Partial purification and properties of acid sphingomyelinase from rat liver

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Abstract Acid sphingomyelinase was purified approximately 5,200-fold from the mitochondria-lysosome-enriched particles of rat liver by sequential chromatography on DEAE-cellulose, octyl-Sepharose, Sephacryl S-300, Concanavalin A-Sepharose, and CM-cellulose. The specific activity of this highly purified enzyme was 3.2 mmol per hr per mg protein. The enzyme was active against 2-hexadecanoylamino-4-nitrophenylphosphorylcholine, but bis-4-methylumbelliferyl-phosphate and bis-*p*-nitrophenyl-phosphate were poor substrates. The preparation was free of Mg^{2+}-dependent neutral sphingomyelinase and eight lysosomal enzymes except for the trace amount of acid phosphatase and β-galactosidase. Apparent molecular weight of the enzyme was 200,000, estimated by Sephadex G-200 filtration in 0.1% Triton X-100. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed three major bands corresponding to molecular weights of 45,600, 44,500, and 40,000 with several minor bands. Characterization of the enzyme revealed almost the same properties as those of human tissues reported by other investigators, including pH optimum, requirement of Triton X-100, effects of metal divalent cations, phosphate ion, EDTA, some thiol blocking reagents, and amphophilic drugs.—**Watanabe, K., N. Sakuragawa, M. Arima, and E. Satoyoshi.** Partial purification and properties of acid sphingomyelinase from rat liver. *J. Lipid Res.* 1983. **24:** 596–603.

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Acid sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12) is an acidic lysosomal enzyme that catabolizes sphingomyelin to phosphorylcholine and ceramide. Niemann-Pick disease (Types A and B) is an inherited lysosomal storage disease, characterized enzymatically by a deficiency of acid sphingomyelinase (1). The purification of this enzyme from human tissues has been reported by several investigators (2–9) but there have been few investigations (10, 11) in regard to the purification and properties of this enzyme from tissues of rats.

Recently, animal models and drug-induced lipidosis mimicking lysosomal storage diseases have been reported, and these models have made possible basic studies concerning the pathogenesis of Niemann-Pick disease and enzyme replacement therapy. Sakuragawa et al. (12) reported previously that the administration of AY 9944, a potent hypocholesterolemic agent, to the suckling rats caused a specific reduction of sphingomyelinase in various tissues and organs with no effects on other lysosomal enzymes and phosphodiesterase. Sphingomyelin accumulated in these animals; it contained fatty acids less than 20 carbon atoms in length. In addition, animal models have been discovered in mice (13) and poodles (14) which appear to be useful animal analogues for investigating the pathogenesis of Niemann-Pick disease.

The present report describes the purification procedures of acid sphingomyelinase from rat liver and some properties of the purified enzyme. This type of experiment would be useful for animal experimentation designed to evaluate the pathogenesis of the animal models and drug-induced lipidosis.

MATERIALS AND METHODS

Reagents

[^{14}Cmethyl]choline-labeled sphingomyelin (45 mCi/mmol) was purchased from New England Nuclear Corp. It was diluted with cold sphingomyelin, obtained from Sigma Chemical Company, to a specific activity (0.15 mCi/mmol or 0.06 mCi/mmol. A chromogenic substrate, 2-hexadecanoylamino-4-nitro-phenylphosphorylcholine (HNP) was the product of Koch-Light Laboratories. Bovine and human serum albumin, α-methylmannoside, and bis-*p*-nitrophenyl-phosphate were purchased from Sigma Chemical Company. Bis-4-MU-phosphate, 4-MU-phosphate, and other 4-MU-glycoside derivatives were the products of Sigma Chemical Com-

Abbreviations: HNP, 2-hexadecanoylamino-4-nitrophenylphosphorylcholine; SDS, sodium dodecyl sulfate; 4-MU, 4-methylumbelliferyl.

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pany and Koch-Light Laboratories. DEAE-cellulose (DE 52) and CM-cellulose (CM 52) were obtained from Whatman Ltd., and octyl-Sepharose, Sephacryl S-300, and Concanavalin A-Sepharose were from Pharmacia Fine Chemicals. Other reagents were purchased from Wako Pure Chemicals.

Enzyme assays

Sphingomyelinase activity was determined by two procedures with [^{14}C]sphingomyelin. This enzyme activity in the chromatographic eluate was routinely assayed by using [^{14}C]sphingomyelin (0.15 mCi/mmol) according to the method of Pentchev et al. (13). The following method was used for the quantitative assay of sphingomyelinase. [^{14}C]Sphingomyelin (0.06 mCi/mmol), 200 nmol in 0.01 ml of methylene chloride–methanol 2:1 (v/v), was put in a small test tube and taken to dryness at 40°C under N_2. After addition of 0.02 ml of 1% (w/v) Triton X-100 and 0.1 ml of 0.2 M sodium acetate (pH 5.0), the mixture was dispersed by ultrasonication (water-bath) for 1 min at room temperature. The incubation mixture contained 0.02 ml of 20 mg/ml bovine serum albumin and enzyme solution in addition to the above solution (total volume, 0.2 ml). The incubation was carried out for 30 min at 37°C and the released [^{14}C]phosphorylcholine was measured by the methods of Pentchev et al. (13). Sphingomyelinase activity against HNP was measured by the method of Gal et al. (15) in the presence of 12.5 mM HNP and 2 mg/ml bovine serum albumin. Acid phosphodiesterase activity was measured according to the methods of Callahan, Lassila, and Philippart (16) by using bis-*p*-nitrophenylphosphate in the presence of 0.1% Triton X-100 and 2 mg/ml of bovine serum albumin. Phosphodiesterase activity (using bis-4-MU phosphate) was measured by the method of Besley (17) in the presence of 0.1% Triton X-100 and 2 mg/ml of bovine serum albumin. The other lysosomal enzyme activities were measured by using corresponding 4-MU-glycoside derivatives. The final concentration of the substrate was between 0.1 mM and 1.0 mM. The reaction mixture contained 0.08 M sodium acetate (pH 5.0), 0.1% Triton X-100, 2 mg/ml of bovine serum albumin, and it was incubated with enzyme solution for 30 min at 37°C. The reaction was stopped by addition of 2.5 ml of glycine-NaOH (pH 10.7) and the released 4-methylumbelliferon was measured by Hitachi type 204 S spectrofluorometer (Ex 365, En 448).

The method of Lowry et al. (18) was used for determining the protein content of the enzyme preparation which contained usually less than 0.1% Triton X-100 (19). The method of Bradford (20) was also used for protein determination of the eluants from the last step of the purification procedure. The bovine serum albumin in the extraction buffer was used as the protein standard.

Purification of sphingomyelinase

Preparation of mitochondria-lysosome-enriched particles. Adult Wistar rats, both male and female, were killed by decapitation and livers were removed and rinsed with cold 0.25 M sucrose containing 0.001 M EDTA (pH 7.0). All procedures were carried out at 4°C unless otherwise stated. The livers (360 g) were minced with a meat grinder and homogenized with 3 vols of 0.25 M sucrose containing 0.001 M EDTA (pH 7.0) with a Potter-Elvehjem homogenizer (three strokes). The homogenate was centrifuged at 2,400 rpm (900 *g*) for 10 min to remove the cell debris and nuclei. This material was homogenized once more with the same volume of the above solution. After centrifugation at 900 *g*, the combined supernatants were centrifuged at 9,500 rpm (15,000 *g*) for 30 min. The pellet was used for the source of sphingomyelinase preparation.

Solubilization and ammonium sulfate fractionation. The sample from the above procedure was resuspended in 1,100 ml of 0.01 M Tris-HCl buffer (pH 7.4), containing, 0.1% Triton X-100, stirred for 1 hr, and centrifuged at 100,000 *g* for 1 hr. Solid ammonium sulfate was added to the supernatant solution and stirred for 30 min. Final saturation of the ammonium sulfate was 50%. After centrifugation at 15,000 *g* for 30 min, the precipitate was dissolved in a minimum volume of extraction buffer and dialyzed against three changes of 5 liters of the same buffer (one change overnight and two changes for 4 hr each).

DEAE-cellulose column chromatography. The dialyzate was applied directly to the column of DEAE-cellulose (5.0 × 30 cm) that was equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100. The column was washed with 600 ml of the equilibration buffer and eluted with 600 ml of equilibrium buffer containing 0.1 M NaCl, followed by 600 ml of equilibrium buffer containing 0.25 M NaCl. The bulk of sphingomyelinase activity was eluted in the fractions containing 0.1 M NaCl. Sphingomyelinase-enriched fractions were combined and concentrated to 90 ml using an Amicon ultrafiltration cell with a PM 10 membrane.

Octyl Sepharose column chromatography. An octyl Sepharose column (3.0 × 19 cm) was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4). The concentrated enzyme preparation was applied directly to the column of octyl Sepharose. All of the sphingomyelinase was bound to this column under these conditions. This column was washed with 420 ml of the equilibration buffer and eluted with the same buffer containing 1.0% Triton X-100. The fractions (90 ml) containing sphingomyelinase activity were combined, concentrated to half volume,

and dialyzed overnight against 2 liters of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and 0.5 M NaCl. The dialyzate was further concentrated to the volume of 8.3 ml and applied to the column of Sephacryl S-300.

Sephacryl S-300 gel filtration. A Sephacryl S-300 column (2.6 × 100 cm) was pre-equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and 0.5 M NaCl. The column was eluted with the same buffer. The fractions containing enzymatic activity were combined (52 ml) and then dialyzed overnight against 2 liters of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.5 M NaCl, and 20% (v/v) glycerol.

Concanavalin A-Sepharose column chromatography. The dialyzate was applied to the column of Concanavalin A-Sepharose (1.0 × 2.0 cm) that was equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.5 M NaCl, and 20% glycerol. A recycling system was introduced for 40 hr with the flow rate of 30 ml/hr. After washing with 44 ml of equilibration buffer, the column was eluted with the same buffer containing 0.75 M α-methylmannoside. The temperature of the column was maintained at 25°C by the thermo-controlled water circulating through the column jacket. The fractions containing sphingomyelinase activity were combined (98 ml), concentrated by ultrafiltration, and dialyzed for 3 hr against two changes of 1 liter of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and 20% glycerol.

CM-cellulose column chromatography. One-half of the above dialyzate was redialyzed overnight against 1 liter of 0.01 M sodium acetate (pH 5.0) containing 0.1% Triton X-100 and 20% glycerol. The dialyzate was then concentrated to 2.3 ml by ultrafiltration and applied to the column of CM-cellulose (0.4 × 9 cm) which contained 0.1% Triton X-100 and 20% glycerol. The column was washed with 7.5 ml of the same buffer and eluted by linear gradient from 0 to 0.5 M NaCl in the same buffer. The fractions containing the highest activity were combined (1.65 ml) and dialyzed against 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and 20% glycerol.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. A part of the final enzyme solution (0.8 ml) was dialyzed against 1 liter of distilled water (one change overnight and one change for 4 hr). The dialyzate was lyophilized and the sample was then denatured by the method of Weber, Pingle, and Osborn (21). Electrophoresis was carried out in 10% polyacrylamide gel (0.6 × 8 cm) with 0.375 M Tris-HCl buffer (pH 8.8) containing 0.1% SDS. The current was constant at 3 mA per gel. The protein bands were stained with Coomassie Brilliant Blue G 250. The molecular weight was estimated using standard proteins of known molecular weight.

RESULTS

Purification of sphingomyelinase

The summary of the purification procedure is shown in **Table 1.** The maximum specific activity was 3,200 μmol of phosphorylcholine released per mg protein per hr with a 5,200-fold purification from the mitochondria-lysosome-enriched fraction. We took into account the following two points in our procedure. The purification was carried out mainly at neutral pH to avoid attack of the lysosomal proteases (cathepsins, etc.). Secondly, we used glycerol in the last two steps of the purification to stabilize the enzyme, because rat liver sphingomyelinase is relatively unstable in the enzyme solution containing lower amounts of protein.

We prepared the mitochondria-lysosome-enriched particle from 360 g of rat liver. Several other trials showed that 80–90% of sphingomyelinase activity in the total tissue was usually recovered. A higher specific activity was obtained in this first step, e.g., 0.619 μmol per mg protein per hr. Tris-HCl buffer was used in the whole procedure because the phosphate ion was inhib-

TABLE 1. Purification of sphingomyelinase from rat liver

Purification Step	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*μmol/hr*	*μmol/hr per mg protein*	*%*	*-fold*
15,000 *g* Particle	19,400	12,000	0.619	100	1
100,000 *g* Supernatant	12,800	11,200	0.875	93.3	1.41
$(NH_4)_2SO_4$ precipitate, 50% saturation	7,400	10,800	1.46	90.0	2.36
DEAE-cellulose	1,040	4,600	4.42	38.3	7.14
Octyl-Sepharose	423	2,330	5.51	19.4	8.90
Sephacryl S-300	115	2,250	19.6	18.8	31.7
Concanavalin A-Sepharose	7.1	1,240	175	10.3	283
CM-cellulose[a]	0.05	160	3,200	1.33	5,170

[a] Forty eight percent of Concanavalin A-Sepharose fraction was used.

itory to sphingomyelinase activity (3); this was confirmed by our study. The enzyme was solubilized by 0.1% Triton X-100 and precipitated by ammonium sulfate. Approximately 90% of total activity could be recovered by these procedures. Sphingomyelinase was bound to DEAE-cellulose and was eluted by the elution buffer containing 0.1 M NaCl and 0.1% Triton X-100. All of the sphingomyelinase activity was bound to the octyl-Sepharose even in the presence of 0.1% Triton X-100 and was eluted by equilibration buffer containing 1.0% Triton X-100. The specific activity was not appreciably increased by this step. Concanavalin A-Sepharose chromatography was used after the Sephacryl S-300 gel filtration. The recycling step was used and elution was carried out at 25°C. About 90% of total activity bound to the column. The purification was increased about 9 times with a 60% yield from the previous step. The specific activity was 175 μmol per mg protein per hr. The purification procedure up to this point was very reproducible; the procedure was performed several times using different amounts of rat liver.

In a preliminary study using CM-cellulose for the last step of the purification procedure, a specific activity of 690 μmol per mg protein per hr with a 1.94% yield was obtained (**Fig. 1**). We modified the original procedure by using larger volumes of the washing buffer, a less steep linear gradient system, and a longer column. With this procedure the highest specific activity, 3,200 μmol per mg protein per hr, was achieved, even though the yield was rather low. In another experiment, we tried Blue Sepharose for the final step of purification; this resulted in a specific activity of 235 μmol per mg protein per hr, with 16.4% yield and 366-fold purification from the starting solution. For the protein determinations, we took into consideration the results of Wang and Smith (19) who noted that accurate protein quantification can be obtained in samples containing as much as 1% Triton X-100. The protein content for the final step was determined by the method of Lowry et al. (18) and by the method of Bradford (20). The value in Table 1 was that of the Lowry method; the value by the Bradford method was 80% of the Lowry value.

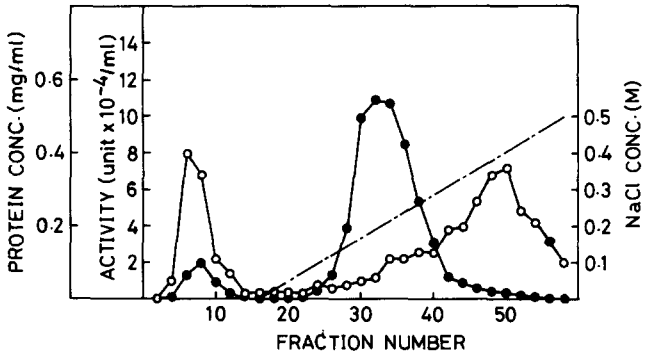

Fig. 1. CM-cellulose column chromatography of partially purified sphingomyelinase of rat liver. Experimental details are described in Materials and Methods. Closed circles indicate sphingomyelinase activity; open circles, protein; and the dotted line, the concentration of NaCl.

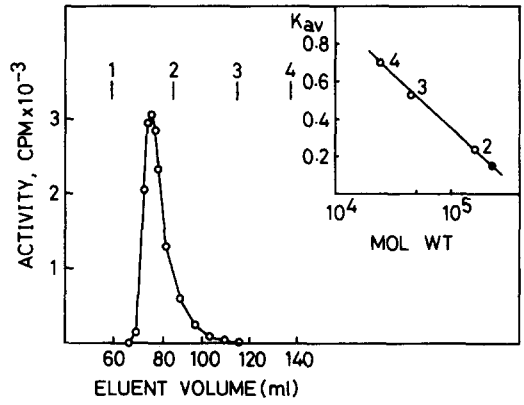

Fig. 2. Sephadex G-200 gel filtration of rat liver sphingomyelinase. Gel filtration was carried out on an analytical column of Sephadex G-200, equilibrated with 0.01 M Tris-HCl buffer (pH 7.2) containing 0.1% Triton X-100. Partially purified sphingomyelinase from the $(NH_4)SO_4$ precipitate was used for the molecular weight estimation. Molecular weight standards: 1, blue dextran; 2, gamma globulin (Mr 160,000); 3, ovalbumin (Mr 45,000); 4, chymotrypsinogen (Mr 24,500). The open circles indicate the sphingomyelinase activity. The inset shows the estimation of the molecular weight of sphingomyelinase (Mr 220,000) from the elution pattern using molecular weight standards.

Enzyme purity and molecular weight of sphingomyelinase

The purified sphingomyelinase preparation contained trace amounts of acid phosphatase and β-galactosidase activities. There was no activity of eight other lysosomal enzymes including α-mannosidase, α-galactosidase, α-fucosidase, β-glucuronidase, β-glucosidase, β-xylosidase, α-arabinosidase, and N-acetyl-β-glucosaminidase. The molecular weight of the rat liver sphingomyelinase was estimated by Sephadex G-200 gel filtration in the presence of 0.1% Triton X-100 by using the partially purified sample from step 3. The apparent molecular weight was estimated to be 220,000 (**Fig. 2**). SDS gel electrophoresis of the purified sphingomyelinase preparation gave three major bands and several minor bands (**Fig. 3**). The molecular weights of the major protein bands were estimated to be approximately 45,600, 44,500, and 40,000.

Properties of purified sphingomyelinase

In order to investigate the substrate specificity of the purified rat liver sphingomyelinase, we examined enzyme activity against several substrates of sphingomyelinase and phosphodiesterase (**Table 2**). The chro-

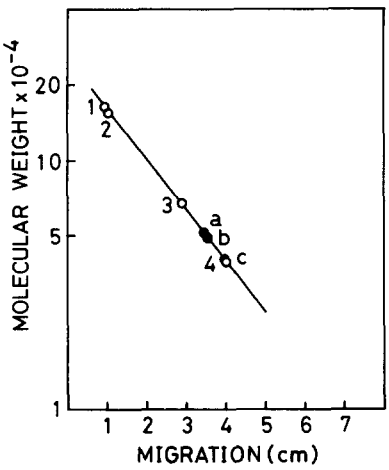

Fig. 3. SDS polyacrylamide gel electrophoresis of purified rat liver sphingomyelinase. Experimental details are given in Materials and Methods. Protein standards: 1, RNA polymerase (Mr 165,000); 2, RNA polymerase (Mr 155,000); 3, bovine serum albumin (Mr 68,000); 4, RNA polymerase (Mr 39,000). Final purified preparation after CM-cellulose chromatography: the three major bands of (a), (b), and (c) represent molecular weights of 45,600, 44,500, and 40,000, respectively.

mogenic sphingomyelin analogue (HNP) was also hydrolyzed by the purified sphingomyelinase. The K_m for [^{14}C]sphingomyelin and HNP were 7.9×10^{-5} M and 2.7×10^{-4} M, respectively. The specific activities against bis-4-MU-phosphate and bis-*p*-nitrophenyl-phosphate were quite low. Purified sphingomyelinase had a pH optimum at about 5.0. The effect of Triton X-100 was examined by addition of various amounts of Triton X-100 to the assay mixture. Sphingomyelinase was activated at a very low concentration of Triton X-100 (0.075%). The degree of activation did not change significantly up to a concentration of 2.0%. The activity of purified sphingomyelinase was linear up to 0.03 μg of protein. Sphingomyelinase activity was also linear with time up to 60 min.

Divalent ions (Mg^{2+}, Mn^{2+}, and Ca^{2+}, 5–50 mM) and EDTA (5–25 mM) had no effect on the sphingomyelinase activity. The activity at pH 7.5 was not stimulated by the addition of 5 mM Mg^{2+} to the assay mixture, which indicated that the purified enzyme was free from Mg^{2+}-dependent neutral sphingomyelinase (**Table 3**). The effect of thiol blocking reagents on purified sphingomyelinase was examined according to the method of Rao and Spence (22). A single concentration for each compound, except for *p*-hydroxymercury-benzoate, was used in order to obtain preliminary information. Sulfhydryl reagents (1.0 mM) and β-mercaptoethanol (10 mM) showed no effect on sphingomyelinase, but dithiothreitol (10 mM) inhibited the enzyme activity to 30% of the control value (**Table 4**).

In addition, we examined the effects of several drugs on the purified sphingomyelinase because these drugs decrease sphingomyelinase activity in various tissues of animals when administered intraperitoneally. AY 9944 and phenethylbiguanide in the range of 0.5–5 mM had no effect on the purified sphingomyelinase in vitro. Chloroquine (5 mM) as a form of diphosphate salt inhibited the enzyme activity about 50%. The inhibitory reaction by chloroquine is possibly due to the phosphate ion itself because the sphingomyelinase was inhibited by the same amount of phosphate ion as that of chloroquine (**Table 5**).

DISCUSSION

To date, highly purified preparations of lysosomal sphingomyelinase have been obtained only from human tissues (3, 4, 6, 7). One of the present authors (9) reported the purification and properties of acid sphingomyelin of human placenta. Properties of the sphingomyelinase from rat tissues have been studied only with crude or partially purified enzyme preparations (10, 11). The highly purified sphingomyelinase of rat tissues could be useful in studies of the animal models of Niemann-Pick disease (12). We purified the sphin-

TABLE 2. Enzymatic activities in the purified sphingomyelinase preparations

Enzyme	Substrate	Assay Condition		Specific Activity	K_m
		pH	Triton		
			%(w/v)	μmol/hr per mg protein	
Sphingomyelinase	sphingomyelin	5.0	0.1	3,200	7.0×10^{-5} M
	2-hexadecanoylamino-4-nitrophenylphosphorylcholine	5.5	0	1,220	2.7×10^{-4} M
	bis-4-MU-phosphate	4.5	0.1	27.4	
	bis-*p*-nitrophenyl-phosphate	5.0	0.1	40.5	
Acid phosphatase	4-MU-phosphate	5.0	0.1	2.41	
β-Galactosidase	4-MU-β-galactosidase	5.0	0.1	0.103	

No activity of the following enzymes was detected: α-mannosidase, α-galactosidase, α-fucosidase, β-glucuronidase, β-glucosidase, β-xylosidase, α-arabinosidase, and N-acetyl-β-glucosaminidase.

TABLE 3. Effect of some ions and EDTA on the activity of purified rat liver sphingomyelinase

Assay Condition	Addition	mM	Relative Specific Activity
pH 5.0	none		100
	Mg^{2+}	5	102
		50	110
	Mn^{2+}	5	106
		50	107
	Ca^{2+}	5	104
		50	104
	PO_4^{3-}	5	62.6
		50	18.7
	EDTA	5	107
		25	104
pH 7.5	none		100
	Mg^{2+}		96.3

TABLE 4. Effect of some thiol-related reagents on the activity of purified rat liver sphingomyelinase

Addition	mM	Relative Specific Activity
None		100
Iodoacetamide	1.0	105
N-ethylmaleimide	1.0	104
p-Chlormercury-benzoate	0.1	96.4
	1.0	96.8
Dithiothreitol	10	67.9
β-Mercaptoethanol	10	102

gomyelinase approximately 5,200-fold from the mitochondria-lysosome-enriched particles from rat liver. The most highly purified preparation had a specific activity of 3.2 mmol per mg protein per hr. This value is approximately 8-fold higher than that of human placenta (9) and approximately 40-fold higher than that of human brain (7); this indicates that the rat liver enzyme has a higher catalytic activity than those of human origin. Very recently, Yamanaka and Suzuki (8) obtained a highly purified homogeneous sphingomyelinase from human brain with a specific activity of up to 800 μmol per mg protein per hr. Although our enzyme preparation was still not homogeneous by SDS gel electrophoresis, this highly purified sphingomyelinase could be used to study some of the properties of the rat liver enzyme.

This purification procedure represents final modifications of several trials including the procedure of several investigators (3, 4, 6, 9). The yield after the Concanavalin A-Sepharose step as described by Yamaguchi and Suzuki (4) was very low in our study. Our modification of the purification included the use of Concanavalin A-Sepharose at the next to the last step and a stepwise elution of DEAE-cellulose and octyl-Sepharose column. In addition, we used glycerol for enzyme stabilization in the last two steps. CM-cellulose was quite useful for this enzyme purification procedure but column condition should be carefully optimized as discussed in Materials and Methods.

SDS gel electrophoresis of the highly purified sphingomyelinase showed three major protein bands corresponding to molecular weights of 45,600, 44,500, and 40,000, and several additional minor bands. Pentchev et al. (3) reported that sphingomyelinase purified from the human placenta was composed of two different molecular subunits corresponding to molecular weights of 36,800 and 28,300. Jones, Shankaran, and Callahan (6) reported that their preparation gave a major band (mol wt 89,100) and two distinct minor components (mol wt 47,500 and 30,800) on SDS gel electrophoresis when reduced and alkylated. Sakuragawa (9) reported that SDS gel electrophoresis revealed two distinct protein bands with molecular weights of 70,500 and 39,800 for the acid sphingomyelinase of human placenta. Yamanaka and Suzuki (8) reported a single protein band with a molecular weight of 70,000 on SDS gel electrophoresis of highly purified human brain sphingomyelinase. The different polypeptides represented by various investigators can be divided into low and high molecular weight components. Three major components of our preparation could represent the low molecular weight components of less than 50,000 daltons. Judging from the single protein band of human brain sphingomyelinase reported by Yamanaka and Suzuki (8), our sphingomyelinase preparation might be still contaminated. However, different molecular weights may indicate species or organ differences. The molecular weight estimation by Sephadex G-200 in the presence of Triton X-100 has been studied by several investigators (3, 7, 23). There must be some limitation to estimation of molecular weights by this method because Triton X-100 and the protein complex would affect behavior on gel filtration. Yedgar and Gatt (24) reported that the rate of hydrolysis of sphingomyelin by partially purified sphingomyelinase of rat brain depended on the concentration of sphingomyelin and Triton X-100. The peak of sphingomyelinase activity at the fixed concentration

TABLE 5. Effect of some drugs on the activity of purified rat liver sphingomyelinase

Drug	mM	Relative Specific Activity
None		100
AY 9944	0.5	102
	5.0	107
Chloroquine	0.5	95.8
	5.0	50.4
Phenethylbiguanide	0.5	101
	5.0	91.1
PO_4^{3-}	1.0	95.7
	10.0	51.0

of Triton X-100 was reached when the ratio between Triton X-100 and sphingomyelin was 4. According to our assay conditions and using the fixed concentration of sphingomyelin, sphingomyelinase activity reached the peak when this ratio was 0.12 and did not change at a ratio of 3.1. This discrepancy may result from the materials used because Yedgar and Gatt (24) used the enzyme purified about 100-fold. The highly purified sphingomyelinase appeared to need a lower concentration of Triton X-100 at the fixed concentration of the substrate.

As to substrate specificity of the purified sphingomyelinase, Pentchev et al. (3) and Sakuragawa (9) reported that the human placental sphingomyelinase hydrolyzed HNP to the same degree as the natural substrate. The purified sphingomyelinase was possibly less active against HNP, in good agreement with the finding of Yamanaka and Suzuki (8). This discrepancy could be suggestive of the presence of species and organ specificity, isozymes, or possibly a phosphodiesterase active against HNP (8). Bis-*p*-nitrophenyl-phosphate appeared to be a poor substrate for the purified sphingomyelinase, in good agreement with the finding of Pentchev et al. (3) and Yamaguchi (4). But Jones et al. (6) and Yamanaka and Suzuki (8) obtained a considerable amount of co-purified enzyme activity toward the bis-*p*-nitrophenylphosphate. Besley (17) noted that the fluorimetric method using bis-4-MU-phosphate was sensitive for the assay of sphingomyelinase activity in the diagnosis of Niemann-Pick disease. Jones et al. (6) and Yamanaka and Suzuki (8) reported that the purified sphingomyelinase was half as active toward this substrate as toward a natural sphingomyelin. Our purified sphingomyelinase was less active toward this substrate. Furthermore, we found in another purification procedure that the bulk of sphingomyelinase was not co-eluted with the activity toward bis-4-MU-phosphate on Sephacryl S-300 and DEAE-cellulose column chromatography.[2] This may indicate the presence of a phosphodiesterase toward this substrate.

The in vivo effect of AY 9944, an inhibitor of 7-dehydrocholesterol reductase, was described by Sakuragawa et al. (12). The injection of AY 9944 caused a specific reduction in sphingomyelinase activity in various tissues of rats without any effect of the phosphodiesterase activity toward bis-*p*-nitrophenylphosphate (12, 25). In additional studies using structurally related compounds such as chloroquine and phenethylbiguanide, the sphingomyelinase activity of various tissues from experimental rats was reduced, although to a lesser extent than with AY 9944.[2] However, AY 9944 and phenethylbiguanide did not inhibit the purified rat liver sphingomyelinase in vitro, which suggested that these agents did not attack the enzyme directly. The other properties of the purified sphingomyelinase were similar to those of partially purified sphingomyelinase from rat and human tissues (3, 4, 22). Even though the present procedure could be modified further to obtain homogeneous sphingomyelinase, the highly purified enzyme of rat liver could be useful for the study of the pathogenesis of drug-induced lipidosis in animal models.

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[2] Watanabe, K. Unpublished data.

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