Biochem. J. (1981) **193**, 275–283
Printed in Great Britain

Studies on the hydrophobic properties of sphingomyelinase

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(*Received 24 June 1980/Accepted 2 July 1980*)

Crude liver lysosomal sphingomyelinase (EC 3.1.4.12) displays a heterogeneous electrofocusing profile. The majority of the enzyme resolves into two major components with acidic pI values near pH 4.6 and 4.8. Several additional minor peaks of activity are seen at more basic pH values (up to pH 8.0). In the presence of 0.1% Triton X-100 (or Cutscum), the location of sphingomyelinase is shifted by about 1 pH unit to more basic pH values. Triton X-100 also increases the apparent heterogeneity of sphingomyelinase. Removal of detergent by treatment with Bio Beads SM-2 restores the acidic pI profile. This behaviour appears to be specific, since it was not shared by six glycosidases several of which hydrolyse sphingolipids. The electrofocusing profile of ^{3}H-labelled Triton X-100 was distinct and separate from sphingomyelinase, suggesting that only a small fraction of detergent interacted directly with the enzyme. To study this behaviour in more detail we examined the effect of detergents on elution of sphingomyelinase from sphingosylphosphocholine–Sepharose. Sphingosylphosphocholine is a competitive inhibitor of sphingomyelinase (K_i 0.5 mM). Binding of enzyme was pH-dependent. Triton X-100, Cutscum and Tween 20 eluted significant amounts of enzyme at 0.01–0.02%. Total elution was achieved with up to 0.1% detergent. These data suggest that sphingomyelinase binds to neutral detergent monomers with a high degree of affinity. In excess detergent (5–7 times the critical micellar concentration) the surface charge on the protein is changed, leading to a pI shift. This behaviour probably does not occur at the active site of the enzyme, since there is no appreciable effect on substrate hydrolysis and substrate analogues were ineffective in eluting the enzyme.

Two distinct forms of sphingomyelinase have been identified in the human (Rao & Spence, 1976; Yamaguchi & Suzuki, 1977). The lysosomal form possesses properties (acidic pH optimum, no metal requirement, stability to mercurials) that distinguish it from the microsomal form (basic pH optimum, metal requirement, inhibited by mercurials). Within the lysosomal sphingomyelinase pool, demonstration of enzyme heterogeneity has proved difficult. The major tool used to date to resolve multiple forms of lysosomal sphingomyelinase has been isoelectric focusing (Besley, 1976, 1977; Callahan *et al.*, 1975; Callahan & Khalil, 1975; Harzer *et al.*, 1977). It has been shown that sphingomyelinase can be partially resolved into several components by using this technique. There is, however, no general agreement that the major components observed actually reflect different species of the enzyme since little information is available on the structure of sphingomyelinases. In addition the electrofocusing behaviour of the enzyme has been affected by the presence of detergents such as Triton X-100. We, for example, observed that the enzyme resolved into two major components with acidic pI values when liver tissue was electrofocused in a low concentration (0.02%) of Triton X-100 (Callahan *et al.*, 1975). Harzer *et al.* (1977) confirmed the presence of two components in liver homogenates prepared in water, but not when prepared in the presence of Triton X-100. Besley (1976, 1977) demonstrated that the isoelectric point of skin fibroblast sphingomyelinase shifted from pI below 5.0 to pI above 7 when electrofocused in the presence of 0.1% Triton X-100. This shift has been confirmed with sphingomyelinase partially purified from human placenta (Callahan *et al.*, 1978).

The results of these three groups indicate that heterogeneity of sphingomyelinase exists, but there is as yet no clear understanding of the influence of Triton X-100 on the electrofocusing behaviour of the enzyme. In the present work we have examined the influence of Triton X-100 and other detergents on

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the electrofocusing and chromatographic behaviour of sphingomyelinase. The interaction of Triton X-100 and sphingomyelinase results in a reversible shift of the isoelectric point and a change in the apparent heterogeneity of the enzyme. Of the seven enzymes examined only sphingomyelinase is affected in this manner. The data suggest that Triton X-100 and sphingomyelinase undergo hydrophobic interaction that results in a conformation change in the enzyme.

Experimental procedures

Materials

Autopsy specimens of liver were obtained within 24h of death and stored at −20°C in closed containers until used. Previous data (Callahan *et al.*, 1975) have shown that sphingomyelinase activity is stable and independent of time of storage under these conditions. Placentae were frozen immediately after delivery and processed as previously described.

Bovine brain sphingomyelin, synthetic glycoside substrates and bovine serum albumin were purchased from Sigma. [*Choline methyl*-^{14}C]-Sphingomyelin and [*phenyl*-^{3}H]Triton X-100 were obtained from New England Nuclear, Boston, MA, U.S.A. Bio Beads SM-2 were a product of Bio-Rad Laboratories, Richmond, CA, U.S.A. Reagent grade sucrose and other chemicals were obtained from BDH, Toronto, Ont., Canada. Carrier ampholines were a product of LKB, Bromma, Sweden.

Purification of sphingosylphosphocholine and coupling to Sepharose 4B

Sphingosylphosphocholine was prepared by the method of Kaller (1961) and purified by chromatography on Silica gel HR. Briefly, 0.74g of sphingomyelin (Sigma type I, bovine brain) was dissolved in a 1:1 mixture of n-butanol/6M-HCl and heated in a boiling-water bath for periods up to 90min. The reaction was monitored by t.l.c. on silica gel G with the solvent butan-1-ol/ethanol/acetic acid/water (80:2:1:3, v/v). Product was detected by 10% phosphomolybdic acid in ethanol. The reaction was virtually complete by 30min. The reaction mixture was cooled in ice/water and evaporated to dryness under vacuum. The residue was twice re-dissolved in 20ml of chloroform/methanol (2:1, v/v) and dried as above. The sample was dissolved in 5ml of chloroform/methanol (2:1, v/v), diluted with 10ml of chloroform and placed on a 25g column (20cm × 2.0cm) of silica gel HR prepared in chloroform. The column was eluted with 200ml of chloroform/1% acetic acid (in methanol) prepared in the ratios 7:3, 1:1, 3:7 (v/v). The bulk of the sphingosylphosphocholine (275mg, 56.1% yield) was eluted in highly purified form with chloroform/1% acetic acid in methanol (3:7). The final product migrated to the same position as authentic sphingosylphosphocholine purchased from Supelco. The phosphate content (17.9%) was in good agreement with the expected value (17.7%) and the ^{13}C-n.m.r. spectrum run in $^{2}H_2O$ was consistent with the expected structure. The purified product was a competitive inhibitor of sphingomyelinase with a K_i of 0.5mM.

Sphingosylphosphocholine was incubated overnight at 20°C with Sepharose 4B, activated by the CNBr technique, in the ratio 14μmol of sphingosylphosphocholine/ml of activated gel (Cuatrecasas, 1970). To block any reactive groups the gel was finally incubated with 1M-ethanolamine at pH8.0. The sphingosylphosphocholine–Sepharose 4B was washed several times with 0.1M-sodium bicarbonate, water and 0.1M-sodium cacodylate, pH6.1. The gel was finally suspended in 0.1M-sodium cacodylate buffer, pH6.1, and several columns were prepared each containing 7–10ml of gel. The sphingosylphosphocholine–Sepharose was stable for at least 6 months when stored in the cold.

Tissue extraction

Liver (1g) was minced with scissors and homogenized in 10vol. of 50mM-citrate/100mM-phosphate buffer, pH4.5, and processed as described previously (Callahan *et al.*, 1975). Placentae were thawed overnight at 2°C, stripped of cord and membranes under running tap water and small pieces were homogenized in 3vol. of 100mM-cacodylate buffer, pH6.1, or as otherwise stated (Callahan *et al.*, 1978).

Isoelectric focusing and processing of enzyme pools

Dialysed and undialysed enzyme extracts were focused in a 110ml column with 2% ampholines and samples were collected in 1–1.5ml fractions. All procedures were carried out between 2 and 4°C. Enzyme activity was measured in the presence of ampholines and sucrose. The distribution of [^{3}H]-Triton X-100 in electrofocusing experiments and in enzyme extracts was determined by counting a 0.5ml portion of the appropriate fraction for radioactivity in 5ml of Bray's scintillant. The pH measurements were obtained from alternate fractions held at 4°C with an ice/water bath. Other conditions such as voltage, time, formation of sucrose gradients and elution of the columns has been described peviously (Callahan *et al.*, 1975; Callahan & Khalil, 1975).

Active fractions were pooled and dialysed for 2–3 days against two changes of 6 litres of 1% glycine. Selected extracts were treated with Bio-Beads to decrease the Triton X-100 concentration. The amount of beads used was based on the volume of the pool and the initial concentration of detergent. In

our hands, 1g of Bio-Beads binds 30mg of Triton X-100 (Callahan *et al.*, 1978).

Chromatography of sphingomyelinase on sphingosylphosphocholine–Sepharose

Portions (2ml) of clear supernatant fluids derived from placenta homogenates were applied to 8ml of sphingosylphosphocholine–Sepharose packed in a 12ml plastic syringe. The column was then washed with 30ml of homogenization buffer. The enzyme was eluted with 20ml of homogenization buffer containing 0.1% (w/v) Triton X-100 or other eluant as indicated. Approx. 1ml fractions were collected until all unadsorbed protein was eluted. Thereafter, 1.8ml fractions were collected.

Enzyme assays

Sphingomyelinase was assayed with both [^{3}H]-sphingomyelin and [^{14}C]sphingomyelin as substrates. The ^{3}H- and ^{14}C-labelled sphingomyelins were purified as described (Callahan *et al.*, 1975, 1978) and radiopurity was checked by radioscanning after t.l.c. The substrates were judged to be at least 99.9% pure. The sphingomyelinase assay with [^{3}H]sphingomyelin has been described (Callahan *et al.*, 1975; Schneider & Kennedy, 1967). The assay with ^{14}C-labelled sphingomyelin was slightly modified from that of Pentchev *et al.* (1977). Each tube contained, in a final volume of 0.2ml, 0.2mg of Triton X-100, 25μmol of sodium acetate buffer, pH5.0, and 25μg of sphingomyelin (25000c.p.m.). The reaction was stopped by adding 1ml of serum albumin (1%, w/v) and 0.3ml of 50% trichloroacetic acid. Blank values were consistently between 50 and 100c.p.m. The following glycosidases were measured by published procedures: methylumbelliferyl β-galactosidase (Callahan & Gerrie, 1975); methylumbelliferyl β-hexosaminidase (Lowden *et al.*, 1973); *p*-nitrophenyl α-fucosidase (Thomas *et al.*, 1973); *p*-nitrophenyl α-*N*-acetylgalactosaminidase

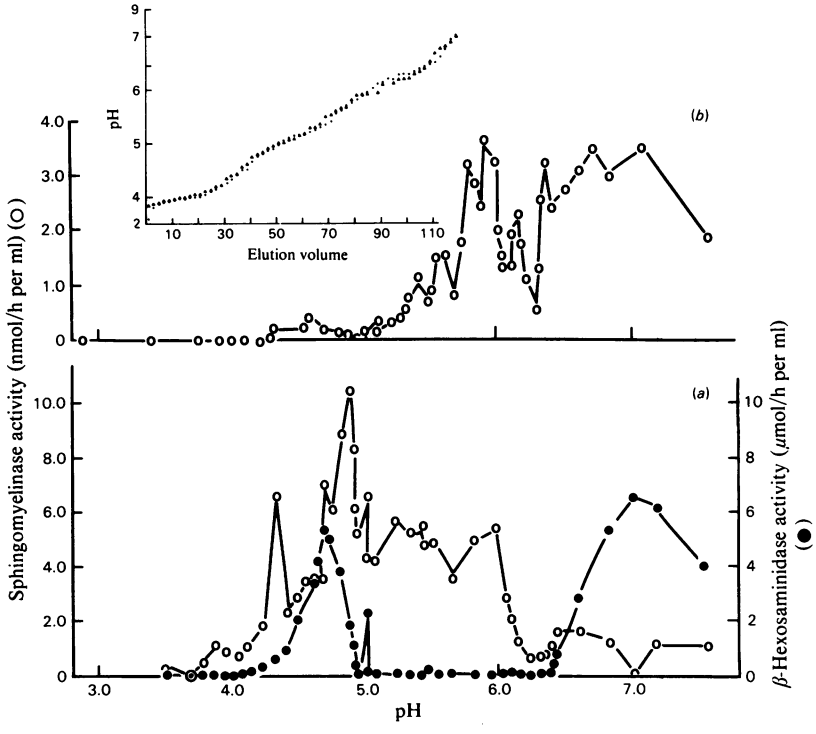

Fig. 1. *Isoelectric-focusing profile of sphingomyelinase with and without Triton X-100*

A dialysed extract of human liver sphingomyelinase (84mg of protein, 344.4 units) was exposed to current for 40h at 600V (maximum) with a pH range of 4–7. The column was eluted from below at a flow rate of 2ml/min. The pH was measured on alternate fractions. In (*a*), sphingomyelinase (○) and an internal control, β-hexosaminidase (●), are shown. No Triton X-100 was included up to this point. Fractions between pI4.3 and 6.3 were pooled and dialysed against 1% glycine. In (*b*) a portion of the extract was re-focused (105 units of activity) under the same conditions, except that a final concentration of 0.1% Triton X-100 was included in the gradient solutions. Sphingomyelinase detected was 87% (*a*) and 70% (*b*). The inset shows the pH gradients, for both experiments, which were identical [▲ refers to (*a*), ● refers to (*b*)].

(Callahan *et al.*, 1973); methylumbelliferyl β-glucosidase (Peters *et al.*, 1976) and methylumbelliferyl α-mannosidase (Thomas *et al.*, 1973). Protein was measured by the method of Lowry *et al.* (1951). One unit of sphingomyelinase activity hydrolyses 1 nmol of substrate/h at 37°C. For the glycosidases, one unit refers to 1 nmol of methylumbelliferone or nitrophenol released per h at 37°C. The amounts of the latter were obtained from standard curves measured under the same conditions.

Results

Electrofocusing behaviour of sphingomyelinase and the effect of Triton X-100

Previously we have shown that homogenization of frozen liver with citrate/phosphate buffer containing 0.25% Triton X-100 solubilizes 85–90% of the enzyme (Callahan *et al.*, 1975). Omitting Triton X-100 from the homogenization medium resulted in lower yields of soluble enzyme (48.8 ± 5.3% of the total). However, for the purposes of the present study it was imperative to obtain enzyme extracts devoid of detergent.

Isoelectric focusing of soluble sphingomyelinase, prepared in the absence of detergent, results in a heterogeneous distribution of the enzyme (Fig. 1*a*). Enzyme activity was distributed throughout the gradient but the major activity peaks were found at pI values of 4.9, 5.2, and 5.9. The activity on the acidic side of the major peak (at pI 4.9) spread to pI less than 4.0 and there was a small peak at pI 4.3. When the fractions from pI 4.3 to 6.2 (about 90% of the total) were pooled, dialysed and re-focused in a gradient containing 0.1% (w/v) Triton X-100, a shift in distribution was observed (Fig. 1*b*). The enzyme at pI 4.3, 4.9 and 5.2 disappeared, whereas the number of peaks and total activity at pI above 5.8 increased. The shift in isoelectric point of the bulk of enzyme activity was approximately one pH unit. When the total original extract was focused under the same conditions the same result was obtained (Fig. 2*a*). This indicates that the shift in isoelectric point is independent of the focusing conditions and the protein composition of the extract. However,

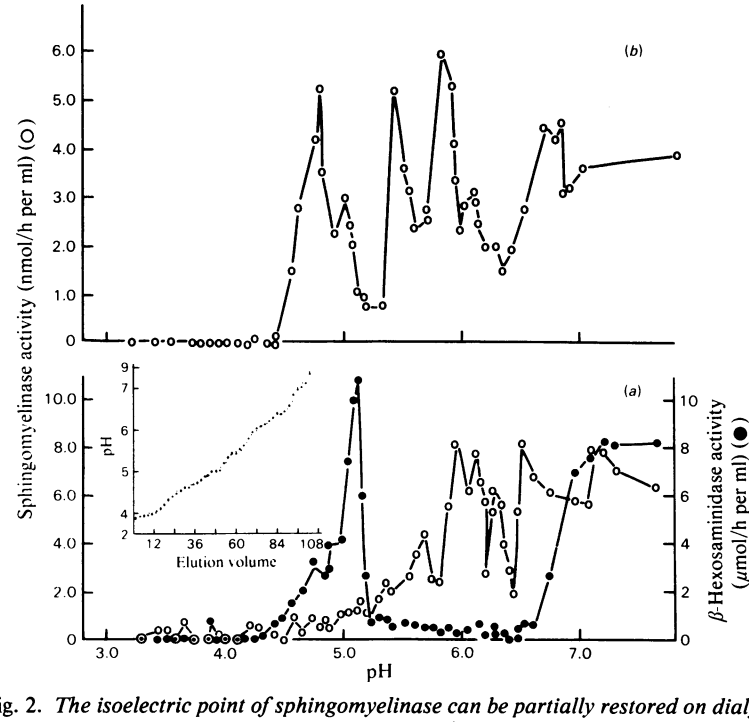

Fig. 2. *The isoelectric point of sphingomyelinase can be partially restored on dialysis*
In (*a*) a liver extract prepared as described was focused initially in a gradient containing 0.1% Triton X-100. Sphingomyelinase (O) was shifted to more basic pI values, whereas β-hexosaminidase (●) was unchanged. The enzyme between pI 5.3 and 7.3 was pooled, dialysed and a portion was re-focused under identical conditions as shown in (*b*). A portion of the sphingomyelinase was shifted back to its initial isoelectric point (pI 4.8). Recovery of activity under both conditions was almost quantitative. Other details are given in the legend to Figure 1. The inset shows the pH gradient for both experiments, which were identical [▲ refers to (*a*), ● refers to (*b*)].

when the enzyme was dialysed and re-focused in the absence of additional detergent, the enzyme profile showed two acidic components (pI 4.8 and 5.0) and distinct peaks at pI values of 5.4, 5.8 and 6.7–6.8 (Fig. 2*b*). This pattern is intermediate between that obtained in the absence (Fig. 1*a*) and in the presence of 0.1% (w/v) Triton X-100 (Figs. 1*b* and 2*a*).

The shift in isoelectric point of sphingomyelinase in the presence of 0.1% Triton X-100 appears to be peculiar to this enzyme. Six glycosidases known to be involved in the catabolism of other sphingolipids (β-galactosidase, β-glucosidase, β-hexosaminidase, α-*N*-acetylgalactosaminidase) and oligosaccharides (α-fucosidase and α-mannosidase) did not display altered isoelectric focusing behaviour in the presence of 0.1% (w/v) Triton X-100 (Table 1). Similarly, neither haemoglobin nor bovine serum albumin were affected. When 0.1% Cutscum was substituted for Triton X-100, the sphingomyelinase pattern was the same as observed for Triton X-100, whereas with sodium taurocholate no shift in isoelectric point from acidic to basic values was observed. The recovery of enzyme with sodium taurocholate was also decreased.

We then determined whether the distribution of sphingomyelinase coincided with Triton X-100 by comparing the profile of [^{3}H]Triton X-100 with sphingomyelinase, measured with ^{14}C-labelled sphingomyelin as substrate (Fig. 3). No coincidence between the enzyme and the detergent was found suggesting that if Triton X-100 and sphingomyelinase interact directly, only a very small portion of the detergent is involved in this reaction. It should be noted here that electrofocusing of sphingomyelin (10mg) in 0.1% Triton X-100 shows coincidence of both substances, the majority of material being located at pI 4.0–4.2 with a small peak at pI 6.3 (results not shown).

The data suggested that the shift in isoelectric point of sphingomyelinase in the presence of detergent was probably reversible. To test this, sphingomyelinase was electrofocused in the presence of 0.1% Triton X-100 (Fig. 4*a*) and the pooled

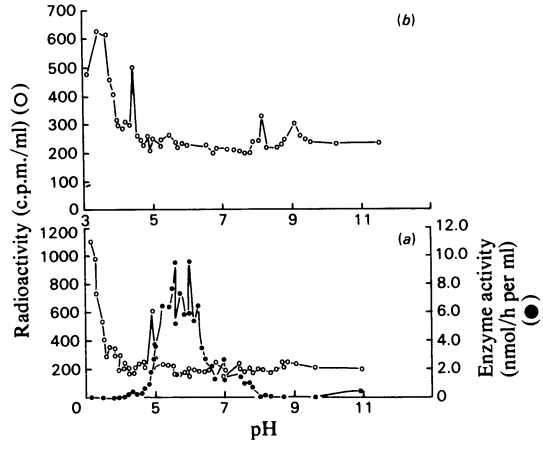

Fig. 3. *Comparisons of the behaviour of sphingomyelinase and [^{3}H]Triton X-100*

Normal human liver was focused as outlined in the legend to Fig. 1 and in the Experimental procedures section, except that the pH gradient was 3.5–10. The gradient was prepared with 0.1% Triton X-100 containing 100×10^3 c.p.m. of [^{3}H]Triton X-100. (*a*) Shows the behaviour of [^{3}H]Triton X-100 (○) (as measured by [^{3}H]Triton X-100) in the presence of liver sphingomyelinase (●). The isoelectric profile of the enzyme and detergent do not correspond. The profile obtained with [^{3}H]Triton X-100 alone is shown in (*b*). There is a shift in the minor peak of Triton X-100 from 4.4 to 5.0, but this does not coincide with a peak of enzyme activity. There is little or no coincidence between the profile of enzyme and detergent between pI 5.0 and 7.0.

Table 1. *Isoelectric points of several lysosomal glycosidases and sphingomyelinase in the presence of Triton X-100*

Liver tissue was processed as described and portions were focused simultaneously in gradients prepared without and with 0.1% Triton X-100. The isoelectric points cited refer to peak enzyme activities. The numbers in parentheses refer to the numbers of peak activities observed. Hexosaminidases A and B are recognized isoenzymes.

	pI	
Enzyme	−Triton X-100	+Triton X-100
β-Galactosidase (2)	3.88, 4.32	4.00, 4.32
β-Hexosaminidases A and B	4.80, 7.00	4.88, 7.24
β-Glucosidase (1)	4.32	4.32
α-*N*-Acetylgalactosaminidase (1)	4.26	4.22
α-Mannosidase (3)	4.86, 5.10, 5.70	4.90, 5.00, 5.70
α-Fucosidase (7)	4.50, 4.76, 4.92	4.56, 4.80, 5.00
	5.12, 5.38, 5.50	5.15, 5.35, 5.67
	5.84	5.76
Sphingomyelinase (5)	3.96, 4.70, 4.90,	5.60, 5.85, 6.10
	5.00, 5.74	6.44, 6.65, 7.20

enzyme was re-focused after removal of the detergent by Bio-Beads and dialysis (Fig. 4*b*). Sphingomyelinase was resolved into multiple peaks of activity at pI values of 4.7, 4.9, 5.6 and 6.5 and with a broad peak at 6.8–7.6. These values are consistent with those in the initial experiments and our previous reports (Callahan *et al.*, 1975; Callahan & Khalil, 1976).

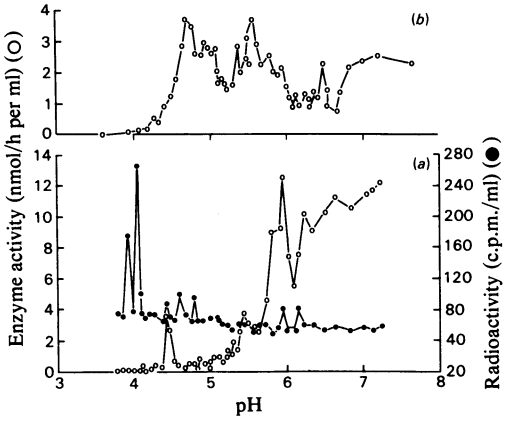

Fig. 4. *Reversibility of the shift in isoelectric point of sphingomyelinase on removal of Triton X-100*
Sphingomyelinase was focused in the presence of 0.1% [^{3}H]Triton X-100 (100×10^3 c.p.m.) and the enzyme between pI values 5.4 and 7.0 was pooled and quickly passed four times through a column of Bio-Beads SM-2 (3.8g). This treatment decreased the Triton X-100 concentration from 0.1% (94×10^3 c.p.m.) to 0.015% (15.5×10^3 c.p.m.). Dialysis decreased the Triton X-100 further to 0.004% (3.6×10^3 c.p.m.). Bio-Bead treatment resulted in a loss of 60% of the activity with about 64% of this detected after isoelectric focusing. (*a*) Shows the isoelectric profile of the enzyme (○) and [^{3}H]Triton X-100 (●) before Bio-Bead treatment, and (*b*) shows the enzyme profile (○) after removal of the detergent. It is noteworthy that the acidic pI value of the major enzyme fractions are restored and enzyme heterogeneity persists.

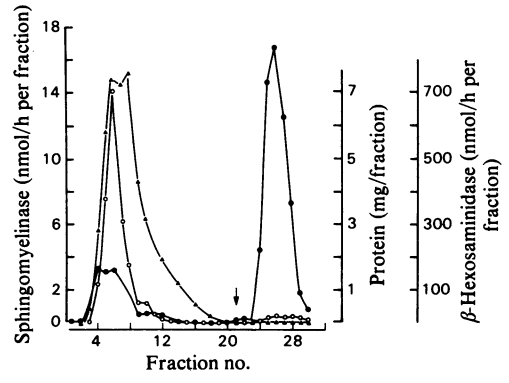

Fig. 5. *Affinity chromatography of placental sphingomyelinase*
Tissue (10g) was homogenized in 20ml of 0.1M-cacodylate buffer, pH 6.1, for six cycles (alternating 1min disintegrating/cooling periods) and then centrifuged at 30000*g* for 30min at 4°C. A portion (2ml) of the supernatant fluid was applied to a 12ml plastic syringe (about 6cm × 1.5cm) containing 8ml of sphingosylphosphocholine–Sepharose. The column was then washed with 30ml of homogenization buffer as described above followed by (arrow) the homogenization buffer containing 0.1% Triton X-100 (20ml). Approx. 1ml fractions were collected until all of the unadsorbed protein was eluted. Thereafter, 1.8ml fractions were collected. Sphingomyelinase (●), β-hexosaminidase (▲) and protein (○) were quantified as described in the text. The elution of β-galactosidase was identical with that of β-hexosaminidase. Recovery of sphingomyelinase was 96%.

Table 2. *Conditions for the binding and elution of sphingomyelinase from Sphingosylphosphocholine–Sepharose*
The placenta was homogenized and the columns (7–10ml) were prepared as described in the text. The data presented below represent separate experiments with different columns. A column could be used several times without appreciable loss of properties. All numbers represent averages of at least duplicate assays.

		Activity (units)			Activity (% of units applied)	
Homogenization buffer	Eluting buffer	Applied	Bound	Recovered	Bound	Recovered
1. Cacodylate (0.1M), pH 6.1	Homogenization buffer + 0.1% Triton X-100	80.0	61.1	58.5	76.4	95.7
2. Maleate (0.1M), pH 6.1	Homogenization buffer + 0.1% Triton X-100	66.0	45.0	44.0	68.2	97.8
3. Acetate (0.1M), pH 5.0	Tris/HCl (0.1M), with 0.1% Triton X-100, pH 8.0	66.0	44.4	37.6	80.7	84.7
4. Hepes* (0.05M), pH 6.8	Tris/HCl (0.1M), pH 8.0	136	24.2	24.3	17.8	100.4
5. Tris/HCl (0.1M), pH 8.0	Homogenization buffer + 0.1% Triton X-100	105	17.3	17.3	16.5	100.0

* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Chromatography of sphingomyelinase on sphingosylphosphocholine–Sepharose

The experiments summarized above show that interaction of Triton X-100 and sphingomyelinase results in reversible changes in pI values and an increase in the apparent heterogeneity. In an attempt to study the enzyme–detergent interaction further experiments were carried out with sphingosylphosphocholine–Sepharose. This ligand has been used as an affinity adsorbent for sphingomyelinase (Sloan, 1972).

Placental extracts were used for these studies since the isoelectric-focusing pattern is similar to that of liver, and the enzyme displays the same altered profile as the liver enzyme in the presence of the detergent (Callahan *et al.*, 1978).

Sphingomyelinase bound to the adsorbent in the absence of Triton X-100. The bulk of the contaminating proteins (A_{280}) and lysosomal hydrolases (β-hexosaminidase and β-galactosidase) were not bound (Fig. 5). The highest binding was observed with acidic pH buffers, whereas at neutral and basic pH values the proportion of enzyme bound was substantially decreased (Table 2). Total recovery of the bound enzyme was achieved with buffer containing 0.05–0.1% Triton X-100. At these concentrations over 80% of the enzyme was readily eluted. Cutscum, Tween-20 and cetylpyridinium chloride were as effective in eluting the enzyme from sphingosylphosphocholine–Sepharose as was Triton X-100 (Fig. 6). Cetylpyridinium chloride was also an inhibitor of sphingomyelinase activity. Sodium taurocholate was a poor eluant for sphingomyelinase. Serine, glycine or choline (0.1 M) were ineffective in eluting the enzyme at pH 6.1, whereas sphingomyelin and phosphate ions were more effective (Table 3). In parallel experiments with [^{3}H]Triton X-100 at least 95% of the detergent was washed out of the column with 3–4 column volumes of buffer. Thus non-specific adsorption of Triton X-100 to sphingosylphosphocholine–Sepharose appears to be minimal.

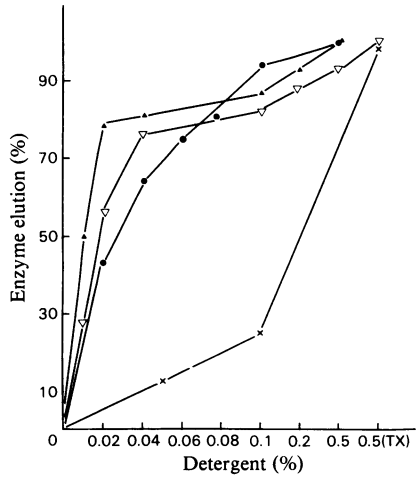

Fig. 6. *Elution of sphingomyelinase from sphingosylphosphocholine–Sepharose by detergents*

The column (0.5 ml) was equilibrated as described in the text. The same amount of enzyme (44 units) was applied to the columns. Columns were eluted stepwise by alternating starting buffer with this buffer containing 0.0–0.5% detergent. The detergents tried were Triton X-100 (●), Cutscum (▽), Tween 20 (▲) and sodium taurocholate (×). The columns were finally washed with buffer containing 0.5% Triton X-100 (TX) to elute any residual enzyme activity.

Table 3. *Effects of substrate and related molecules on the elution of sphingomyelinase from sphingosylphosphocholine–Sepharose*

Conditions are as described in the Experimental procedures section and in the legends to Fig. 5 and Table 2. Note that each column was eluted with the substance in the buffer (20 ml) as indicated. The column was then washed with 20 ml of 0.1 M-cacodylate, pH 6.1, before the next eluant. Activity eluted in the wash was added to the total for the compound used.

Condition	Activity (units)	Recovery (%)	Condition	Activity (units)	Recovery (%)*
Enzyme bound	25.4	100.0	Enzyme bound	44.1	100.0
Eluted with 0.1 M-cacodylate, pH 6.1, containing:			Eluted with cacodylate (0.1 M), pH 6.1, containing:		
Serine (0.1 M)	1.3	5.2	Sphingomyelin (0.2 mM)	1.55	3.6
			(0.3 mM)	2.94	6.7
Glycine (0.1 M)	1.3	5.2	Oleic acid (0.3 mM)	0.35	0.8
Choline (0.1 M)	0.0	0.0	Phosphate (10.0 mM)	0.20	0.4
			(15.0 mM)	2.50	6.0
Triton X-100 (0.1%)	24.5	92.2	Triton X-100 (0.1%)	16.65	37.8
Total	27.1	107.6		24.14	55.3

* The lower recovery in this experiment is attributed to phosphate ion, which is known to inhibit the enzyme (about 50%) at this concentration (15 mM).

Discussion

Most workers have used neutral detergents such as Triton X-100 or Cutscum in homogenization fluids to achieve maximum solubilization of sphingomyelinase (Rao & Spence, 1976; Yamaguchi & Suzuki, 1977; Callahan *et al.*, 1975; Schneider & Kennedy, 1967; Pentchev *et al.*, 1977). During our attempts to develop a procedure for the purification of sphingomyelinase from human placentae and liver we noted shifts in the isoelectric point of the enzyme (Callahan *et al.*, 1978, 1980). The majority of the buffers included Triton X-100. Besley (1976), however, was the first to directly demonstrate an effect of Triton X-100 on the isoelectric point of sphingomyelinase. On the basis of the present experiments, the altered electrofocusing behaviour appears to be reversible, but there is loss of enzyme activity during removal of the detergent. The sphingomyelinase–Triton X-100 interaction likely results in an alteration in the surface charge of the enzyme. This is presumed to occur through hydrophobic interaction between the detergent and a hydrophobic domain in the protein. Other lysosomal hydrolases do not show an altered isoelectric point suggesting the interaction is specific. Bovine serum albumin that binds fatty acids and haemoglobin, two proteins that have been shown to bind very small amounts of Triton X-100 (Clarke, 1977) do not show altered pI values.

Where in the protein hydrophobic interactions occur cannot be assessed at this time. However, the enzyme binds to octyl-Sepharose and can be eluted by 0.5% Triton X-100, whereas it is not bound to lower-chain-length-alkane–agaroses (C. S. Jones & J. W. Callahan, unpublished work).

Enzyme binding to sphingosylphosphocholine–Sepharose may occur at the active site of the enzyme, since binding to this ligand was highest at acidic pH values, consistent with the pH optimum of the enzyme. Substances that form part of the sphingomyelin structure (choline and phosphate) were surprisingly ineffective as eluants as were saturating concentrations of sphingomyelin. Neutral detergents such as Triton X-100, Cutscum and Tween 20 on the other hand were the most effective at 0.05–0.10%.

The interaction of Triton X-100 with proteins has been studied by Helenius & Simons (1972), Makino *et al.* (1973) and Clarke (1975, 1977) among others. Soluble proteins such as alcohol dehydrogenase, haemoglobin, catalase and serum albumin bind little detergent, whereas recognized membrane proteins bind appreciably more. Since sphingomyelinase requires the presence of surface-acting agents for complete solubilization and binds to hydrophobic adsorbents it is reasonable to assume that it possesses a high degree of lipophilic character. The alteration in isoelectric point of sphingomyelinase was observed in the presence of Triton X-100 and Cutscum concentrations, which were about 5–7 times the critical micelle concentration of the detergent. Shifts in the isoelectric points were not seen at concentrations at or below the critical micellar concentration of 0.01–0.02% (Callahan *et al.*, 1975; Callahan & Khalil, 1975). We have attempted to demonstrate direct binding of the enzyme and Triton X-100 by means of the sphingosylphosphocholine–Sepharose column, but have been unsuccessful. However, at concentrations below the CMC, a portion (10–15%) of the Triton X-100 (as determined with [^{3}H]Triton X-100) is retained and can be eluted with the bound enzyme. As shown in Fig. 6, these concentrations of detergents (0.01–0.02%) elute significant proportions of the enzyme from sphingosylphosphocholine–Sepharose. It should be noted that Nakajin *et al.* (1979) reported that Triton X-100 binds to cytochrome *P*-450, leading to a shift in the isoelectric point from 6.5 to 8.2. They also observed a change in the absorbance properties consistent with a conformational change in the protein.

A change in the conformation of sphingomyelinase after Triton X-100 binding would explain not only the shift in isoelectric point, but also the increase in apparent heterogeneity and the elution of the enzyme from sphingosylphosphocholine–Sepharose. It is also possible that the interaction of sphingomyelinase and Triton X-100 at hydrophobic regions on the enzyme leads to an increased net negative charge by affecting the state of aggregation of the proteins. Since the amino acid and subunit composition of the enzyme is unknown it is not possible to predict the number of hydrophobic domains in the protein and the response of these to detergent binding. The final answer to this question must await purification of the enzyme and studies of the spectral changes in the presence of detergents, such as Triton X-100.

The present data confirm and extend our previous reports on isoelectric focusing of sphingomyelinase (Callahan *et al.*, 1975; Callahan & Khalil, 1975). In these reports we demonstrated the presence of two major partially resolved peaks of activity in liver and brain, both of which had acidic pI values. Identification of the structural properties of the protein in each of the isoelectric forms requires further study.

We thank Dr. J. A. Lowden and Dr. D. Mahuran for helpful discussion and criticism and Mrs. S. Mohamed for typing the manuscript. This work was supported by a program grant from the Medical Research Council of Canada, Grant PG-4.

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