

The subcellular localization of neutral sphingomyelinase in rat liver

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Abstract The subcellular distribution of neutral sphingomyelinase activity has been determined in rat liver. Neutral sphingomyelinase is present in the plasma membrane. This enzyme requires either Mg^{2+} or Mn^{2+} for full activity; these cations cannot be replaced by Co^{2+} or Ca^{2+} . The plasma membrane sphingomyelinase is strongly inhibited by Hg^{2+} . A small amount of neutral sphingomyelinase activity appears to be present in microsomes. No neutral sphingomyelinase activity is present in liver mitochondria or cytosol. Lysosomal sphingomyelinase is fully active at pH 4.4–4.8 without added divalent cations. However, between pH 5.0 and 7.5, lysosomal sphingomyelinase activity is stimulated by Mg^{2+} , Mn^{2+} , Co^{2+} , and Ca^{2+} . Below pH 4.8, Mg^{2+} inhibits the reaction. In contrast to the results obtained with the neutral sphingomyelinase activity of plasma membranes and microsomes, lysosomal sphingomyelinase is unaffected by sulfhydryl inhibitors.—Hostetler, K. Y., and P. J. Yazaki. The subcellular localization of neutral sphingomyelinase in rat liver. *J. Lipid Res.* 1979. **20**: 456–463.

Supplementary key words acid sphingomyelinase · lysosomes · plasma membrane · microsomes · mitochondria

Earlier studies have demonstrated the presence of a sphingomyelinase in liver with a pH optimum of 4.8; it is unaffected by divalent cations or sulfhydryl inhibitors (1, 2) and is localized in the lysosomes (3, 4). This sphingomyelinase has been shown to be absent or severely reduced in the tissues of patients with Niemann–Pick disease, a condition which is characterized in part by marked accumulation of sphingomyelin in liver and spleen (5, 6).

In 1967, a neutral sphingomyelinase that requires magnesium ions was reported in passing as being present in homogenates of human liver and spleen tissue (6). More recently, a neutral sphingomyelinase that requires Mg^{2+} for maximal activity was found in rat brain microsomes (7). Neutral sphingomyelinase was also present in much smaller amounts in the homogenates of other rat organs, including liver, spleen, and kidney (2.3, 0.6, and 4.6% of the activity of brain, respectively) (7). It was not clear in this latter study whether or not the activity could be accounted

for by plasma membrane contamination of the microsomal fraction. Rao and Spence (8) partially purified a neutral sphingomyelinase from human brain extracts which differed from acid sphingomyelinase in its pH optimum and in its response to divalent cations and inhibitors. Yamaguchi and Suzuki (9) purified two acid sphingomyelinases from extracts of human brain (enzymes A and B) which were stimulated by Mg^{2+} at neutral pH. In liver, only the B enzyme was found to be present (10). In the studies mentioned above (6–10), the subcellular localization of the neutral sphingomyelinase activity was not extensively characterized; hence the subcellular localization of neutral sphingomyelinase in liver was unknown.

In this publication, we report the subcellular localization of neutral sphingomyelinase activity in rat liver. Neutral sphingomyelinase is primarily localized in liver plasma membranes, although a small amount of activity appears to be present in a microsomal fraction. Lysosomes have an acid sphingomyelinase that exhibits considerable activity at a neutral pH, particularly in the presence of divalent cations. Evidence is presented showing that the neutral sphingomyelinase activity in the plasma membrane and microsomes is distinct from lysosomal acid sphingomyelinase.

MATERIALS AND METHODS

Preparation of subcellular fractions from rat liver

Male rats of the Sprague–Dawley strain were fasted overnight, killed with a blow, and the livers were removed. The homogenization and subcellular fractionation were carried out as previously described (11). Lysosomes were prepared by the method of Trouet (12); plasma membranes were isolated by the method of Neville (13). The membrane fractions were taken up in 0.25 M sucrose containing 5 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4) and stored at $-60^{\circ}C$ until use. Protein was determined by the method of Lowry et al. (14).

Marker enzyme determinations

Succinate dehydrogenase was determined by the method of Green, Mii, and Kohout (15), acid phosphatase by the method of Gianetto and deDuve (16) using glycerol-2-phosphate as a substrate, rotenone-insensitive NADPH-cytochrome *c* reductase by the method of Sottacasa et al. (17), and 5'-nucleotidase by the method of Touster et al. (18).

Preparation of [³H]sphingomyelin

Sphingomyelin (bovine brain) was tritiated by a non-reductive catalytic exchange method by the New England Nuclear Corporation, Boston, MA. The [³H]-sphingomyelin was further purified by silicic acid chromatography. Contamination of the final product with tritium-labeled ceramide and fatty acid was less than 0.5%. The specific activity was adjusted by the addition of unlabeled sphingomyelin. [³H]Sphingomyelin was completely hydrolyzed by phospholipase C from *Clostridium welchii*; less than 1% of the total tritium counts were found in the phosphocholine moiety. Therefore, the specific activity of [³H]ceramide was taken to be the same as that of the parent compound, [³H]sphingomyelin, in making calculations.

Sphingomyelinase assays

Plasma membrane sphingomyelinase activity was measured as follows. The incubation medium contained 50 mM mono[tris(hydroxymethyl)aminomethane]maleate, pH 7.1, 2.0 mM [³H]sphingomyelin, specific activity 9.9–19.5 mCi/mmol, 2.5 mg/ml Triton X-100, and 0.2 mg/ml membrane protein; 40 mM MgCl₂ was added last. The final volume was 200 μl. The mixture was stirred on a Vortex mixer and incubated for 20 min (or as noted) at 37°C and was assayed for the release of [³H]ceramide by the method of Schneider and Kennedy (6). Mg²⁺-dependent sphingomyelinase was calculated by subtracting [³H]ceramide released in the absence of divalent cations. Endogenous plasma membrane sphingomyelin was 1% of the [³H]sphingomyelin added. The reaction was linear for 20 min and continued at a substantial rate for 120 min. The reaction was linear with protein to at least 0.37 mg/ml.

For measurement of lysosomal sphingomyelinase, the incubation medium consisted of 50 mM sodium acetate, pH 4.8; 1.0 mM [³H]sphingomyelin, sp act 3.5–12.8 mCi/mmol; 2.5 mg/ml Triton X-100, and 60 μg/ml of lysosomal protein in a final volume of 200 μl. In experiments with fractions of liver homogenate, 1 mg/ml of protein was used. After a 30-min incubation at 37°C, the release of [³H]ceramide was measured as noted above. Endogenous lysosomal sphingomyelin represented 3% of the added [³H]sphingomyelin.

Lysosomal sphingomyelinase activity was linear with protein to at least 60 μg/ml and with time to 45 min.

For measurement of Mg²⁺-dependent neutral sphingomyelinase in microsomes, the incubation medium contained 50 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.2; 0.4 mM [³H]sphingomyelin, sp act 3.5–12.8 mCi/mmol; 2.5 mg/ml Triton X-100; 1 mM EDTA; 10 mM MgCl₂; and 1 mg/ml microsomal protein in a final volume of 200 μl. The protein in buffer solution was preincubated for 10 min at 37°C in the presence of EDTA to remove traces of endogenous divalent cations. The [³H]ceramide release was measured as noted above. The Mg²⁺-dependent sphingomyelinase activity was corrected for sphingomyelin hydrolysis occurring in the absence of added divalent cations. Microsomal sphingomyelin was 5% of the added [³H]sphingomyelin substrate. Microsomal sphingomyelin hydrolysis was linear with protein to 2 mg/ml and with time to 60 min.

Characterization of reaction products

The reaction products were identified by thin-layer chromatography as described by Schneider and Kennedy (6). Layers of silica gel H (0.25 mm) were activated for 60 min at 110°C. The samples were spotted at the origin and the plates were developed to a height of 15 cm with chloroform–methanol–glacial acetic acid 90:4:4 (by volume). The plates were dried with nitrogen and developed to a height of 7 cm with chloroform–methanol–2M ammonium hydroxide 70:30:4 (by volume). The plate was scanned with a Panax thin-layer radiochromatogram scanner (Panax Equipment Ltd., Redhill, Surrey, England). With lysosomes, plasma membranes, and microsomes, a single radioactive peak was found which cochromatographed with reference ceramide (*R_f* 0.60 vs. 0.58).

Chemicals

Phospholipase C (*Cl. welchii*), NADP, 5'-AMP, *sn*-glycerol-2-phosphate, disodium succinate, and tris(hydroxymethyl)aminomethane-HCl were purchased from Sigma Chemical Company, St. Louis, MO; Triton X-100 and *p*-chloromercuribenzoate were from Calbiochem, La Jolla, CA; sphingomyelin was from Avanti Biochemicals, Birmingham, AL; Triton WR-1339 was from Supelco, Inc., Bellefonte, PA; silica gel H and silicic acid, Type 60 were obtained from EM Reagents, Elmsford, NY. All solvents were redistilled before use; other chemicals were of analytical reagent grade.

RESULTS

Plasma membranes, lysosomes, microsomes, and mitochondria were prepared from rat liver by estab-

TABLE 1. Marker enzyme and sphingomyelinase activities in purified subcellular membranes from rat liver

Purified Fraction	5'-Nucleotidase ^a		Rotenone-insens. NADPH-Cyt. <i>c</i> Reductase ^a		Acid Phosphatase ^a		Lysosomal Sphingomyelinase ^b		Microsomal Sphingomyelinase ^b		Plasma Membrane Sphingomyelinase ^b	
	Sp Act	R.S.A. ^c	Sp Act	R.S.A.	Sp Act	R.S.A.	Sp Act	R.S.A.	Sp Act	R.S.A.	Sp Act	R.S.A.
Mitochondria	n.d. ^d		2.36	3.6	1.05	0.61	1.6	0.03	0	0	n.d. ^d	
Microsomes	4.41	5.6	65.6	100	3.27	1.9	13.6	0.26	10.47	5.1	11.9	1.08
Plasma membrane	78.66	100	7.49	11.4	0.85	0.5	14.9	0.28	60.0	29.1	96.5	8.75
Lysosomes	13.24	16.8	0.43	0.66	172	100	5,309.0	100	206.0	100	1,103	100

^a nmol mg⁻¹ min⁻¹.

^b nmol mg⁻¹ hr⁻¹.

^c Relative specific activity; the most active fraction has been set to equal 100.

^d Not determined.

lished methods and the purity of these fractions was assessed with marker enzymes. The results are shown in **Table 1**. Microsomal contamination was determined by measurement of rotenone-insensitive NADPH-cytochrome *c* reductase; the microsomal contamination of mitochondria was 3.6%, of plasma membranes 11.4%, and of lysosomes 0.66%. Plasma membrane contamination of the microsomal fraction was 5.6% and of lysosomes 16.8%, based on 5'-nucleotidase. Using acid phosphatase, lysosomal contamination of mitochondria was 0.61%, of microsomes 1.9%, and of plasma membrane 0.5%. Lower values for lysosomal contamination were obtained when acid sphingomyelinase was used as the lysosomal marker enzyme. The remainder of the results shown in Table 1 will be discussed below.

Preliminary experiments indicated the presence of neutral sphingomyelinase activity in the plasma membrane, lysosomes, and microsomes. Experiments were carried out to determine the optimal conditions for

assaying each of these activities and the results are described below.

Plasma membrane sphingomyelinase

Fig. 1 shows the effect of pH on liver plasma membrane sphingomyelinase. The optimal pH was between 7.0 and 7.2 and virtually no activity was found below pH 5.0. **Fig. 2** shows the dependence of plasma membrane sphingomyelinase on the concentration of divalent cations. Both Mg²⁺ and Mn²⁺ supported the reaction while Ca²⁺ and Co²⁺ were inactive (data not shown). Higher reaction rates were obtained with 4 mM Mn²⁺ (95 nmol mg⁻¹ hr⁻¹) than that found at the optimal Mg²⁺ concentration (82 nmol mg⁻¹ hr⁻¹). Above 4 mM Mn²⁺ the rate of [³H]ceramide release from [³H]sphingomyelin declined to about 50% of the maximal rate. Since the optimal concentration for Mn²⁺ occupies a rather narrow range, all experiments with the liver plasma membrane sphingomyelinase were done with 40 mM Mg²⁺. The dependence of plasma sphingomyelinase on the concentration of [³H]sphingomyelin is shown in **Fig. 3**. At concentra-

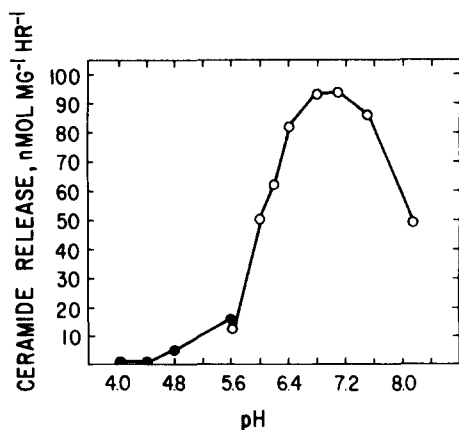


Fig. 1. The effect of pH on plasma membrane sphingomyelinase. The incubation conditions were as described in Methods except that the buffer concentration was 100 mM. The closed circles represent sodium acetate buffer; the open circles are mono[tris(hydroxymethyl)aminomethane]maleate buffer.

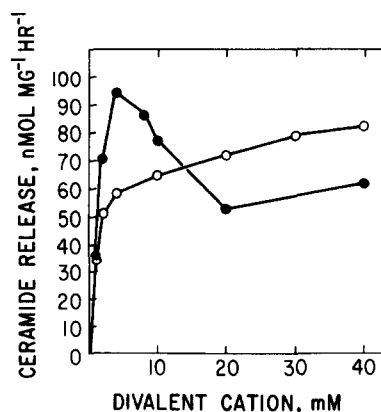


Fig. 2. The dependence of plasma membrane sphingomyelinase on divalent cations. The open circles represent Mg²⁺; the closed circles are Mn²⁺. Incubation conditions were described in Methods.

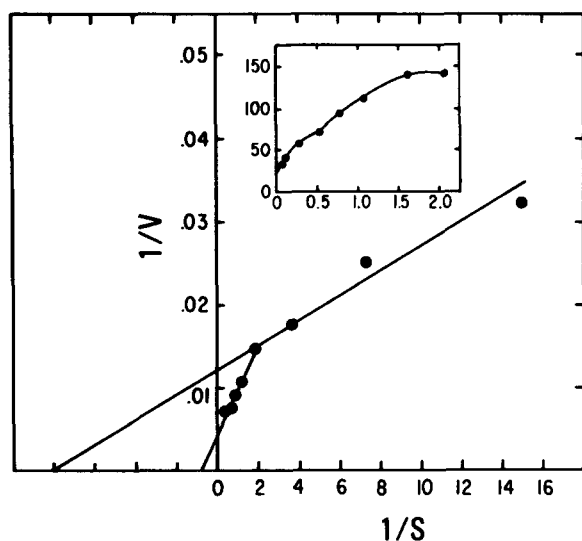


Fig. 3. The effect of sphingomyelin concentration on plasma membrane sphingomyelinase activity. Incubations were carried out as described in Methods. The concentration of Triton X-100 was held constant as the sphingomyelin concentration was varied. The results represent the average of two experiments. In the Lineweaver-Burke plots the data are represented as $1/S$ (mM) and $1/V$ ($\text{nmol mg}^{-1} \text{hr}^{-1}$). The position of the lines in this and other double reciprocal plots was determined by the method of least squares. In the velocity-substrate plot (insert) the units are $\text{nmol mg}^{-1} \text{hr}^{-1}$.

tions of sphingomyelin less than 0.5 mM a lower apparent K_m for sphingomyelin was obtained than that found at concentrations of sphingomyelin between 0.5 and 2.0 mM ($1.2 \times 10^{-4} \text{ M}$ vs. $1.3 \times 10^{-3} \text{ M}$, respectively). At sphingomyelin concentrations less than 0.5 mM the V_{max} was $81 \text{ nmol mg}^{-1} \text{hr}^{-1}$; above 0.5 mM the V_{max} was $235 \text{ nmol mg}^{-1} \text{hr}^{-1}$.

Lysosomal sphingomyelinase

Fig. 4 shows the dependence of lysosomal sphingomyelinase on pH in the presence and absence of 125 mM Mg^{2+} . In the absence of Mg^{2+} , the optimal pH was 4.4–4.8. The activity declined sharply between pH 5.0 and 7.0, although significant activity remained at pH 7.0 (18% of maximal) and at pH 7.5 (11% of maximal). When 125 mM Mg^{2+} was present in the incubation medium, the maximal sphingomyelinase activity shifted to a slightly less acidic pH (Fig. 4A). The effect of the added 125 mM Mg^{2+} was also recalculated by difference from the results in Figure 4A and the data are plotted in Fig. 4B. Above pH 5.0, it can be seen that Mg^{2+} stimulated lysosomal sphingomyelinase and that this effect was maximal at pH 6.0–6.4. The stimulation by Mg^{2+} was about $2.4 \text{ } \mu\text{mol mg}^{-1} \text{hr}^{-1}$. Below pH 5.0, Mg^{2+} caused a considerable inhibition of lysosomal sphingomyelinase. At pH 3.8, the lowest pH examined, Mg^{2+} inhibited the reaction by 98%. The results obtained with 125 mM Mg^{2+} did not differ from the data obtained with 100 mM Mg^{2+} .

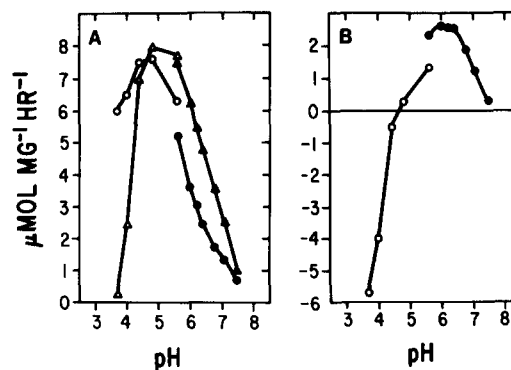


Fig. 4. The effect of pH and 125 mM Mg^{2+} on sphingomyelin hydrolysis by lysosomes. Panel A: the dependence of lysosomal sphingomyelinase on pH is shown in the presence of 125 mM Mg^{2+} (triangles) or without added divalent cations (circles). Open symbols represent sodium acetate buffer; closed symbols refer to mono[tris(hydroxymethyl)aminomethane]maleate buffer. Panel B: inhibition or stimulation of lysosomal hydrolysis of sphingomyelin by 125 mM Mg^{2+} . The effect of Mg^{2+} was calculated by subtracting the rate of sphingomyelin hydrolysis in the absence of the added divalent cation. Except as noted above, standard incubation conditions were used as noted in Methods.

Fig. 5 shows the stimulation of lysosomal sphingomyelinase by divalent cations at pH 6.2. In contrast to the neutral sphingomyelinases found in liver plasma membrane, Co^{2+} and Ca^{2+} , as well as Mg^{2+} and Mn^{2+} , were active in stimulating lysosomal sphingomyelinase. The shape of the velocity vs. cation concentration curves was remarkably similar for all four divalent cations. Maximal rates of 5.3 – $5.7 \text{ } \mu\text{mol mg}^{-1} \text{hr}^{-1}$ were obtained at 100 mM concentrations of divalent cation while the rate without added cations was $3.0 \text{ } \mu\text{mol mg}^{-1} \text{hr}^{-1}$. Thus, a stimulation of approximately 82% was attained with 100 mM Co^{2+} , Ca^{2+} , Mn^{2+} , or Mg^{2+} .

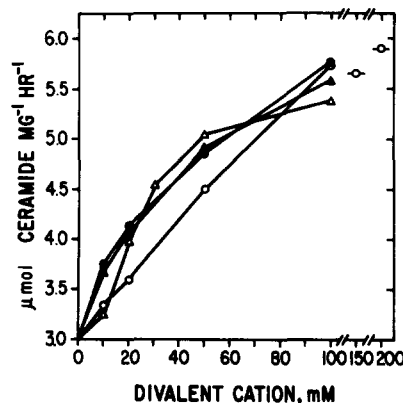


Fig. 5. Stimulation of lysosomal sphingomyelinase by divalent cations at pH 6.2. The incubations were done as described in the methods except that the buffer was 50 mM mono[tris(hydroxymethyl)aminomethane]maleate-HCl, pH 6.2. Divalent cations were added as noted: open circles, Mg^{2+} ; open triangles, Ca^{2+} ; closed circles, Mn^{2+} ; closed triangles, Co^{2+} .

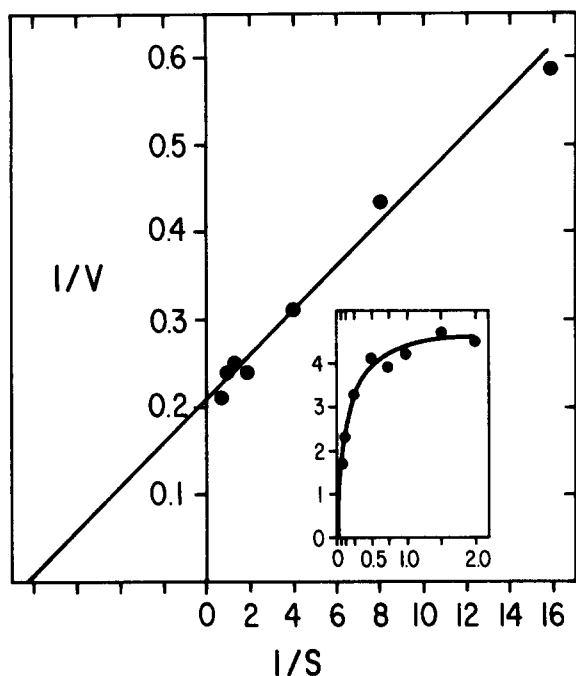


Fig. 6. The dependence of lysosomal sphingomyelin hydrolysis on the substrate concentration. The incubations were done at pH 4.8 as described in Methods. The amount of Triton X-100 was held constant as the sphingomyelin concentration was increased. The results are given as $\mu\text{mol mg}^{-1} \text{hr}^{-1}$ and mM sphingomyelin (inset) or as the reciprocals in the Lineweaver-Burke plot.

Velocity-substrate plots of lysosomal sphingomyelinase at pH 4.8 in the absence of divalent cations are shown in **Fig. 6**. The apparent K_m for sphingomyelin was 1.2×10^{-4} M and the maximal velocity was $4.9 \mu\text{mol mg}^{-1} \text{hr}^{-1}$ based on the double reciprocal plot.

Microsomal sphingomyelinase

The pH optimum of microsomal sphingomyelinase was 7.2 (data not shown). Microsomal sphingomyelinase was activated by both Mg^{2+} and Mn^{2+} , whereas Co^{2+} and Ca^{2+} were not active. Maximal sphingomyelinase activity was attained at 2 mM Mn^{2+} and 10 mM Mg^{2+} , respectively (data not shown). These results are generally similar to those obtained with the plasma membrane except that the optimal concentrations of Mn^{2+} and Mg^{2+} are lower for the microsomal sphingomyelinase (2 mM vs. 4 mM for Mn^{2+} and 10 mM vs. 40 mM for Mg^{2+}). The dependence of microsomal sphingomyelinase activity on sphingomyelin concentration is shown in **Fig. 7**. The apparent K_m for sphingomyelin calculated from the double reciprocal plot was 6.2×10^{-5} M. The maximal velocity for microsomes was about $8 \text{ nmol mg}^{-1} \text{hr}^{-1}$. However, some variation was observed in different preparations. Sphingomyelinase activities as high as $15 \text{ nmol mg}^{-1} \text{hr}^{-1}$ were found in some microsomal preparations.

Subcellular localization of sphingomyelinase activities

Table 1 shows the results of sphingomyelinase assays at the optimal conditions in subcellular membrane fractions from rat liver. The data have been expressed in terms of specific activity and in terms of the relative specific activity where the most active fraction has been set equal to 100. Each fraction was also assayed at optimal conditions for the other sphingomyelinases in order to be able to calculate the amount of sphingomyelinase activity in each fraction due to cross contamination of the respective membrane fractions.

Plasma membrane sphingomyelinase assayed at optimal conditions gave a specific activity of $96.5 \text{ nmol mg}^{-1} \text{hr}^{-1}$. Lysosomes gave $1103 \text{ nmol mg}^{-1} \text{hr}^{-1}$ under these assay conditions. Thus, the specific activity of the plasma membranes relative to purified lysosomes is 8.75, where the lysosomal activity has been set to 100. The contamination of plasma membranes with lysosomal activity was very slight, amounting to a relative specific activity of only 0.5 (based on acid phosphatase)

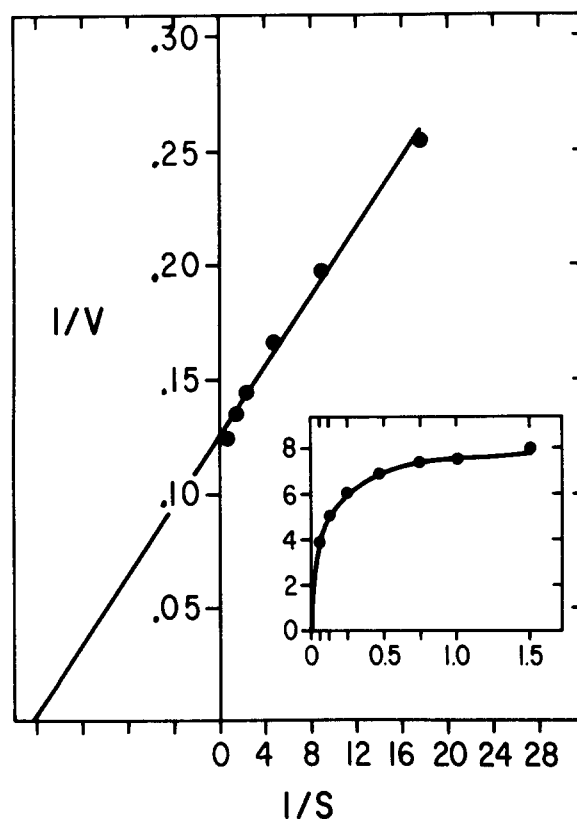


Fig. 7. The effect of sphingomyelin concentration on microsomal sphingomyelin hydrolysis. The incubations were done as noted in Methods. Triton X-100 concentration was maintained constant. In the double reciprocal plot and in the velocity-substrate plot (inset) the results have been expressed in **Fig. 3**.

or 0.28 (based on acid sphingomyelinase). Therefore, the sphingomyelinase activity of the plasma membrane cannot be explained by lysosomal contamination.

The specific activity of microsomal sphingomyelinase was 10.47 nmol mg⁻¹ hr⁻¹ under optimal assay conditions. Lysosomes gave 206 nmol mg⁻¹ hr⁻¹ under these conditions; thus, the relative specific activity of microsomes is 5.1, where the specific activity of lysosomes has been set to 100. The relative specific activity of the lysosomal marker enzymes contaminating the microsomes is 0.26, based on acid sphingomyelinase, or 1.9, based on acid phosphatase. Thus, the lysosomal contamination of microsomes accounts for 5.1% or 37% of the total microsomal sphingomyelinase activity, depending on which marker enzyme one uses for the calculation. As shown in Table 1, plasma membrane contamination of the microsomal fraction is 5.6% based on 5'-nucleotidase. Therefore, plasma membrane contamination accounts for 32% of the observed microsomal sphingomyelinase activity $([0.056 \times 29.1/5.1] \times 100)$. Thus, the sphingomyelinase activity of microsomes cannot be completely accounted for by contamination with lysosomes and plasma membranes.

Gradient-purified mitochondria did not have significant sphingomyelinase activity. When assayed at the conditions optimal for microsomes, no activity was detected. At pH 4.8, sphingomyelinase activity of 1.6 nmol mg⁻¹ hr⁻¹ was measured in the purified mitochondrial fraction. This activity can be accounted for completely by the presence of lysosomal contamination. Finally, the cytosol did not have significant neutral sphingomyelinase activity (data not shown).

The total protein represented by the plasma membrane and lysosomal fractions can be estimated from the enrichments of the respective marker enzymes over those in the homogenate if one assumes that the assay conditions are completely comparable. The specific activity of 5'-nucleotidase in the plasma membrane is 40 times greater than that of the homogenate and the specific activity of acid phosphatase in the purified lysosomes is 46-fold greater than that of the homogenate (not shown). From these data, one can calculate that plasma membrane fraction represents 2.5% and the lysosomal fraction represents 2.2% of liver protein. Thus, at pH 7.4 in the presence of 40 mM Mg²⁺, the total neutral sphingomyelinase activity of the plasma membranes is only 10% of the lysosomal sphingomyelinase activity measured at these conditions $([96.5 \times 0.025/1,103 \times 0.022] \times 100)$.

Effects of inhibitors

Table 2 shows the effects of various inhibitors and activators on the sphingomyelinase activity of the

TABLE 2. Effect of inhibitors and activators on liver sphingomyelinases

Conditions	Sphingomyelinase		
	Lysosome	Plasma Membrane	Microsome
Control	100	100	100
0.05 mM Hg ²⁺	95	2	7
1 mM <i>p</i> -chloromercuribenzoate	89	2	24
10 mM AMP	2	70	109
70° × 10 min	4	8	0
Triton X-100 omitted	1	9.6	0
Mg ²⁺ omitted		0	20
<i>n</i> -Butanol extraction	127	0	n.d.

Sphingomyelinase assays were carried out with each fraction at optimal conditions. The control incubation in each case contained all additions as noted in the methods; this value has been set to 100. Other incubation conditions were varied as noted above and the results expressed relative to the control. Control values were: lysosome, 5,106 nmol mg⁻¹ hr⁻¹; plasma membrane, 95.4 nmol mg⁻¹ hr⁻¹; microsome, 11.9 nmol mg⁻¹ hr⁻¹; n.d., not determined.

three subcellular membrane fractions. The control activities have been set equal to 100 and represent the activity at the optimal assay conditions for each preparation. The neutral sphingomyelinase activity of plasma membranes and microsomes was strongly inhibited by Hg²⁺ and *p*-chloromercuribenzoate (98 and 93%, respectively). These sulfhydryl inhibitors had little effect on lysosomal sphingomyelinase. Conversely, 10 mM AMP inhibited lysosomal sphingomyelinase by 98% while much smaller effects were noted on the activity of the plasma membrane (30% inhibition) and the microsomal fraction (9% stimulation). Heating to 70°C for 10 min resulted in a loss of activity in all three preparations. None of these membrane-bound sphingomyelinases had significant activity in the absence of Triton X-100. Liver plasma membrane sphingomyelinase was inactive without added Mg²⁺; however, with microsomes, a basal activity representing 20% of the control was noted without Mg²⁺, which probably reflects the lysosomal contamination of this membrane preparation. Extraction with *n*-butanol destroyed the plasma membrane activity but had little effect on the lysosomal sphingomyelinase.

Heat stability

Fig. 8 shows the effect of heating the plasma membrane and lysosomes at 55°C for varying periods of time followed by standard assay for sphingomyelinase. Plasma membrane neutral sphingomyelinase activity was heat labile. After 10 min at 55°C only 15% of the control activity remained compared with 78% remaining in lysosomes. Only 3% of plasma mem-

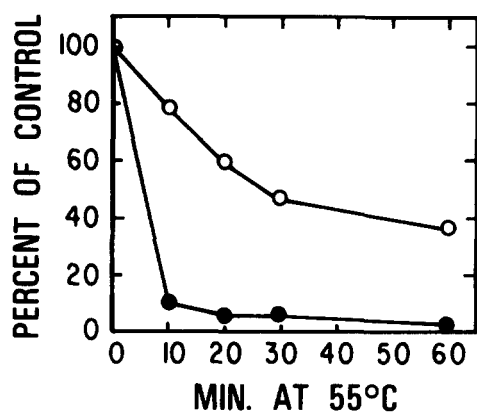


Fig. 8. Effect of heating on the sphingomyelinase activity of plasma membranes and lysosomes. Plasma membrane or lysosomal protein was heated at 55°C for various times as noted. After heating, sphingomyelinase incubations were carried out at standard conditions as noted in Methods. The results are expressed as percent of the unheated control. Control values for unheated membranes were: plasma membrane, 125 nmol mg⁻¹ hr⁻¹; lysosomes, 1,500 nmol mg⁻¹ hr⁻¹. Closed circles, plasma membrane; open circles, lysosomes.

brane activity remained after 60 min at 55°C vs. 37% of the control activity remaining in lysosomes.

DISCUSSION

These studies have demonstrated that neutral sphingomyelinase activity is present in the plasma membrane (and microsomes) of rat liver. This neutral sphingomyelinase differs from lysosomal sphingomyelinase in several ways including its subcellular localization, response to divalent cations, pH optimum, apparent K_m for sphingomyelin, heat stability, and in its response to inhibitors.

In addition, it has been shown that, above pH 5.0, lysosomal sphingomyelinase is stimulated by the divalent cations Ca²⁺, Co²⁺, Mg²⁺, and Mn²⁺. Little or no stimulation by divalent cations was found between pH 4.4 and 4.8, the pH at which lysosomal sphingomyelinase is usually measured. This may account for the earlier reports which found no effect of Ca²⁺ on the reaction velocity (1, 2). The stimulation by divalent cations seemed to be nonspecific because all four of the divalent cations tested gave similar velocity vs. concentration curves. The maximum stimulatory effect was found at pH 6.0–6.4 and the stimulation above the basal level was considerable, representing an 82% increase over the control. Below pH 4.4, divalent cations strongly inhibited lysosomal sphingomyelin hydrolysis. Thus, it seems apparent that variations in the pH or in the concentration of divalent cations may play an important role in the regulation of lysosomal sphingomyelinase activity.

Few differences could be observed between the neutral sphingomyelinase activities of the plasma membrane and microsomal fractions. They are both inhibited by sulfhydryl reagents and are not significantly inhibited by 10 mM AMP. They differ only in their apparent K_m for sphingomyelin and their requirements for Mg²⁺. Much of the microsomal activity can be accounted for by contamination with lysosomes and plasma membranes. Nevertheless, about 50% of the microsomal neutral sphingomyelinase activity cannot be explained by contamination and it seems reasonable to assume that a small amount of endogenous sphingomyelinase is present in microsomes. This small pool of activity might represent newly formed enzyme destined for incorporation into the plasma membrane.

At the present time, the role of the neutral sphingomyelinase in plasma membranes and microsomes is uncertain. Van Golde et al. (19) have shown that sphingomyelin biosynthesis takes place in the rough and smooth endoplasmic reticula (maximal velocity 43 nmol mg⁻¹ hr⁻¹). However, the plasma membrane appears to be devoid of *de novo* sphingomyelin biosynthesis (19) and, at the present, it is not certain how the plasma membrane content of sphingomyelin is acquired or renewed. Nevertheless, it seems reasonable to assume that plasma membrane sphingomyelinase might serve to regulate the concentration of this lipid in the plasma membrane. Similarly, microsomal neutral sphingomyelinase might act to prevent the local accumulation of newly formed sphingomyelin.

Sphingomyelin is an important component of the phospholipids of liver plasma membranes and red cell membranes. The membrane content of cholesterol correlates well with the sphingomyelin content in many natural membranes, and Demel et al. (20) have presented evidence of a preferential interaction between sphingomyelin and cholesterol. Natural sphingomyelins are high-melting; studies employing differential scanning calorimetry of aqueous dispersions of sphingomyelin have shown a broad gel–liquid crystalline phase transition from 20 to 45°C (20–23). Since the membrane content of sphingomyelin and cholesterol may be an important determinant of membrane fluidity, plasma membrane neutral sphingomyelinase might play an important indirect role in this process by regulating the membrane content of sphingomyelin.

In certain forms of Niemann–Pick disease, sphingomyelin accumulates in tissues although the activity of acid (lysosomal) sphingomyelinase appears to be normal or slightly reduced (24). The relation of liver neutral sphingomyelinase to the pathogenesis of these diseases is not known, but it is possible that sphingomyelin accumulation might be caused by the absence

of neutral sphingomyelinase activity in body tissues. This possibility remains to be investigated. ■■

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