 20 mM $CaCl_2$ and 200 mM NaCl was used (Fig. 2c).

ATP concentrations up to 4 mM stimulated the galactosylceramide sulfotransferase activity as shown in Fig. 3. The activation in percent was similar

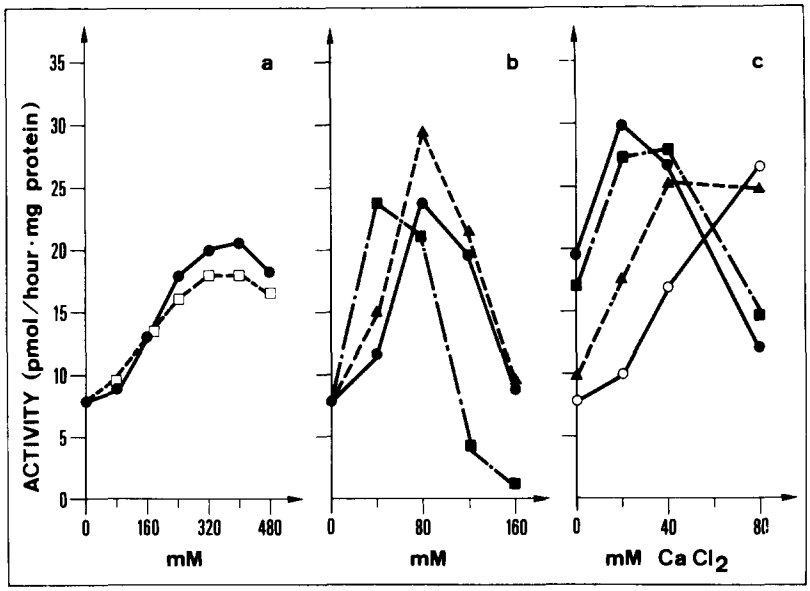
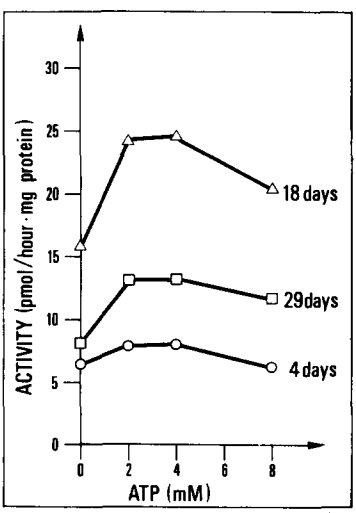


Fig. 2. The effect of electrolytes on the galactosylceramide sulfotransferase activity of brain homogenates (10%, w/v) of 18-day-old mice. The reaction mixtures consisted of 80 mM imidazole buffer, pH 7.0, 2 mM ATP, 100 μM cerebrosides from bovine brain, 0.4% Triton X-100, 100 μl brain homogenate and 200 nM [^{35}S]PAPS. (a) Effect of NaCl (●) and KCl (□). (b) Effect of $MgCl_2$ (●), $CaCl_2$ (▲) and $MnCl_2$ (■). (c) Effect of different concentrations of NaCl in the presence of variable amounts of $CaCl_2$. NaCl concentrations: 0 mM (○), 80 mM (▲), 160 mM (■), 200 mM (●).



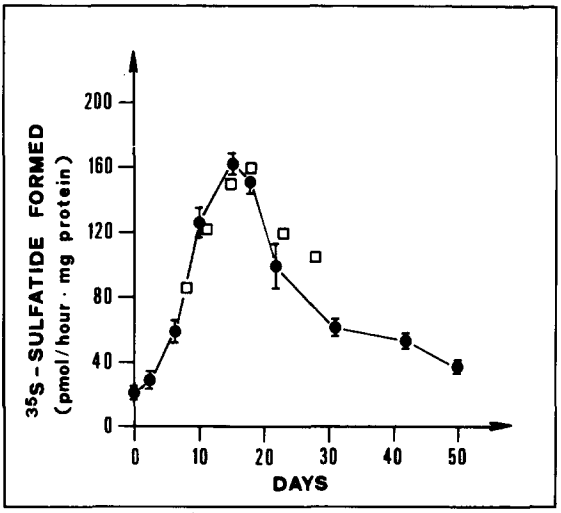


Fig. 3. The effect of ATP on the galactosylceramide sulfotransferase activity of brain homogenates of different developmental stages. All assay conditions were the same as described in Material and Methods with the exception, that the ATP containing imidazole buffer solution was adjusted to pH 7.0.

Fig. 4. In vitro galactosylceramide sulfotransferase activity in homogenates and microsomes of mouse brain during the postnatal development. The changes of the specific enzyme activity using 10% aqueous brain homogenates as the enzyme source (●) are compared with the specific activity pattern derived from isolated microsomes (□). For the enzyme activities of crude homogenates the means ± S.D. of 3—8 determinations are plotted. The data of microsomal enzyme activities are mean values of three independent experiments. Assay conditions were the same as described in Materials and Methods except that the [^{35}S]PAPS concentration was 1400 nM (specific activity 0.66 Ci/mmol).

TABLE I

EFFECT OF ENDOGENOUS AND EXOGENOUS CEREBROSIDES ON THE IN VITRO GALACTOSYLCERAMIDE SULFOTRANSFERASE ACTIVITY OF MIXTURES OF HOMOGENATES FROM BRAINS OF DIFFERENT DEVELOPMENTAL STAGES

Brain homogenates (10 and 5%, respectively) of mice aging 6, 17 and 40 days, respectively, were mixed in the proportion of 1 : 1 (v/v). The expected activities of the mixtures, as calculated from the activities of unmixed aliquots, were taken as 100%. The tabulated data are means of three independent experiments.

Mixed homogenates (1 : 1, v/v)		No bovine cerebrosides (1)			50 μM bovine cerebrosides (2)		
Age in days	Concentration (w/v)	Expected * activity (EA)	Observed * activity (OA)	$\frac{OA}{EA} \times 100$	Expected * activity (EA)	Observed * activity (OA)	$\frac{OA}{EA} \times 100$
6/17	10%	1030	875	85%	1535	1535	100%
	5%	405	322	82%	775	760	98%
17/40	10%	1495	1855	124%	1850	1905	103%
	5%	645	955	148%	950	997	105%
6/40	10%	625	945	151%	785	800	102%
	5%	255	357	140%	375	390	104%

* pmol [^{35}S]sulfatide/h per g fresh weight.

in homogenates of 4-, 18- and 29-day-old brains. 1.2 mM APS in the presence of 2 mM ATP and 1 mg exogenous cerebrosides per assay stimulated the enzyme activity of 35%, whereas under the same conditions 1 mM ADP inhibited the reaction.

Reliability of the method

In control experiments, the addition of different concentrations of Triton, electrolytes (NaCl, $CaCl_2$) and ATP to the assay after incubation did not change the distribution of labelled sulfatide in the biphasic separation system. If in addition to these components inorganic [^{35}S]sulfate (800 000 dpm, carrier free) and [^{35}S]PAPS, respectively, were added after incubation, the same results were obtained.

Without the addition of exogenous substrate, the in vitro galactosylceramide sulfotransferase activity depended markedly on the amount of endogenous substrate present, as demonstrated by mixing equal volumes of brain homogenates of different developmental stages (Table I, column 1). Under the final assay conditions, however, substrate saturation was given for each age group (Table I, column 2, and Fig. 1a) and the influence of endogenous factors during development was eliminated (Table I, column 2).

Applicability of the method

Fig. 4 shows the developmental activity patterns of galactosylceramide sulfotransferase in mouse brain, as determined in vitro using homogenate or microsomal fraction as the enzyme source. Similar developmental activity patterns were found for the enzyme of brain homogenate and microsomes, and specific enzyme activities were highest around day 16 to 18 post-partum.

Discussion

The enzymic transfer of sulfate from [^{35}S]PAPS to galactosylceramides and the identification of the sulfated reaction products as sulfatides have been established in sheep brain [8], in rat brain [4,9] and in mouse brain [6,10].

The presented assay system allows a reliable and sensitive determination of galactosylceramide sulfotransferase activity of brain homogenates during development without apparent interferences by tissue intrinsic factors.

At 30° C, the reaction is linear with time up to 30 min and with protein concentrations up to 1 mg per assay. The effect of the increasing amount of endogenous substrate during development on the in vitro enzyme activity has been eliminated.

Non-linear enzyme reaction in homogenate at 37° C was previously described [9] and attributed to a lack of endogenous substrate. In our experiments, neither the presence of up to 1 mg cerebrosides per assay nor the readdition of [^{35}S]PAPS after 10 min of incubation could restore activity at 37° C. The readdition of fresh homogenate to the assay, however, restored the linearity of the reaction.

The presence of 50 μM exogenous cerebrosides was found to be necessary for optimal substrate saturation, especially in young animals. The [^{35}S]PAPS concentration used in our assay system was below saturation. However, since

not more than 1% of [^{35}S]sulfate was transferred to galactosylceramides during incubation, the decrease of the PAPS concentration may be negligible.

A stimulation of the galactosylceramide sulfotransferase activity by electrolytes has been previously observed for the enzyme from microsomes [9] and for a solubilized and partly purified enzyme [20] of rat brain. To find the same stimulation effect in homogenates, about four times higher ionic concentrations were necessary.

A 200 mM NaCl concentration was used in the assay system, because it is known, that the cerebrosidesulfate sulfatase activity is inhibited by this NaCl concentration in homogenates of cultured human skin fibroblasts [21]. The presence of high salt concentrations in the assay did not affect the distribution of [^{35}S]PAPS, [^{35}S]sulfate or [^{35}S]sulfatides.

The developmental activity patterns of mouse brain galactosylceramide sulfotransferase derived from homogenate and microsomes are similar (Fig. 4). This pattern is in good agreement with the developmental activity patterns found in microsomes of rat brain [4,5], as well as with the activity patterns found in homogenates [10] and in the microsomal fraction [6] of mouse brain. In addition, the determined in vitro galactosylceramide sulfotransferase activity pattern correlates well with the rate of the in vivo sulfatide formation in the developing mouse [7] and rat brain [4]. The similarity of the developmental activity patterns of the enzyme from homogenate and microsomes indicates, that in homogenates an interference of the enzyme cerebrosidesulfate sulfatase with the in vitro sulfatide formation is negligible, and that homogenate is a valid enzyme source for the study of galactosylceramide sulfotransferase activity.

The high sensitivity, due to optimal amounts of substrate, detergent, ATP and electrolytes present in appropriate relations in the assay, allows a reliable determination of the in vitro galactosylceramide sulfotransferase activity down to 20 μg homogenate protein per assay.

The close relationship between myelination and galactosylceramide sulfotransferase activity [4,6] has rendered this enzyme to a marker for this physiologically important process. The described method may help to get more information on myelin disorders and specially on disorders of the sulfatide metabolism in the developing brain.

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