Sphingomyelinase activity at pH 7.4 in human brain and a comparison to activity at pH 5.0

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Abstract A hitherto undescribed sphingomyelinase (sph'ase 7.4) of human brain has been studied in crude and partially purified (3- to 4-fold) extracts of grey matter, and compared to the known sphingomyelinase with an acid pH optimum (sph'ase 5.0). Its specificity for sphingomyelin as substrate is similar to that of sph'ase 5.0, but it differs from sph'ase 5.0 in its pH optimum (7.4 vs 5.0) and in a requirement for Mg^{2+} for optimal activity. Other properties of sph'ase 7.4 that distinguish it from sph'ase 5.0 include (*a*) its lack of appreciable solubilization during dialysis of crude homogenates; (*b*) a more marked concentration in grey matter than in white matter (9- to 13-fold vs 1.5- to 2-fold for sph'ase 5.0); (*c*) inhibition by Ca^{2+} and Cd^{2+} ions, and by EDTA; (*d*) stimulation by dithiothreitol, and inhibition by cysteine, *N*-ethylmaleimide, and *p*-hydroxymercuribenzoate; (*e*) lack of inhibition by nucleotides (AMP, ADP, and ATP) and by NAD plus NADH; and (*f*) relative instability to storage or manipulation between −20°C and 40°C. These differences indicate that sph'ase 7.4 is a different enzyme protein from sph'ase 5.0. Unlike sph'ase 5.0, which is widely distributed in mammalian tissues, sph'ase 7.4 occurs predominantly in grey matter and little activity was observed in spleen, liver, or leukocytes. The high levels of this enzyme in brain suggest a role related to the specific functions of this organ or to the need for a more stringent control of sphingomyelin catabolism in brain as compared to other organs.

Supplementary key words lipid metabolism · sphingolipid metabolism · dithiothreitol · liver · spleen · kidney · leukocytes · sphingomyelin hydrolysis · grey matter

Previous reports on sphingomyelin-cleaving activity in mammalian tissues have dealt almost exclusively with an enzyme that has maximal activity in vitro at pH 5.0 (sph'ase 5.0, refs. 1–10). Sphingomyelin hydrolysis maximal at neutral or alkaline pH has also been reported. Nilssen (11), who described a sphingomyelin-cleaving activity at alkaline pH that is enriched in brush-border preparations of rat intestinal mucosa, also demonstrated activity in human duodenal contents and meconium and suggested that this enzyme degrades dietary sphingomyelin. Schneider and Kennedy (4) briefly mention a Mg^{2+}-stimulated sphingomyelin-cleaving activity at pH 7.4 that was elevated above normal levels in spleens of patients with types A and B Niemann-Pick disease. The latter have a marked deficiency in sph'ase 5.0 activity (4, 10). A sphingomyelinase from chicken erythrocyte plasma membranes with optimal activity between pH 7.0 and 9.0 has been reported; activity could not be detected in human erythrocytes (12).

To our knowledge the present report is the first to describe detailed studies of mammalian sphingomyelinase from normal human tissues that has maximal activity in vitro at pH 7.4 (sph'ase 7.4) and is enhanced by Mg^{2+}. Evidence is presented that the primary substrate for this enzyme is sphingomyelin, and that the activity resides in a different protein from that which hydrolyzes sphingomyelin at acid pH (sph'ase 5.0).

MATERIALS AND METHODS

[^{3}H]Sphingomyelin was prepared by New England Nuclear Corp. (Boston, Mass.) by reductive tritiation of bovine brain sphingomyelin (Sigma Chemical Co., St. Louis, Mo.). Initial supplies were a generous gift of Dr. J. A. Lowden, Hospital for Sick Children, Toronto, Ontario. The [^{3}H]sphingomyelin was repurified monthly by preparative thin-layer

Abbreviations: bis-*p*NPP, bis-*para*-nitrophenylphosphate; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GPC, glycerylphosphorylcholine; Pel extract, partially purified fraction enriched in sphingomyelinase activity at pH 7.4; Sn extract, partially purified fraction enriched in sphingomyelinase activity at pH 5.0; sph'ase, sphingomyelinase [E.C.3.1.4.12; sphingomyelin cholinephosphohydrolase]; TLC, thin-layer chromatography.

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chromatography (13) in $CHCl_3$–CH_3OH–H_2O–CH_3COOH 70:30:4:1 (by volume—solvent system A) followed by silicic acid column chromatography (14, 15), using a discontinuous gradient of CH_3OH in $CHCl_3$. Sphingomyelin was eluted with $CHCl_3$–CH_3OH 1:4 (by volume).

The material was identified as >98% [^{3}H]sphingomyelin by TLC in four solvent systems: $CHCl_3$–CH_3OH–7N NH_4OH–H_2O 85:15:0.5:0.5 (B); $CHCl_3$–CH_3OH–CH_3COOH 94:2:4 (C); $CHCl_3$–CH_3OH–H_2O 14:6:1 (D); and $CHCl_3$–CH_3OH–CH_3COOH–H_2O 25:15:4:2 (E). Treatment of the [^{3}H]sphingomyelin (16) with purified phospholipase C [E.C.3.1.4.3; phosphatidylcholine cholinephosphohydrolase] from *Clostridium welchii* (Sigma Chemical Co.) produced a tritiated product that cochromatographed with ceramide in three solvent systems: B, C, and D. Larger amounts of [^{3}H]ceramide were prepared in a similar manner, and purified by preparative TLC in the first solvent system. Identity was confirmed by chromatography in the other two solvent systems. Radioactivity on TLC plates was determined by scanning on a thin-layer radiochromatograph scanner (Actigraph III, Nuclear Chicago, Des Plaines, Ill.) or by suspending the scraped gel in 4% Cab-o-Sil/dioxane-based scintillation fluid and counting in a liquid scintillation spectrometer (Mark II, Nuclear Chicago).

[^{14}C]Lecithin labeled in the fatty acid portion was prepared by incubation of rat brain microsomes with [1-^{14}C]oleic acid and lysolecithin (17). [U-^{14}C]Lecithin, New England Nuclear Corp., Montreal, Que., was shown to be free of radiolabeled monoglyceride, diglyceride, and free fatty acid by TLC in diethyl ether–petroleum ether–acetic acid 10:40:1 (F).

Nonradioactive lipids were purchased from Sigma Chemical Co., Applied Science Laboratories, State College, Pa., and Serdary Research Laboratories, London, Ont. Aquacide II was obtained from Calbiochem, La Jolla, California. Other chemicals were obtained from Fisher Scientific Co., Dartmouth, N.S. All solvents were ACS grade and were further purified and distilled before use (18).

Tissues

For most of the studies, human tissues from persons of either sex were obtained at necropsy within 8–24 hr of death. Tissues that were not processed immediately were wrapped in Saran Wrap and stored frozen at −20°C. Two samples of fresh brain were obtained at craniotomy for excision of a deep tumor in one case and pre-frontal lobotomy in the other.

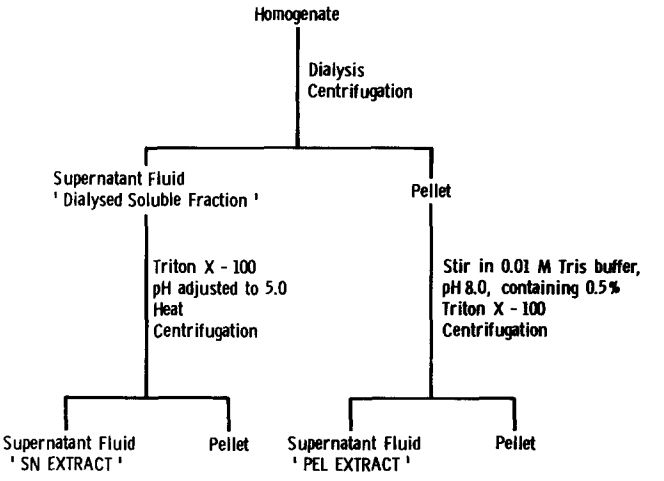

Fig. 1. Procedure for preparation of partially purified fractions of sph'ases 5.0 and 7.4. SN EXTRACT is the fraction used in most studies of sph'ase 5.0 and is free of sph'ase 7.4 activity. PEL EXTRACT is the fraction used in most studies on sph'ase 7.4; it contains some sph'ase 5.0 activity.

Preparation of enzyme (Fig. 1)

Unless noted otherwise, all steps were conducted at 0–4°C. Fresh or frozen human tissue was homogenized in 10 vol of 0.01 M Tris-HCl buffer, pH 8.0, with a closely-fitting Teflon and glass homogenizer. The resulting crude homogenate fraction was dialyzed for 22 hr at 4°C against 120 volumes of the same buffer, then centrifuged at 40,000 *g* for 1 hr.

Sn extract

Triton X-100 (2 mg/ml) was added to the supernatant fluid, and the pH was adjusted to 5.0 with 0.1 M CH_3COOH. The mixture was heated at 45°C for 10 min, cooled to 4°C, and centrifuged at 10,000 *g* for 10 min; the resultant supernatant fluid, designated Sn extract, was the partially purified fraction used in most studies of sph'ase 5.0.

Pel extract

The pellet was rehomogenized in 0.01 M Tris-HCl buffer containing 0.5% (w/v) Triton X-100 and stirred overnight at 4°C. The mixture was centrifuged at 40,000 *g* for 1 hr. The supernatant, designated Pel extract, was used for most of the studies of sph'ase 7.4. The residual sph'ase 5.0 activity in this fraction was also examined.

Protein was determined by the method described by Lowry et al. (19) with the use of bovine serum albumin as standard.

Sphingomyelinase assay (sph'ase 5.0)

Except where indicated otherwise, the incubation mixture contained substrate (40 nmoles of [^{3}H]-

TABLE 1. Effect on activity of sph'ase 7.4 of ions added to the incubation mixture

Cation[a]	Conc.	Relative Specific Activity[b]
	mM	
None	0	1.00
Mg^{2+}	6.0	4.43
Mg^{2+c}	6.0	4.12
Mn^{2+}	6.0	2.39
Ca^{2+}	6.0	0.17
Ca^{2+} + Mg^{2+}	6.0 + 6.0	2.70
Ca^{2+} + Mg^{2+}	0.2 + 6.0	4.08
Cd^{2+} + Mg^{2+}	0.2 + 6.0	2.57

[a] The salts tested were added to the incubation mixture immediately before assay.
[b] Specific activity relative to activity in the absence of ions taken as 1.00:28.3 nmoles [^{3}H]ceramide produced/mg protein/hr.
[c] $MgSO_4$; all other ions were added as chloride salts.

sphingomyelin, sp act 80,000 dpm/nmole), 30 μmoles acetate buffer at pH 5.0, Triton X-100 (1 mg/ml), and tissue preparation containing 50–100 μg of protein in a total volume of 0.2 ml. Incubation was for 1 hr at 37°C. Each experiment included blanks containing all components except substrate or enzyme; incubation in the presence of boiled enzyme resulted in blank levels of radioactivity (generally less than 1% of sample tubes). Before addition to the assay mixture, the substrate was solubilized in buffer or water containing Triton X-100 (1 mg/ml) by agitation at 50–55°C. The reaction was terminated and the tritiated product isolated essentially as described by Schneider and Kennedy (4). The recovery of [^{3}H]ceramide, 60.3 ± 1.1% (N = 18) was taken into consideration in the calculation of sphingomyelinase activity.

Sph'ase 7.4

The assay system was similar to that for sph'ase 5.0, except for the substitution of 30 μmoles of Tris–histidine or Tris-HCl buffer, pH 7.4, and the inclusion of 1.2 μmoles of $MgCl_2$. The usual incubation was for 20 min.

Lecithin hydrolysis

Tissue preparations were assayed for lecithin-cleaving activity as for sphingomyelinase except that [U-^{14}C]- or [oleate-^{14}C]lecithin was substituted for [^{3}H]sphingomyelin in the assay mixture and incubation was extended to 2 or 4 hr to facilitate detection of low rates of hydrolysis. The reaction was stopped by adding 5 ml of $CHCl_3$–CH_3OH 2:1 and washing (20). The reaction products were identified by radio–TLC in system F with the use of lecithin, diglyceride, and free fatty acid as standards. Results were expressed as percentage values of rates obtained with [^{3}H]sphingomyelin as substrate in simultaneous incubations.

RESULTS

Sph'ases 7.4 and 5.0 were assayed concurrently in most of these studies, to ascertain differences between their activities. Sn extracts were used for most studies of sph'ase 5.0 activity, and Pel extracts for sph'ase 7.4. The residual sph'ase 5.0 activity remaining in the Pel extract was also examined; it had properties similar to sph'ase 5.0 of the Sn extract under all conditions tested.

Identification of products as ceramide and phosphoryl choline

TLC of the product phase in three solvent systems (B, C, and D) showed >99% of the radioactivity to chromatograph identically to [^{3}H]ceramide prepared by ourselves or cold ceramide (Sigma Chemical Co.). [^{3}H]Sphingomyelin accounted for <0.4% of the radioactivity. Insignificant radioactivity was recovered from areas of the plate corresponding to sphingosine and fatty acid.

In order to determine that the other product of the reaction was phosphorylcholine, [choline methyl-^{14}C]sphingomyelin (New England Nuclear Corp., diluted to a specific activity of 1.52 nCi/mmole) was incubated in the same way as the tritiated substrate. The reaction was stopped with 5 ml of $CHCl_3$–CH_3OH 2:1; 1 ml of 0.1 M KCl containing 1 mM phosphorylcholine was added, and the phases mixed. Following removal of the upper phase, the lower phases were washed twice with 1 ml of $CHCl_3$–CH_3OH–H_2O 3:48:47. The upper phases were pooled and extracted with 3 ml $CHCl_3$–CH_3OH 85:15. TLC chromatographic examination of the upper phase in CH_3OH–H_2O–7N NH_4OH 6:3:1 (G), and $CHCl_3$–CH_3OH–H_2O–CH_3COOH 14:6:1:0.1 (H) and system B showed all the radioactivity to cochromatograph with standard phosphorylcholine. TLC examination of the lower phases in $CHCl_3$–CH_3OH–CH_3COOH–H_2O 100:45:20:7 (I), and systems B and H showed all lower phase radioactivity to cochromatograph with sphingomyelin.

Grey matter/white matter gradient for sph'ases in brain

The grey matter to white matter ratio of the specific activity of sph'ase 7.4 was 7.6 ± 3.2 (N = 7) in crude homogenates. For sph'ase 5.0, the ratio was 1.6 ± 0.3 (N = 7). The specific activities were unaffected by age or sex. Because activity was

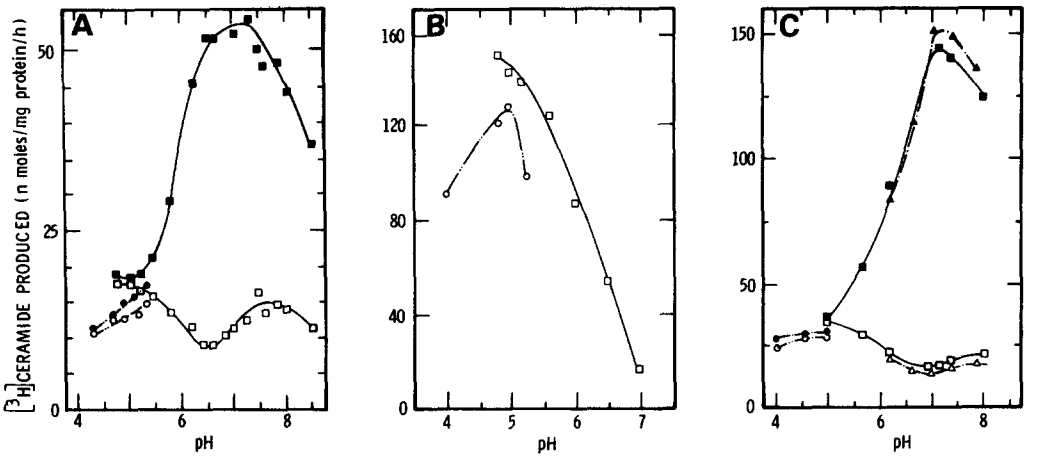

Fig. 2. Effect of variation in pH on sphingomyelin hydrolysis by preparations of human brain at $MgCl_2$ concentrations of zero (open symbols) and 6 mM (closed symbols). A, crude homogenate of whole brain (9-year-old male); B, Sn extract of whole brain (9-year-old male); C, Pel extract of grey matter (55-year-old male). Buffers: acetate (—··—○—●—··—), Tris–histidine (—□—■—), imidazole–HCl (—·—△—▲—·—).

higher in grey matter, this tissue was used as the source of enzyme for most of the studies.

Ion requirement

Activity measurable in brain at pH 7.4 was stimulated by Mg^{2+} ions (**Table 1**). The stimulation became maximal between 2.0 and 2.5 mM and no inhibition was observed up to 10 mM. Mn^{2+} was less stimulatory; Ca^{2+} and Cd^{2+} inhibited activity. EDTA (10 mM) reduced Mg^{2+}-stimulated activity at pH 7.4 by 71% and nonstimulated levels by 55%; EGTA had no effect. Sph'ase activity at pH 5.0 was unaffected by any of the ions or chelating agents tested.

pH, time, protein, substrate and detergent effects

In the absence of Mg^{2+}, pH curves on whole-brain crude homogenate were biphasic with activity optima around pH 5.0 and 7.4. In the presence of Mg^{2+}, activity was minimally affected at pH 5.0 but rose with increasing pH to an optimum at 7.2–7.4 (**Fig. 2A**). The optimal pH for sphingomyelin hydrolysis in the Sn extract (Fig. 2B) was 4.8–5.0 in acetate or Tris–histidine buffers. In the Pel extract, Mg^{2+}-stimulated activity was optimal at pH 7.1–7.4 (Fig. 2C) in imidazole–HCl, Hepes, Tris-HCl and Tris–histidine buffers; rates in potassium phosphate and citrate phosphate buffers were only 15% and 5%, respectively, of rates with the other buffers. In the absence of Mg^{2+}, activity was maximal at pH 5.0, due to the residual sph'ase 5.0 in this fraction. Activities at pH 5.0 and 7.4 were unaffected by variation in buffer concentration between 25 and 300 mM. Linearity with protein concentration was observed to 0.9 mg/ml with sph'ase 5.0 and to 0.6 mg/ml with sph'ase 7.4. Lineweaver–Burk plots of activity vs substrate concentration curves (**Fig. 3**) indicated an apparent K_m for sph'ase 5.0 of 1.0×10^{-4} M, and for sph'ase 7.4 of 6.6×10^{-5} M. At all concentrations tested (0.02–0.30 mM), dihydrosphingomyelin and sphingomyelin were hydrolyzed at similar rates.

Triton X-100 (1 mg/ml) was included in the assay system to disperse the substrate. The addition of Cutscum or cholate (1 mg/ml) to the Triton-containing incubation mixture reduced sph'ase 7.4 activity (37%), and variably enhanced sph'ase 5.0 activity (5–25%). Taurocholate addition reduced both activities by approximately 20%.

Lipids as substrates or inhibitors

Assays with both [U-^{14}C]- and [oleate-^{14}C]lecithin as substrate for crude homogenates of normal brain

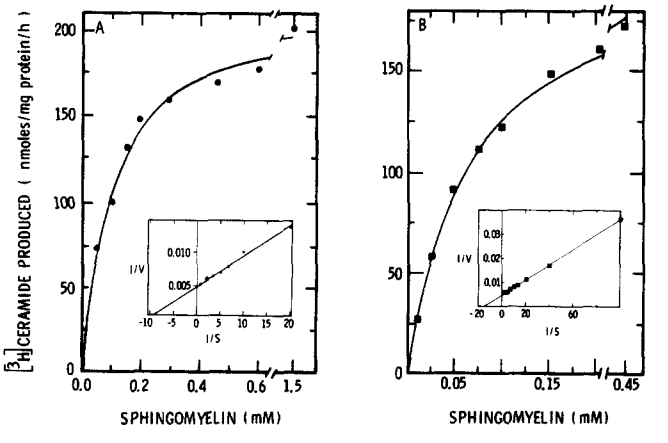

Fig. 3. Variation in sphingomyelin-hydrolyzing activity with substrate concentration. A, Sph'ase 5.0 of Sn extract of whole brain; B, Sph'ase 7.4 of Pel extract of grey matter.

TABLE 2. Effect of nucleotides added to the incubation mixture on sphingomyelin hydrolysis by sph'ases 5.0 and 7.4

Nucleotide	Conc.	Relative Specific Activity[a]	
		Sph'ase 5.0	Sph'ase 7.4
	mM		
None		1.00	1.00
AMP	0.2	0.39	0.97
	2.0	0.05	1.00
ATP	0.1	1.01	1.00
	2.0	0.21	1.05
cAMP	0.2	1.04	0.93
ADP	0.2	0.76	0.96
NAD + NADH	1.0 + 1.0	0.63	0.93
NADP + NADPH	1.0 + 1.0	0.68	0.73
GTP	0.2	1.00	0.99

[a] Activity relative to specific activity in the absence of added nucleotide taken as 1.00: sph'ase 5.0, 146.0 and sph'ase 7.4, 119.2 nmoles [^{3}H]ceramide produced/mg protein/hr. Sph'ase 5.0 was measured in Sn extract and sph'ase 7.4 in Pel extract of normal grey matter.

under conditions optimal for sph'ase 7.4 and for sph'ase 5.0 indicated that lecithin was hydrolyzed at no more than 10% of the rate of hydrolysis of sphingomyelin. Lecithin (0.15 mM) added to standard incubations did not reduce either activity. Addition of the products of the reaction, ceramide (0.10 mM) and phosphorylcholine (0.05–0.20 mM), resulted in <10% inhibition of activity at pH 5.0 and 7.4. Glycerylphosphorylcholine (GPC) as the cadmium salt inhibited sph'ase 7.4 activity (29% at 0.10 mM and 50% at 0.20 mM GPC), and assay of sph'ase 7.4 with similar concentrations of $CdCl_2$ showed the same degree of inhibition (Table 1). GPC had no effect on sph'ase 5.0 activity. Cardiolipin (0.09 mM) decreased sph'ase 7.4 activity by 23% but sph'ase 5.0 by only 7%. By contrast, phosphatidic acid (0.08 mM) seemed to inhibit sph'ase 5.0 (13%) more than sph'ase 7.4 (2%). Phosphatidylethanolamine (0.10 mM) reduced both activities by 8%.

Nucleotides as substrates or inhibitors

Some nucleotide phosphodiesterases have alkaline pH optima and their activity is enhanced by Mg^{2+} (21). It seemed possible, therefore, that the sph'ase 7.4 activity might be due to a nucleotide phosphodiesterase or phosphatase able to hydrolyze sphingomyelin also. Therefore, several nucleotides were tested for their ability to inhibit sphingomyelinase activity at pH 5.0 and 7.4. With the exception of NADP–NADPH mixtures, none of the nucleotides tested appreciably affected sph'ase 7.4 activity (**Table 2**). By contrast, most nucleotides inhibited sph'ase activity at pH 5.0.

Hydrolysis of bis-nitrophenyl phosphate (bis-*p*NPP) compared with sphingomyelin

Enzyme activity in brain at pH 7.4 with the artificial substrate bis-*p*NPP has been reported to be stimulated by Mg^{2+} and cysteine (22). The natural substrate for this enzyme or enzymes is unknown. To determine whether bis-*p*NPP might be a substrate for sph'ase 7.4, activity with the artificial substrate was compared with sphingomyelin in brain homogenates. Two major differences were observed. First, the grey/white matter ratio of sphingomyelin hydrolysis was 7–9 and the ratio of bis-*p* NPP hydrolysis was 0.5. Second, assay of activity at 30 and 90 min incubation indicated a loss of 46% of sphingomyelin-hydrolyzing activity but only 9% of bis-*p*NPP-hydrolyzing activity. Hence sph'ase 7.4 may have some bis-*p*NPP-hydrolyzing activity, but it is unlikely to be the principal enzyme hydrolyzing the artificial substrate.

Effects of compounds containing and/or reacting with sulfhydryl groups

The effect of several thiol reagents on sph'ases 5.0 and 7.4 was tested by preliminary incubation of enzyme preparations with each reagent for 10 min at room temperature at pH ~7.5, then assay of activity over 20 min at 37°C. With the exception of dithiothreitol, all reagents tested inhibited sph'ase

TABLE 3. Effect of thiol reagents on sph'ases 5.0 and 7.4 of human grey matter

Reagent	Conc.	Relative Specific Activity[a]	
		Sph'ase 5.0	Sph'ase 7.4
	mM		
None		1.00	1.00
Iodoacetamide	1.0	1.01	0.90
p-OH-Mercuribenzoate	0.1	1.39	0.03
p-OH-Mercuribenzoate	1.0	1.30	0.02
N-Ethylmaleimide	1.0	0.99	0.14
Coenzyme A	0.4	0.82	0.90
Coenzyme A	2.0	0.03	0.47
Dithiothreitol	10	0.53[b]	1.21[b]
Cysteine	10	0.94	0.80

[a] Activity relative to specific activity in the absence of thiol reagent taken as 1.00: sph'ase 5.0, 24.3 and sph'ase 7.4, 144.1 nmoles [^{3}H]ceramide produced/mg protein/hr. Both activities were measured in Pel extract of normal grey matter.

[b] Results of a single experiment. Studies of this effect with several preparations of sph'ases 5.0 and 7.4 demonstrated $P < 0.001$ by sign test, and $P < 0.01$ by paired t test (30). Other additions were made only three times and were not analysed statistically. However, the direction of change, when present, was consistent.

TABLE 4. Partial purification of sphingomyelinase activity at pH 5.0 and 7.4 from human brain

Fraction	Sph'ase 5.0			Sph'ase 7.4		
	S.A.[a]	Purif.	Recovery	S.A.	Purif.	Recovery
Crude homogenate	14.5	1.0	100.0	41.2	1.0	100.0
Dialyzed soluble fraction	53.7	3.7	82.6	3.0	0.1	1.5
Sn extract	143.5	9.9	78.4	0		0
Pellet of dialyzed soluble fraction	10.2	0.7	20.1	64.5	1.6	80.4
Pel extract	21.8	1.5	20.1	134.0	3.3	60.5

[a] S.A. is specific activity in nmoles [^{3}H]ceramide produced/mg protein/hr.

7.4 (**Table 3**). By contrast only coenzyme A and dithiothreitol inhibited sph'ase 5.0. Although coenzyme A was inhibitory to both enzymes, the effect at 2.0 mM was much greater on sph'ase 5.0 (97%) than on sph'ase 7.4 (53%). Direct addition of dithiothreitol (10 mM) to the incubation mixture inhibited sph'ase 5.0 activity by 50% at 20 min of incubation. Activity at pH 7.4 was enhanced by 20–40%, with maximal effect at 5 mM and no indication of inhibition to 15 mM. Direct addition of cysteine (10 mM) did not affect sph'ase 5.0, but caused varied loss of activity at pH 7.4 (<15%).

Separation of sphingomyelin-hydrolyzing activities at pH 5.0 and 7.4 during partial purification

After centrifugation of the dialyzed homogenate at 40,000 *g*, the supernatant fluid contained 70–80% of the sph'ase 5.0 activity but virtually no sph'ase 7.4 activity (**Table 4**). A supernatant (Sn extract) obtained from this fraction after acidification and heating in the presence of Triton X-100 was enriched 7- to 10-fold in sph'ase 5.0 activity over crude homogenate. No sph'ase 7.4 activity was detected in this fraction. Treatment of the pellet of the dialyzed homogenate with buffer containing Triton X-100 released sph'ase 7.4 and the residual sph'ase 5.0. This pellet extract (Pel extract) was purified 3- to 4-fold over crude homogenate with respect to sph'ase 7.4 activity; recovery of activity was 60–75% in various preparations (Table 4). Several detergents were tested for their ability to solubilize sph'ase 7.4. Cutscum (1% w/v) and Triton X-100 (0.5% w/v) were equally effective in releasing both enzymes into Pel extract fractions, whereas taurocholate (0.5% w/v) or cholate (0.5% w/v) treatment released sph'ase 5.0 but not sph'ase 7.4.

Further purification of sph'ase 7.4 from Pel extract fractions and its separation from residual sph'ase 5.0 were attempted. Ammonium sulfate fractionation (23, 24) resulted in substantial losses of sph'ase 7.4, and after butanol extraction (24), no sph'ase 7.4 and only 15% of sph'ase 5.0 were recovered. Sph'ase 7.4 activity was reduced 30–60% after concentration of fractions by lyophilization, and 25% after concentration against Aquacide II. Smaller, varied losses of sph'ase 7.4 activity were observed during prolonged dialysis.

Various attempts were made to stabilize the enzyme during purification. The presence of Mg^{2+} (6 mM) did not appear to protect sph'ase 7.4 activity but did alter the relative distribution of sph'ases 5.0 and 7.4. Normally, >90% of recovered sph'ase 7.4 activity was solubilized by Triton extraction of the pellet of the dialysed homogenate (Fig. 1 and Table 4). In the presence of Mg^{2+}, only 45% of recovered activity was solubilized. The presence of cysteine during purification reduced sph'ase 7.4 activity by 61%; pyridoxal phosphate added to the assay mixture did not reactivate the cysteine-treated enzyme. Preliminary incubation of enzyme preparations for 15 min at 37°C with pyridoxal phosphate resulted in 98% loss of activity.

Conditions affecting enzyme stability

Storage at −20 and 0°C. Sphingomyelinase activity measured at pH 5.0 was similar in fresh and frozen tissue specimens, and was unchanged in solid tissues during frozen storage (−20°C) for one year, and in crude homogenates and Sn extracts stored frozen for at least one month. Lyophilization did not alter activity.

Sphingomyelinase activity at pH 7.4 in fresh tissue or necropsy specimens was unaffected by frozen storage for up to one year, when activity was determined in Pel extracts (Fig. 1). In crude homogenates freshly prepared from the same tissues over the same period, however, activity decreased 50–60%, but was restored by the subsequent dialysis and Triton X-100 extraction steps for preparation of the Pel extract. (Since most assays of sph'ase 7.4 were conducted on Pel extracts, this activity loss in the crude homogenates was not a problem.) Crude homogenates and extracts stored at −20°C lost 40–45% of their initial activity in 28 days.

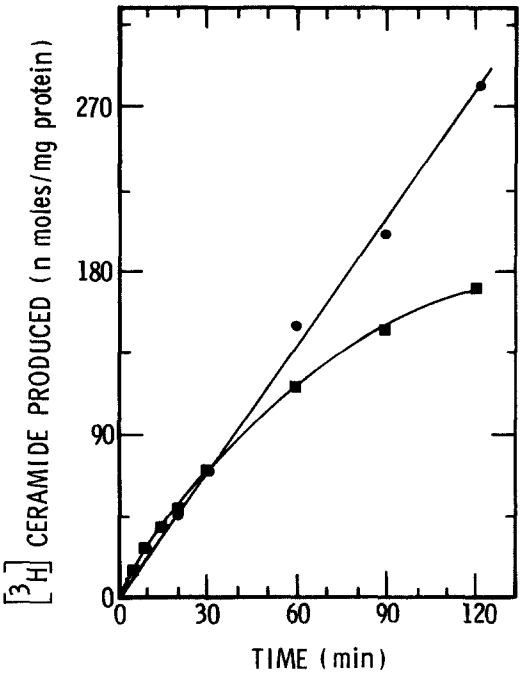

Fig. 4. Effect on sphingomyelin-hydrolyzing activity of variation in time of incubation. Sph'ase 5.0 of Sn extract of whole brain (—●—) and sph'ase 7.4 in Pel extract of grey matter (—■—).

Rapid freezing and thawing (7 cycles between −70°C and 37°C) had little effect on sphingomyelinase activity at pH 5.0 or 7.4. Sph'ase 5.0 activity in Sn extracts or the residual sph'ase 5.0 activity in Pel extracts was unaffected by storage at 0–4°C for 4 hr; sph'ase 7.4 activity in Pel extracts decreased by 22%. Dithiothreitol (10 mM) did not retard the loss of 7.4 activity, but did cause a temperature-related loss of sph'ase 5.0 activity. Enzyme exposed to dithiothreitol for 4 hr at 0–4°C showed a 50% loss of activity; this loss increased with time of preliminary incubation at 37°C, so that activity was barely detectable at 60 min.

Enzyme stability at 37°C in the presence of substrate. Sphingomyelin hydrolysis by sph'ase 5.0 in Sn extracts observed after 2 hr was >85% of that predicted from the hydrolysis rate at 10 min, if there had been no denaturation (**Fig. 4**). By contrast, hydrolysis by sph'ase 7.4 after 2 hr was only 50% of that predicted on the same basis.

Enzyme stability at 37°C in the absence of substrate. Denaturation of both enzymes at 37°C was examined under several conditions (**Fig. 5**). There was nonlinear loss (65%) of sph'ase 7.4 activity over 4 hr, in the presence or absence of added dithiothreitol. Lyophilization (followed by reconstitution to original volume with distilled H_2O) markedly decreased activity, which further declined over 4 hr.

Sph'ase 5.0 activity was unaffected by preliminary incubation at 37°C for 4 hr, unless dithiothreitol was added.

Enzyme stability above 37°C. The greater stability of sphingomyelinase activity at pH 5.0 as compared to 7.4 was even more apparent as the temperature of preliminary incubation was raised (**Fig. 6A**). The activity at pH 5.0 is remarkably heat stable, even at 55°C in both the Pel extract (Fig. 6B) and Sn extract (not shown). The activity at pH 7.4, on the other hand, rapidly decreases under the same conditions. Maximum activity of sph'ase 5.0 was observed at 55–60°C during incubation periods of 10 min to 1 hr. Maximal activity of sph'ase 7.4 was observed at 45–50°C during 10 and 20 min incubations. This difference in temperature optima between the two activities must be interpreted cautiously due to the increased lability of sph'ase 7.4 at elevated temperatures.

Enzyme activity in extracerebral tissues

Several preparations of normal human liver, spleen, kidney, erythrocytes and leukocytes were examined for sph'ase activity at pH 7.4 in the presence of Mg^{2+} and dithiothreitol. Liver, spleen, and white cells had very low Mg^{2+}-stimulated activity (<2 nmoles/mg protein/hr); activity in kidney (3.9 nmoles/mg protein/hr) was severely inhibited by dithio-

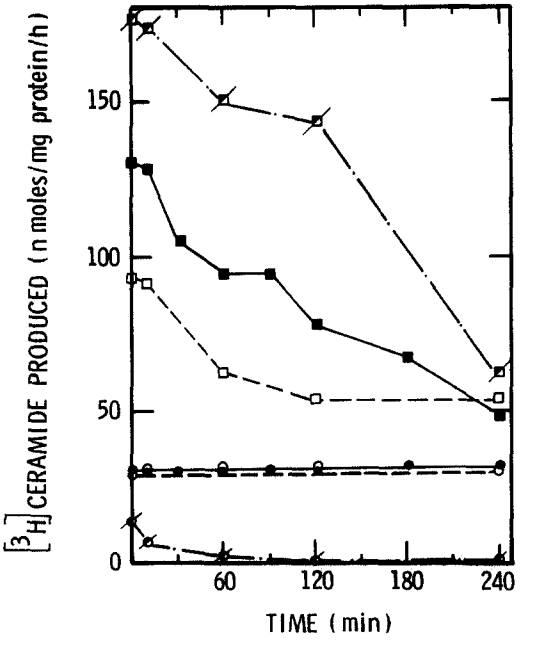

Fig. 5. Effect of variation in time of preliminary incubation at 37°C without substrate on the specific activity (assayed over 20 min at 37°C) of sph'ases 5.0 (circles) and 7.4 (squares) in untreated (—●—, —■—), lyophilized (— ○ —, — □ —), and dithiothreitol-treated (—Ø—·—, —◪—·—) Pel extract of grey matter of normal human brain (55-year-old male).

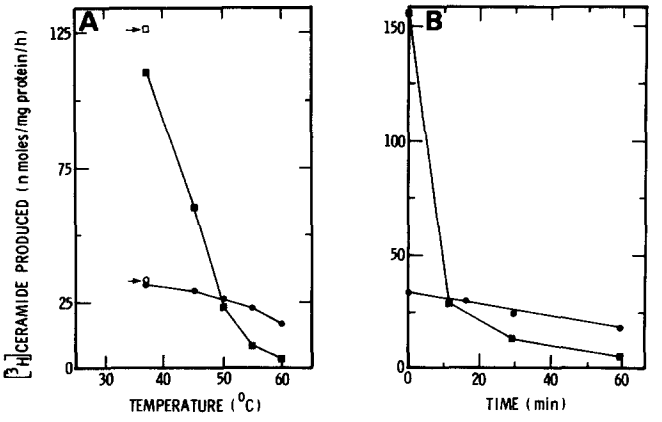

Fig. 6. Effect of preliminary incubation at elevated temperature on the specific activity (assayed over 20 min at 37°C) of sph'ases 5.0 (●) and 7.4 (■) in Pel extract of grey matter of normal human brain (55-year-old male). A, Effect of variation in temperature of preliminary incubation (for 10 min) in the absence of substrate (→ denotes activity with no preliminary incubation). B, Effect of variation in time of preliminary incubation at 55°C.

threitol (>75%). No activity was observed in erythrocytes. By contrast, sph'ase 5.0 activity is ubiquitously distributed. The range of activities (nmoles/mg protein/hr) observed in organs of several normal individuals were: liver (5 subjects) 6.3–9.0, spleen (3) 1.8–4.3, kidney (3) 11.2–54.7, and white blood cells (5) 2.7–5.0.

DISCUSSION

Human cerebral grey matter contains two specific sphingomyelinase activities with pH optima at 5.0 and 7.4. Results of measurements of lecithin-cleaving activity and of the ability of various phosphodiesters to inhibit sphingomyelin hydrolysis indicate that the substrate specificity of the activity at pH 7.4 is comparable to that at pH 5.0. While there were some slight differences in the effects of other lipids on the two activities, none were sufficiently great to suggest a possible role as substrate or specific inhibitor, particularly in such crude systems. Although preparations with sph'ase 7.4 activity hydrolyzed the artificial substrate, bis-*p*NPP, the differences in distribution in grey and white matter and stability during incubation of the activities indicate that sph'ase 7.4 was probably not the principal enzyme species active on the artificial substrate. Thus, we propose that the role of sph'ase 7.4 in vivo, like that of sph'ase 5.0, is the hydrolytic cleavage of phosphoryl choline specifically from sphingomyelin.

Some of the differences between the activities at pH 5.0 and 7.4 are summarized in **Table 5.** The activities are separable by relatively simple physical techniques, e.g., response to solubilization following dialysis (Table 4). The tissue distributions are different—activity at pH 5.0 is ubiquitous, whereas the activity at pH 7.4 is low in most tissues except brain. Within brain, also, the distribution of these two activities differed.

There were major differences in the response of the two enzymes to a wide variety of compounds added to incubation mixtures, e.g., divalent cations, nucleotides, sulfhydryl reagents. Although it seems unlikely, it is possible that some of the differential effects of added reagents might be due to different effects on the substrate micelles. In all these assays, the substrate was probably present both as sphingomyelin monomers and mixed micelles of Triton X-100 and sphingomyelin (25). Triton X-100 is non-ionic, and the charge of sphingomyelin is essentially unchanged between pH 5.0 and 7.4. Accordingly, the micellar properties are probably similar under both assay conditions. Coupled with the additional differences in properties indicating that sph'ases 5.0 and 7.4 are different proteins, it seems most probable that the primary effects of these reagents are on the enzyme protein, and not on the substrate micelle.

The cause(s) of the instability of sph'ase 7.4 activity during purification, storage, and incubation is not known. The possibilities include combination with

TABLE 5. Some differences between sph'ase 5.0 and sph'ase 7.4 in brain

Parameter or Reaction Condition	Sph'ase 5.0	Sph'ase 7.4
Centrifugation of dialysed crude homogenate	Predominantly in supernatant fluid	Predominantly in pellet
Grey matter/white matter activity	1.5–2.0	9.0–13.0
pH optimum	4.8–5.2	7.2–7.4
Ion effects[a]		
Mg^{2+}	0	Stimulation
Mn^{2+}	0	Stimulation
Ca^{2+}	0	Inhibition
Cd^{2+}	0	Inhibition
EDTA	0	Inhibition
Thiol reagents		
Dithiothreitol	Inhibition	Stimulation
Cysteine	0	Inhibition
N-ethylmaleimide	0	Inhibition
p-Hydroxymercuribenzoate	Stimulation	Inhibition
Nucleotides		
AMP, ADP, ATP	Inhibition	0
NAD + NADH	Inhibition	0
Stability	Relatively stable at −20° to 40°	Unstable

[a] 0, no detectable effect.

an endogenous inhibitor, dissociation of subunits, and metal ion-catalyzed oxidation of sulfhydryl groups by molecular oxygen.

Four studies have reported sphingomyelinase activity optimal at neutral to alkaline pH in tissues from vertebrates. Schneider and Kennedy (4) commented on the presence of a Mg^{2+}-stimulated sphingomyelinase, with pH optimum at 7.4 in spleen. The activity is much lower than that we observed in brain. The Mg^{2+}- and Mn^{2+}-stimulated sphingomyelinase of chicken erythrocyte (12) has a broader pH optimum (7.0–9.0) and appears to be more stable than the sph'ase 7.4 of human brain. The intestinal sphingomyelin-cleaving activity reported by Nilssen (11) had a much higher pH optimum (9.2) than sph'ase 7.4 and was also more stable. The effect of ions on this enzyme was not reported. Gatt (26) has recently briefly described a Mg^{2+}-stimulated enzyme with a slightly alkaline pH optimum in rat brain that appears to differ from human sph'ase 7.4 in stability and response to Ca^{2+}. He also observed sphingomyelin hydrolysis at pH 7.4 in the presence of Mg^{2+} in human brain. The increased stability of the intestinal, erythrocyte, and rat brain enzymes suggests that they may be different from sph'ase 7.4 of human brain. The relationship of the latter to the human spleen enzyme is unknown.

Sph'ase 5.0 is primarily a lysosomal enzyme (27–29) but may occur in other subcellular organelles (27). Degradation of material by incorporation into lysosomes is a mechanism well suited for turnover of cellular constituents in bulk, but not for turnover of individual molecules of various subcellular structures and membranes in situ. Although the pH optimum of sph'ase 7.4 suggests that it is not localized in lysosomes, its sedimentation at 40,000 *g* and 'solubilization' by detergent indicates that it is a membrane-bound enzyme. Sphingomyelin, like other phospholipids, is primarily a constituent of membranes and has been found in varying quantities in most subcellular organelles. Sph'ase 7.4 may function in sphingomyelin turnover in the intact structures, not requiring their incorporation into lysosomes.

The higher sph'ase 7.4 activity observed in brain than in other tissues may reflect greater sensitivity of this organ to changes in composition, resulting in more stringent requirements for clearance of sphingomyelin. Alternatively, the high brain activity may relate to a specific role for the enzyme in the special functions of this organ. For instance, transient removal of the charged phosphoryl choline group from sphingomyelin may be important in the dynamic alterations of membrane structure that underlie nervous activity. Thus, the major role of sph'ase 7.4 may not be in normal sphingomyelin degradation, but rather in the transient removal (and exchange) of phosphoryl choline moieties among components of cerebral tissues.

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