Sphingomyelinase in normal human spleens and in spleens from subjects with Niemann-Pick disease

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ABSTRACT This paper describes the purification and some of the properties of an enzyme from human spleen that catalyzes the hydrolysis of sphingomyelin with the formation of ceramide and phosphoryl choline. The enzyme, which is located in the subcellular particulate fraction that sediments between 700 and 8500 *g*, is readily made soluble and has been partially purified. Its pH optimum is between 4.5 and 5.0. It is unaffected by divalent cations, chelating agents, and sulfhydryl reagents, but is inhibited by phosphate. The enzyme attacks sphingomyelin and dihydrosphingomyelin, but is inactive toward sphingosine phosphoryl choline, *O*-acetylsphingomyelin, and lecithin. In some of its properties, the enzyme from human spleen is different from the previously studied sphingomyelinase from rat tissues.

The enzyme is absent or markedly reduced in spleens from patients with classical and visceral varieties of Niemann-Pick disease, but is present in normal amounts in the late infantile type of the disease. In the present study another enzyme, this one magnesium-dependent, capable of catalyzing the cleavage of sphingomyelin has been detected in the spleens of patients with the classical form of Niemann-Pick disease. Some implications of these findings for theories of the metabolic defect in Niemann-Pick disease are discussed.

KEY WORDS spleen · sphingomyelinase · man · sphingomyelin accumulation · Niemann-Pick disease · lipid storage disease

ALTHOUGH SPHINGOMYELIN is an important constituent of the lipids of mammalian tissues, little has been known until recently about pathways for its catabolism in the mammal. It is well known that phospholipase C from various bacterial sources, especially *Clostridium perfringens*, attacks sphingomyelin, with the cleavage of phosphoryl choline and formation of a ceramide (1). Such phospholipases are relatively nonspecific: they also attack lecithin and "cephalins." In the present report, we describe the partial purification and some of the properties of a sphingomyelinase from human spleen that is specific for sphingomyelin and dihydrosphingomyelin, and that hydrolyzes the bond between ceramide and phosphoryl choline. Human spleen was chosen for these studies because this organ is relatively easily obtained in fresh and undiseased condition. On the other hand, the spleen is also prominently involved with abnormal accumulation of sphingomyelin in Niemann-Pick disease, and the study of the biochemistry of this condition is of interest not only for its own sake but also within the general topic of the catabolism of sphingomyelin in the intact organism. For this reason, we have also examined the sphingomyelinase activity of spleens from patients suffering from Niemann-Pick disease of several types.

Early reports of sphingomyelinase activity in various mammalian tissues neither characterized the enzyme (2, 3) nor demonstrated its specificity (4).

Recent papers by Kanfer, Young, Shapiro, and Brady (5) and Heller and Shapiro (6) have described a sphingomyelinase from rat liver. Some of the properties of the enzyme from human tissues, described here for the first time, are different from those of the rat enzyme.

In a brief communication, Brady, Kanfer, Mock, and Fredrickson (7) have reported that the level of sphingomyelinase is greatly reduced in tissues derived from patients with Niemann-Pick disease. In a more comprehensive study of the sphingomyelinase activity in spleens from patients with this disease, we have found that the enzyme is greatly reduced or absent from spleens of patients with the classical and visceral forms of the disease, but present in significant amounts in late infantile forms. We have also detected in spleens from

Abbreviation: μU, micro (international enzyme) unit(s).

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patients with the classical form of the disease another, magnesium-dependent, enzyme capable of catalyzing the hydrolysis of sphingomyelin, the presence of which had not previously been reported.

MATERIALS AND METHODS

Bovine heart sphingomyelin was obtained from the Sylvana Chemical Company (Millburn, N.J.) and was subjected to alkaline hydrolysis for 1 hr at 37°C in 0.4 M methanolic KOH. An excess of HCl was added (final concentration of acid, 0.4 M) and hydrolysis was continued for another hour, after which $CHCl_3$ and water were added. The $CHCl_3$ phase was washed several times with water and dried under a stream of nitrogen. The sphingomyelin was crystallized twice from cyclohexane–acetone. This preparation gave a single spot with an R_f of 0.18 on thin-layer chromatography, which was performed on Silica Gel H (E. Merck, A.G., Darmstadt, Germany) in an unlined glass cylinder of 6 cm diameter with the solvent $CHCl_3$–CH_3OH–2 M NH_4OH 70:30:4.

Dihydrosphingomyelin was prepared from sphingomyelin by catalytic reduction with H_2 over platinum oxide in ethanol at room temperature and atmospheric pressure. The product was purified by crystallization from cyclohexane–acetone or by column chromatography on silicic acid.

Dihydrosphingomyelin-^{3}H was prepared from sphingomyelin by reduction with tritium gas at atmospheric pressure in dibutyl ether at 65°C in the presence of platinum oxide as catalyst. Labile tritium was removed by adding and distilling off ethanol several times. The product was stored in ethanol at −20°C and purified before use by thin-layer chromatography. Unlabeled dihydrosphingomyelin was added to adjust the specific activity to about 5μc/μmole. Degradation studies showed that about 10% of the tritium was located in the amide-linked fatty acid and the remainder in the sphingosine moiety.

Ceramide and dihydroceramide were prepared from the respective parent compounds by the action of phospholipase C from *Clostridium perfringens* (8) and purified by chromatography on a silicic acid column from which these products were eluted by 5% CH_3OH in $CHCl_3$.

Sphingosine phosphoryl choline was prepared according to Kaller (9).

O-Acetylsphingomyelin was prepared by treatment of 0.5 g of sphingomyelin dissolved in 7 ml of pyridine with 5 ml of acetic anhydride for 3 days at 37°C. Water was added and the mixture dried under reduced pressure. The product was purified by column chromatography on silicic acid. On thin-layer chromatography, in the same system as that used for sphingomyelin (see above), the *O*-acetylsphingomyelin ran as a single spot with an R_f of 0.30.

Lecithin labeled with ^{14}C in the choline methyl group was prepared enzymatically from diglyceride and cytidine diphosphate-choline-^{14}C (10).

N-Octanoyl *threo*-sphingomyelin also labeled with ^{14}C in the choline methyl group was likewise prepared enzymatically (8).

Phosphorus was determined by the method of Bartlett (11). Protein was measured according to Lowry (12). If the final solution was turbid because of high tissue lipid content, it was washed with $CHCl_3$ just before the color was read. Tissue sphingomyelin was determined as lipid phosphorus that resisted hydrolysis by 0.4 M KOH and 0.4 M HCl.

Tritium activity was measured in a Packard scintillation counter, with toluene containing 0.4% diphenyloxazole and 0.01% 1,4-bis[2(5-phenyloxazolyl)]benzene as scintillator fluid for dihydroceramide, and a similar solution containing one-third ethanol (13) for dihydrosphingomyelin. Efficiency corrections were made with internal standards. A Nuclear-Chicago gas flow counter with an ultra-thin window was used to measure ^{14}C.

Human spleens were obtained at surgery. They had been removed for a variety of reasons (trauma, palliation of aplastic anemia, or incidental to other surgery), and were histologically normal unless otherwise stated. A series of spleens from patients with Niemann-Pick disease and other infiltrative disorders was kindly provided by Dr. Allen Crocker (Children's Medical Center, Boston).

Assay

The assay for sphingomyelinase activity is a modification of the method of Roitman and Gatt (4). The enzyme was incubated in a total volume of 0.2 ml containing 1.5 mM dihydrosphingomyelin-^{3}H, Triton X-100 [a nonionic detergent, mixture of *p*, *t*-octyl poly(phenoxyethoxy) ethanols, Rohm & Haas, Philadelphia, Pa.], 2.5 mg/ml, and 50 mM sodium acetate buffer, pH 4.8. The dihydrosphingomyelin and Triton mixture was warmed to about 50°C to dissolve the substrate and allowed to cool before addition of the enzyme. The buffer must be added last when any enzyme is used that has not been brought past the purification step of acid precipitation (see below). The reaction was stopped by the addition of 5 ml of heptane–isopropanol 1:1, containing 2×10^{-7} M dihydroceramide carrier. 1 ml of water was added and the system mixed and centrifuged. The upper (heptane) phase, which contained most of the released dihydroceramide, was taken, and the remaining traces of dihydrosphingomyelin were removed by the addition of silicic acid (0.25 g), freshly activated at 110°C. After thorough mixing and centrifugation a

2 ml aliquot was transferred to a scintillation vial with 10 ml of the toluene-based scintillator for determination of tritium activity.

Each assay included a "zero time" control which amounted to 100–300 cpm, or about 0.01% of the total activity of the substrate. Since the dihydroceramide is not extracted completely by the heptane phase, a control was also included in which dihydroceramide-^{3}H replaced the dihydrosphingomyelin-^{3}H in the standard incubation mixture. This latter control also corrected for small losses of dihydroceramide by adsorption on the silicic acid. Over-all recovery was about 60%. The amount of dihydroceramide formed was calculated from the specific activity of the substrate. The activity of enzyme is expressed in international enzyme units (μmole/-min). A net count of 200 cpm in a 2 hr assay is equivalent to about 1 unit (μU).

The assay is linear with time up to 2.5 hr. It is linear with amount of crude tissue homogenate up to 0.6 mg of protein and with amount of purified enzyme up to at least 200 μU. Unless otherwise stated, enzyme activity was measured at 37°C. The standard deviation of the assay corresponds to 3% or less for enzyme levels above 10 μU.

The above assay system seems to be inherently more sensitive than one depending on the chemical measurement of water-soluble phosphorus released from sphingomyelin (5). It also seems to be somewhat more sensitive than the previously reported assay utilizing the release of water-soluble ^{14}C from choline-^{14}C labeled sphingomyelin (6) since the higher specific activity of our tritium-labeled substrate provides approximately a 10-fold higher final count rate per μmole of product (the zero control is increased only 4-fold). No data are given in previous reports to allow assessment of precision.

RESULTS

Identification of the Product

To identify the reaction product actually measured in our assay as dihydroceramide-^{3}H, we concentrated the final heptane phase derived from an assay under a stream of nitrogen and applied the solution in a spot to a thin layer of Silica Gel H. The chromatogram was developed first with $CHCl_3$–CH_3OH–CH_3COOH 90:4:4 to a height of 15 cm, then dried and developed with $CHCl_3$–CH_3OH–2 M NH_4OH 70:30:4 to 7 cm. Under these circumstances fatty acid, dihydroceramide, dihydrosphingosine, and dihydrosphingomyelin travel with R_f values of 0.77, 0.55, 0.25, and 0.07, respectively; these compounds were run concurrently as markers. Bands were scraped from the thin-layer plate and eluted with $CHCl_3$–CH_3OH 2:1. The eluates were dried and counted in a scintillation counter. Total recovery of applied counts was 98%. Of the recovered counts 93% was found in the dihydroceramide band. Fatty acid, dihydrosphingosine, and dihydrosphingomyelin together accounted for only 1% of the activity, 4% was in the lower front, and the remaining 2% was in the upper front and in the areas between the defined bands.

To characterize the other reaction product we washed the aqueous phases derived from an assay (and a zero time control) three times with 2 ml of $CHCl_3$, and took aliquots of each washed phase for analysis of both total phosphorus and orthophosphate. Of the water-soluble phosphorus released during the reaction, less than 1% was orthophosphate, the remainder being organic phosphate, presumably phosphoryl choline. For further identification, the aqueous phase derived from an assay was washed with $CHCl_3$, concentrated, applied as a spot to Whatman No. 43 paper, and chromatographed in an ascending system of 60% ethanol with 0.02 M acetic acid. Marker compounds were added to zero time controls of the assay system, processed similarly, and run concurrently. The chromatogram was stained with the Hanes-Isherwood spray for phosphorus. The phosphorus released during the reaction formed a single spot running with the R_f of phosphoryl choline (0.52) and ahead of phosphoryl ethanolamine and orthophosphate.

Distribution of Enzyme in Subcellular Fractions

A 10% (w/v) homogenate of fresh human spleen in 0.25 M sucrose was prepared in a Potter-Elvehjem homogenizer and fractionated by differential centrifugation as previously described (14). The fractions, their specific activities, and enzyme content are listed in Table 1. The highest specific activity is in the hard-packed pellet that sediments at 8500 *g*, i.e., presumably the mitochondrial fraction. The enzyme easily becomes soluble and this may account for the substantial amount in the supernatant fraction of the 100,000 *g* centrifugation. Because of this property, the tissue was homogenized very gently, which could account for the high per-

TABLE 1 INTRACELLULAR DISTRIBUTION OF SPHINGOMYELINASE IN HUMAN SPLEEN

Fraction	Specific Activity	% of Total Activity
	μU/mg protein	
Homogenate	257	100
"Nuclei" (700 *g* sediment)	112	27
"Mitochondria" (8500 *g*, hard-packed sediment)	1030	13
"Intermediate" (8500 *g*, loosely-packed sediment)	795	26
"Microsomes" (100,000 *g* sediment)	327	16
Supernatant (100,000 *g*)	139	18

centage of enzyme remaining in the 700 *g* sediment in unbroken cells.

Enzyme Preparation and Purification

A 20% (w/v) homogenate of fresh or frozen human spleen was made in 0.01 M Tris buffer, pH 8, with the use of a Potter-Elvehjem homogenizer with a loosely fitting glass pestle for the first rough grinding, followed by 10 passes in a closely fitting Teflon–glass homogenizer. The homogenate was dialyzed overnight at 4°C against 0.01 M Tris buffer, pH 8, to bring the enzyme into solution. The preparation was then centrifuged for 1 hr at 40,000 *g*.

To the supernatant fraction was added 1 μg/ml each of ribonuclease and deoxyribonuclease, and 2 μmoles/ml of $MgCl_2$. The mixture was incubated for 1 hr at 37°C to degrade the nucleic acids, which interfered somewhat with subsequent ethanol fractionation. After incubation 2 mg/ml of Triton X-100 was added and the pH was adjusted to 5 with 2 M acetic acid. The mixture was heated to 45°C for 10 min, cooled to 4°C, and centrifuged at 10,000 *g* for 10 min. The supernatant fluid was fractionated with ethanol at a temperature between −5° and −10°C. The precipitate that formed between 30 and 40% ethanol was recovered and resuspended in a few milliliters of 0.01 M Tris, pH 8. The material that would not dissolve was removed by centrifugation and the soluble portion was taken for column chromatography.

About 20–30 mg of protein was placed on 2–3 g of ECTEOLA-cellulose (Cellex-E, Bio-Rad) in a column of 1 cm diameter and 11–17 cm height. The sample was washed onto the column with 10 ml of 0.01 M Tris, pH 8, and elution continued with a solution of 0.01 M Tris, pH 8, 0.04 M KCl. Fractions of 2–3 ml were collected. After a red (hemoglobin) peak had been eluted (about 50 ml), the eluent was changed to 0.01 M Tris, pH 7, 0.1 M KCl, 0.1 mg/ml of Triton X-100. The enzyme appeared after about 6 ml of this last eluent had been added and fractions with the highest specific activity were pooled.

Table 2 presents the results of an enzyme purification procedure. The method is reproducible if the same spleen is used but there is some variation in purity and yield among different spleens. In the studies on partially purified enzyme reported here, preparations with a specific activity of 10,000–14,000 μU/mg of protein were used.

TABLE 2 ENZYME PURIFICATION

Fraction	Specific Activity	Degree of Purification	Yield
	μU/mg protein	*-fold*	
Crude homogenate	210	1	100
Soluble fraction	220	1.05	69
pH 5 Supernate	520	2.5	67
30–40% Ethanol precipitate	4,050	19	31
ECTEOLA column eluate	15,900	76	7

* Standard assay system, at 37°C and pH 4.8.

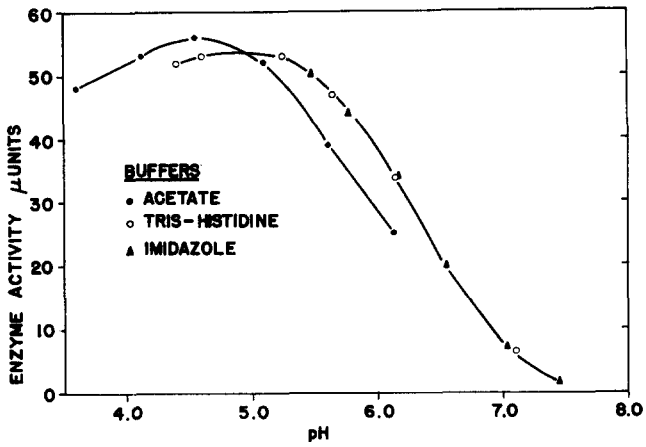

FIG. 1. Effect of pH on enzyme activity. Partially purified sphingomyelinase was incubated for 2 hr at 37°C with concentrations of 1.5 mM dihydrosphingomyelin, 2.5 mg of Triton X-100 per ml, and 50 mM buffer of the kind indicated. The shift between the curves along the abscissa is reproducible.

Enzyme activity in the crude homogenate is quite stable and remains constant for several months if the preparation is frozen, but the purer and more dilute enzyme loses a considerable amount of activity on freezing (especially if Triton X-100 is present) unless it is protected by the presence of at least 0.05 M sucrose. The enzyme was therefore stored between purification steps at 4°C, a temperature at which it is stable for several days. For prolonged storage the partially purified enzyme was lyophilized after the solution had been made 0.05 M in sucrose; this does not, however, entirely prevent inactivation.

Effect of pH on Activity

The activity of the partially purified enzyme as a function of pH with various buffers is displayed in Fig. 1. The shift of the curves from one buffer to another is a reproducible phenomenon but in either case the pH optimum is between 4.5 and 5.0.

Effect of Temperature on Activity

Fig. 2 shows the effect of temperature on the reaction. The incubation time for the reaction was 1 hr, and during this time at the higher temperatures the enzyme loses activity. The effect of this nonlinearity, as determined by extrapolating the enzyme activity measured at 15 min, is that measurement at 1 hr indicates an activity 4% too low at 50°C, 11% at 55°C, and 34% at 60° and 65°C. The peak shown is thus lower than if true initial reaction rates had been used, but its maximum is at the correct temperature.

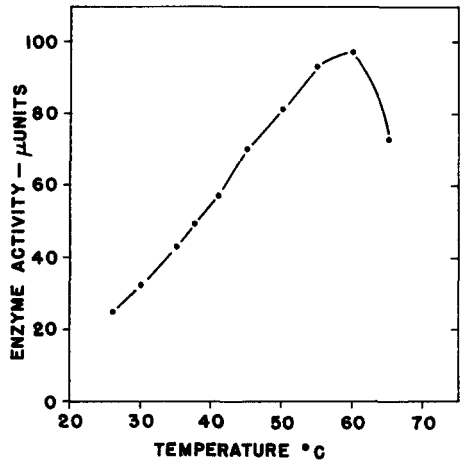

FIG. 2. Effect of temperature on enzyme activity. Partially purified sphingomyelinase was incubated for 1 hr at the indicated temperature with 1.5 mM dihydrosphingomyelin, 2.5 mg of Triton X-100 per ml, and 50 mM acetate buffer, pH 4.8.

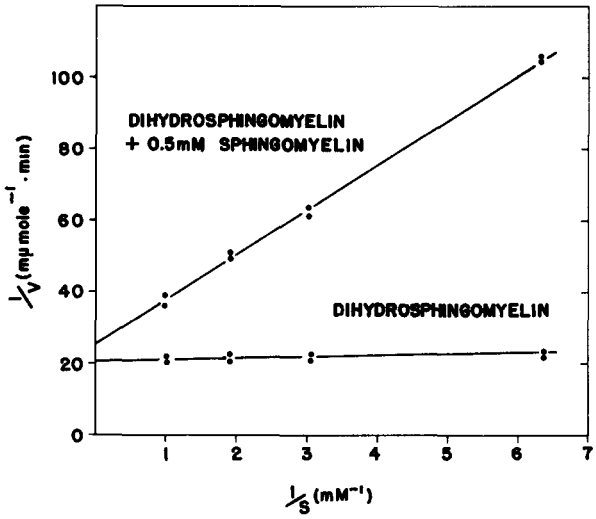

FIG. 3. The Lineweaver-Burk, double reciprocal, plot of enzyme activity and substrate concentration. The partially purified sphingomyelinase was incubated for 1 hr at 37°C with 2.5 mg of Triton X-100 per ml, 50 mM acetate buffer, pH 4.8, and various substrate concentrations to give the lower line. Conditions for determination of the upper line were the same except that 0.5 mM sphingomyelin was present in each assay. The K_m for dihydrosphingomyelin calculated from the lower line is 3.2×10^{-5} M and the K_m for sphingomyelin from the upper line is 3.7×10^{-5} M.

If the enzyme is incubated (without substrate and detergent before assay, it loses 50% of its activity (measured subsequently at 37°C) in 15 min at 55°C. Partial protection against inactivation is afforded by the presence of both substrate and detergent. Substrate alone is ineffective, presumably because of its insolubility.

Effects of Ions and Sulfhydryl Reagents

At a concentration of 25 mM in the standard assay mixture, $MgCl_2$, $MnCl_2$, $CaCl_2$, citrate, and ethylenediaminetetraacetate (EDTA) had no effect on activity. Phosphate, on the other hand, caused a 60% inhibition at 10 mM and 75% at 25 mM.

Preincubation of the enzyme with 1 mM *N*-ethylmaleimide or iodoacetamide for 10 min at pH 7.5–7.8 at room temperature had no significant effect on enzyme activity. Under the same conditions *p*-hydroxy mercuribenzoate enhanced the activity by about 10%.

Specificity of Enzyme

Lecithinase activity of the enzyme was measured in the standard assay mixture, with the dihydrosphingomyelin replaced by 0.25 mM lecithin-^{14}C labeled in the choline moiety. The release of water-soluble radioactivity was measured by extraction of the lecithin into $CHCl_3$ and measurement of the ^{14}C remaining in the water phase. A crude homogenate of spleen with 100 μU of sphingomyelinase activity showed 25 μU of lecithinase activity. However, 200 μU of the partially purified enzyme had no detectable activity against lecithin. This demonstrates that although a lecithinase exists in the spleen, it is distinct and separable from the sphingomyelinase.

Activity towards *O*-acetylsphingomyelin, sphingomyelin, and sphingosine phosphoryl choline was measured as the release of water-soluble phosphorus in a standard assay system with these potential substrates replacing the dihydrosphingomyelin at a concentration of 1.5 mM. The reaction was stopped with 2.5 ml of $CHCl_3$–CH_3OH–*n*-C_4H_9OH, 2:2:1 and 0.8 ml of 0.02 M KOH. The upper phase so formed was separated and washed with fresh "lower phase" and the phosphorus in the upper phase was determined. This partitioning procedure was designed primarily for the sphingosine phosphoryl choline but served adequately for the other two substrates. In concurrent determinations a sample of partially purified enzyme displayed 250 μU of activity against sphingomyelin but no detectable activity against the other two compounds.

N-Octanoyl *threo*-sphingomyelin labeled with choline-^{14}C was tested as substrate by measurement of the release of water-soluble radioactivity as in the lecithinase assay, with a substrate concentration of 0.25 mM. Compared to activity against similar amounts of dihydrosphingomyelin, the enzyme was about half as active against the *threo* compound (the amount of *erythro*-sphingomyelin contributed to the *threo* preparation by the microsomal enzyme used in its biosynthesis was taken into account in this estimate).

Determination of the K_m

Fig. 3 shows a Lineweaver-Burk plot of enzyme action on dihydrosphingomyelin substrate. A K_m of 3.2×10^{-5} M was calculated from this experiment but the value ranged up to 4.3×10^{-5} M in others. Since the

TABLE 3 EFFECT OF VARIOUS SUBSTANCES ON ENZYME ACTIVITY

Substance	Enzyme Activity
	% of control
Glucose-6-phosphate	96
Glyceryl phosphoryl choline	96
Phosphoryl choline	93
Ceramide (bovine heart)	96
Sphingosine phosphoryl choline	50
O-Acetylsphingomyelin	70
Lecithin (egg yolk)	90
erythro-Dihydrosphingosine*	80
threo-cis-Sphingosine*	45
threo-trans-Sphingosine*	35
erythro-cis-Sphingosine*	45
erythro-trans-Sphingosine	35
Hexadecylamine	50
Octylamine	105
Palmitic acid	40

60–70 μU of enzyme were incubated in the standard assay mixture (0.2 ml) with 1.5 mM dihydrosphingomyelin (substrate) and 1.5 mM test substance, except that only 0.5 mM ceramide was used.

* These synthetic compounds were the gift of Ciba Pharmaceutical Co.

substrate in the assay mixture is undoubtedly in the form of micelles, at least in part, the true concentration of the reactive species, be it monomer or even a unit of the micelle, is not known. Hence, our K_m, calculated from total substrate amounts, remains only an "apparent K_m."

The upper line in Fig. 3 is the result of an experiment in which a concentration of 0.50 mM sphingomyelin was present in each of a series of assay mixtures, together with various amounts of dihydrosphingomyelin. From this line the K_i of sphingomyelin treated as a competitive inhibitor can be determined. If such an inhibitor is actually also a substrate for the enzyme, as is sphingomyelin (see above), the K_i becomes equivalent to the K_m for that substrate. Theoretically, the two lines in Fig. 3 should meet at the ordinate; the discrepancy found may indicate a noncompetitive inhibition, due perhaps to an impurity in the sphingomyelin preparation. If this is regarded as the result of a separate noncompetitive inhibitor, the calculated K_m for sphingomyelin is 3.8×10^{-5} M. If, on the other hand, the intercept on the ordinate is accepted as indicating the true V_{max}, the K_m for sphingomyelin is 3.7×10^{-5} M. Thus, the error that may occur because the lines do not meet is small. At any rate, the K_m values of the enzyme for the saturated and unsaturated substrates are not significantly different.

Effect of Certain Related Compounds

The effects of several compounds on enzyme activity are listed in Table 3. The substances that were not water-soluble were added to the assay mixture before the enzyme and dispersed by mechanical agitation (sonication in the case of ceramide) and heating to about 65°C. Upon such treatment all mixtures formed clear solutions except for those containing ceramide, dihydrosphingosine, or palmitic acid; these last three formed stable fine dispersions.

In the absence of other information, the results of these trials must be considered of empirical value only, since the mechanism of inhibition is not known: it could be either an effect on the enzyme or an effect on the micellar disposition of the substrate. Hexadecylamine is an amphipathic molecule at pH 5 and is known to be surface-active. Similarly, the sphingosines, which are long-chain amines, should also be surface-active. The smaller effect of dihydrosphingosine could be attributed to its lower solubility compared with that of the unsaturated sphingosines. That amines in general do not inhibit the enzyme is shown by the inactivity of octylamine.

It would be most interesting to know if sphingosine phosphoryl choline and *O*-acetylsphingomyelin actually compete for an enzyme site with the substrate they resemble, but here again a surface-active effect on the substrate cannot be excluded.

Species and Organ Distribution

Sphingomyelinase activity under our standard assay conditions has been found in various tissues, the enzyme levels of which are listed in Table 4. The tissues were all assayed as crude homogenates prepared in 0.01 M Tris buffer, pH 8. The human spleens had all been frozen for 2 wk–10 months before assay but no correlation of activity with this period of storage was observed. In a few instances assays were done on fresh spleen and later after freezing and showed no significant differences. Two spleens which were fibrotic and enlarged as an effect of portal hypertension had activities of 220 and 170 μU/mg of protein, well within normal limits.

The Enzyme in Niemann-Pick Disease

Sphingomyelinase activity was measured in homogenates of a number of spleens from cases of Niemann-Pick disease and, as controls, in some other cases of infiltrative disease. Table 5 lists the time for which the spleens were

TABLE 4 SPHINGOMYELINASE ACTIVITY IN VARIOUS ORGANS

Tissue	Activity
	μU/mg protein
Rat liver	1300
Rat spleen	270
Rat kidney	690
Chicken liver	100
Human liver	240
Human kidney	430
Human spleen*	200 (140–290)

* Activity given is the mean of six spleens and the range is indicated in parentheses.

TABLE 5 SPHINGOMYELINASE ACTIVITY IN SPLEENS OF NIEMANN-PICK DISEASE

Patient	Age	Time Frozen	Sphingomyelin	Sphingomyelinase Observed	Sphingomyelinase Corrected
	yr	*yr*	*mμmole/mg protein*	*μU/mg protein*	
Type A ("classical")					
S. Sp.	1.6	3	979	0.4	1.0
R. N.	1	11	679	0.4	1.4
Type B ("visceral")					
S. So.	11	6	712	1.2	3.0
J. K.	3.7	6	645	4.2	7.6
J. P.	25	3.5	508	2.0	3.3
Type C ("late infantile")					
D. K.	6	4	230	154	181
P. M.	5.7	3	134	73	88
C. A.	3	11	153	120	152
L. W.	4.7	1.5	314	188	254
A. L.*	0.7	0.7	144	100	117
Type D ("Nova Scotia")					
A. B.	7	8.5	253	27	36
Gaucher's disease					
C. Z.	1.3	10		65	
P. B.	1.3	2.5		83	
K. S.	6.2	8		124	
Hunter-Hurler syndrome					
L. L.	10.9	2.5		201	
D. K.	7.5	0.1		181	

* Considered atypical by Dr. A. Crocker but best described as type C.

frozen prior to assay, their sphingomyelin content, and the enzyme activity. In the Niemann-Pick cases the sphingomyelinase activity could, theoretically, be corrected to account for substrate dilution in the assay system by the endogenous tissue sphingomyelin. However, under the conditions of the assay a considerable fraction of the endogenous sphingomyelin does not go into solution but remains particulate, and hence does not equilibrate with the labeled substrate. Therefore, the actual enzyme activity lies between the observed activity and the "corrected" activity. For this reason, both values are given in the table.

These spleens are all from the collection of Dr. Allen Crocker and are listed in the order of his classification (15). The enzyme levels in type A, the classical infantile Niemann-Pick disease, are not significantly different from zero in our assay. Type B disease, characterized by the same visceral involvement as type A but with a normal nervous system, shows enzyme levels that are very low (and hence imprecise), but still significantly above zero. Type C disease appears in late infancy with neurological and visceral disturbances similar to those in type A but with less visceral sphingomyelin accumulation. These cases all have normal or only mildly depressed enzyme levels. Type D disease is a variant found in several Nova Scotian families that is characterized by onset in middle childhood and death in adolescence. The case we assayed had a lower than normal enzyme content.

The type C and D Niemann-Pick cases, the Gaucher's cases, and those of the Hunter-Hurler syndrome all serve as controls for the type A and B cases. They show that neither prolonged freezing nor general infiltration of the spleen can account for the virtual absence of enzyme activity in type A and B disease. In particular, the type C cases show that the mere presence of sphingomyelin itself in the spleen does not lead to a low assay.

The presence of an inhibitor was disproved by the addition of partially purified enzyme to the system for assay of Niemann-Pick spleens; in each case the increment of activity caused by the added enzyme was no less than expected.

The possibility exists that in type A and B disease the enzyme is present but is somehow occluded or complexed by the sphingomyelin, especially by the part insoluble in the assay system, and thus not measurable. This possibility was excluded as follows. Spleen homogenates were lyophilized and then extracted with absolute ethanol at −5°C. The residue was suspended in 0.01 M Tris, pH 8, containing 2 mg/ml of Triton X-100, and incubated for 10 min at 37°C. This preparation was then centrifuged at 100,000 *g* for 20 min, and the supernatant fraction and sediment were assayed for enzyme. This treatment extracted 80–95% of the sphingomyelin into the ethanol; 2–10% remained in the sediment, and the remaining 2–10% was found in the supernatant fraction. This procedure solubilized more than 97% of the enzyme present in type C spleens. In type B spleens, essentially all of the small amount of enzyme present was also solubilized, but in both types A and B enzyme activity did not significantly increase. Extraction of most of the sphingomyelin should have released at least some enzyme were it complexed to the lipid. That no extra activity was observed seems good evidence that no occult enzyme was present in either type A or B spleens.

It was striking, on the other hand, to find that at pH 7.4 a sphingomyelinase activity could be demonstrated in spleens of type A and B disease. The presence of Mg^{++} ion was required for this activity. The nature of this enzyme is not clear at present but preliminary studies suggest it to be a separate entity, similar to a Mg^{++}-dependent microsomal sphingomyelinase (phospholipase C) which we have found in chicken liver. The levels of this enzyme in type A and B spleens appear to be well above the levels of this enzyme in normal spleens.

DISCUSSION

A deficiency of sphingomyelinase activity in the livers of classical (type A) Niemann-Pick disease has been

recently reported (7). Our findings in the spleen confirm the lack of this enzyme in such cases. We have also found a marked reduction of the enzyme in a group of patients (type B) who have visceral sphingomyelin accumulation but no neurological defects. On the other hand, the enzyme is present in normal or less drastically reduced amounts in the late infantile (type C) and "Nova Scotian" (type D) disease. The deficiency of enzyme in type A and B disease adequately explains the abnormal visceral accumulation of sphingomyelin in those cases. In turn, the accumulation of that lipid in the absence of the enzyme serves as evidence that this enzyme is physiologically important and that the first step of sphingomyelin catabolism in the human is a breakdown to ceramide and phosphoryl choline.

We have found higher than normal levels of a Mg^{++}-dependent sphingomyelinase in spleens of type A and B disease and this may represent some sort of compensatory mechanism for lack of the primary enzyme. It is evident, however, that the Mg^{++}-dependent enzyme fails to prevent sphingomyelin accumulation.

The question then becomes, why does sphingomyelin accumulate in type C and D disease, in which enzyme levels are not depressed? Synthesis of a substance at rates above normal is a rare cause of biochemical lesions and although this is not definitely excluded in these cases, decreased degradation is more likely. Faulty disposal of sphingomyelin in the presence of adequate amounts of the catabolic enzyme could be due to an enzyme inhibitor, but our studies in vitro have not disclosed any inhibition by tissue homogenate. Another possibility is that enzyme–substrate interaction is prevented by some derangement of cellular organization or lack of some cofactor. In relation to this last possibility we can point out only that our enzyme–substrate interaction in vitro requires detergent and that theoretically a lack of some similar natural substance, such as lipoprotein, might prevent interaction in vivo.

The relation between sphingomyelin metabolism and the cerebral defect in Niemann-Pick disease remains unresolved since cerebral sphingomyelin accumulation is not abnormal in this disease, except in the gray matter of type A cases, and then only moderately so. One could hypothesize that a slower cerebral turnover of sphingomyelin causes less accumulation there than in the viscera of type A cases. By the same token, in type B disease a slow cerebral turnover may permit very small, though definite, amounts of enzyme to keep pace with the demands of sphingomyelin catabolism and avoid neurological lesions. In type C and D disease the cerebral as well as the visceral defect remains unclear. It is also possible that in all Niemann-Pick cases the visceral and cerebral aspects are associated though distinct phenomena, as has been suggested (15).

The recently described rat liver sphingomyelinase (5, 6) appears to be similar to the human spleen enzyme described in this report, insofar as it is specific for sphingomyelin and dihydrosphingomyelin, has a pH optimum of about 5, and is primarily located in the subcellular particles sedimenting between 600 and 9000 *g*. On the other hand, the rat liver enzyme has a reported K_m for sphingomyelin between $1.8–9 \times 10^{-4}$ M (5, 6), whereas the human enzyme exhibits a K_m of 3.8×10^{-5} M. The human spleen enzyme is markedly inhibited by phosphate ion and is hardly affected by lecithin while the rat liver enzyme (5) appears insensitive to phosphate but is competitively inhibited by lecithin. These significant differences suggest that these enzymes are not identical.

We are indebted to Dr. Marilynne Swanson for samples of normal spleens and to Dr. Allen Crocker for the lipidotic spleens.

This work has been supported by grants from the U. S. Public Health Service, National Institute of Neurological Diseases and Blindness, NB-02946 and the National Institute of General Medical Sciences, GM-13952, and the Life Insurance Medical Research Foundation.

Manuscript received 31 October 1966; accepted 18 January 1967.

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