HYDROLYSIS OF SPHINGOMYELIN LIPOSOMES BY SPHINGOMYELINASE

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1. Introduction

The enzymic utilization of complex lipids, which belong to the group defined as "insoluble, swelling amphiphiles" [1], usually requires a detergent [2]. This group contains compounds such as lecithin, cephalins, sphingomyelin and the neutral glycosphingolipids [1]. While the detergent could be omitted in some cases i.e., using lecithin dispersions, all the reactions of hydrolysis or biosynthesis of the sphingolipids by mammalian enzymes, were done in the presence of a detergent. The latter was either nonionic (i.e., Triton X-100 or Cutscum) or anionic (i.e., sodium cholate or taurocholate); in some cases a mixture of these two detergents was used (reviewed in [2]).

Detergents are required to attain maximal reaction rates, but they markedly affect the components of the reaction. The most drastic effect is the disruption of membranes. Therefore, addition of a detergent precludes studies on intact, membrane-bound enzymes. Detergents affect the physical state of the substrate and frequently also that of the enzymic protein, they also might impart a charge to the surface of the mixed, substrate–detergent micelle. Furthermore, kinetic analyses are complicated by the introduction of one further component to the ternary system of enzyme substrate–water. This work aimed to prepare dispersions of water-insoluble complex lipids which will be utilized by their respective enzymes, in the absence of a detergent. For this purpose we studied the conditions required for preparation of stable liposomes of sphingomyelin by ultrasonic irradiation. These were used as substrates for soluble or particulate preparations of rat brain sphingomyelinase.

2. Materials and methods

2.1. *Enzyme*

Three preparations rat brain sphingomyelinase were used; all were prepared according to Barenholz et al. [3] or Gatt and Barenholz [4], as follows: Rat brain was homogenized in 9 vol of 0.25 M sucrose–1 mM EDTA, pH 7 and particles were sedimented between 800 and 20,000 *g*. These particles were suspended in sucrose–EDTA and subjected to sonic irradiation for 8 min at 10 Kc. The suspension was centrifuged at 25,000 *g* and the sediment ("sonically disrupted particles") was suspended in isotonic sucrose–EDTA. To solubilize the enzyme, the sonically disrupted particles were frozen and rethawed 10 times, the mixture was centrifuged for 30 min at 25,000 *g* and the supernatant was retained.

2.2. *Sphingomyelin*

A solution of 800 mg of bovine spinal cord sphingomyelin in 40 ml chloroform–methanol, 1:1 was hydrogenated with 45 mCi tritium gas, in the presence of palladium on charcoal. (This hydrogenation was done by the Nuclear Research Center, P.O. Box 9001, Beer Sheva, Israel). After removal of exchangeable hydrogens, the specific activity was 225 mCi/mmole. An aliquot was mixed with non radioactive spingomyelin, which had previously been hydrogenated, using palladium, on charcoal at a pressure of 50 psi. The lipid solution was filtered and chromatographed twice on columns of alumina [5]. The tritium labeled sphingomyelin migrated as a single, radioactive spot on thin-layer plates of silica gel, using chloroform–methanol–water, 75:25:4. Its specific activity was 760,000 dpm

per μmole. The tritium-labeled sphingomyelin was hydrolyzed [6] and the products were separated using standard procedures. 66% of the radioactivity resided in the fatty acid moiety and 34% in the sphingosine bases (mostly dihydrosphingosine).

2.3. *Chemicals*

Cholesterol, dicetylphosphate (sodium salt), methyl oleate and monoolein were purchased from Sigma and octadecylamine from Koch-Light. Lecithin and lysolecithin were prepared from egg yolks.

Several batches of phosphatidic acid (PA) were used. i) "Egg PA" was prepared from egg lecithin by treatment with phospholipase D [7]. ii) Dilauryl-PA and dipalmitoyl-PA were synthesized by Drs. Barzilay and Lapidot [8]. iii) A "mixed PA" containing oleic and palmitic acid residues in equimolar amounts, was synthesized as above [8].

2.4. *Methods*

The quantity of the ceramide formed by the enzymic hydrolysis of sphingomyelin was determined according to Barenholz and Gatt [3, 4]. Microelectrophoresis was done using a cylindrical microelectrophoresis apparatus (Rank Bros., Bottisham, Cambridge, England) according to Bangham et al. [9]. Phosphorus was determined according to Bartlett [10] and protein according to Lowry et al. [11].

2.4.1. Preparation of "sonicated" liposomes

A solution of sphingomyelin (and any added lipid) in chloroform–methanol, 2:1 was evaporated to dryness in a glass tube of 8–10 mm diameter. Water was added and the tube was subjected to ultrasonic irradiation in a Braun-Sonic 300 (Braun, Melsungen, Germany) using a microtip of a diameter of 4 mm, at an output of 35% of the maximal scale. In several experiments the tube was centrifuged for 30 min at 25,000–100,000 *g* and the supernatant was retained.

2.4.2. Preparation of non-sonicated liposomes [12]

A solution of sphingomyelin (and any added lipid) in chloroform–methanol, 2:1, was evaporated to dryness in a round-bottom 25 ml flask. Water and one glass bead were added, the content was vigorously stirred on a cyclomixer and then slowly agitated on a rotatory shaker for 18 hr at 26°.

Units: 1 unit of enzymic activity is defined as nmoles ceramide released by one mg protein in 1 hr. 1 mobility unit is defined as $\mu sec^{-1} \times volt^{-1} \times cm$.

3. Results

3.1. *Comparison of sonicated and non-sonicated liposomes*

The preparation of these two types of sphingomyelin liposomes is described under Materials and methods. The sonicated liposomes were small (about 0.1 μm) and a considerable portion was single bilayered

Table 1

Effect of lipid additives on the rates of hydrolysis of liposomal sphingomyelin.

Additive	Molar ratio additive: sphingomyelin	Relative reaction rate (%)
A) Sonicated liposome		
–	–	100*
Phosphatidylcholine (egg)	0.15	102
Lysophosphatidylcholine (egg)	0.10	50
Phosphatidic acid (egg)	0.15	230
Phosphatic acid (dipalmitoyl)	0.15	220
Phosphatic acid (dilauroyl)	0.15	210
Phosphatic acid (mixed)	0.15	226
Dicetylphosphate	0.15	223
Octadecylamine	0.10	137
Methyl oleate	0.25	242
Methyl palmitate	0.25	250
Cholesterol	0.15	151
Glycerol monooleate	0.15	86
Triton X-100	0.20	152
Triton X-100	2.70	415
B) Non-sonicated liposome		
–	–	30
Phosphatidic acid (mixed)	0.15	96
Methyl oleate	0.15	35
Triton X-100	0.20	150
Triton X-100	2.70	415

Sphingomyelin (1 mM) was mixed with the additive and liposomes were prepared as described under Materials and methods. Incubations and assays were done as described under Materials and methods. 20–75 μg of the soluble enzyme was used.
* The rate of 100% is equal to 308 units.

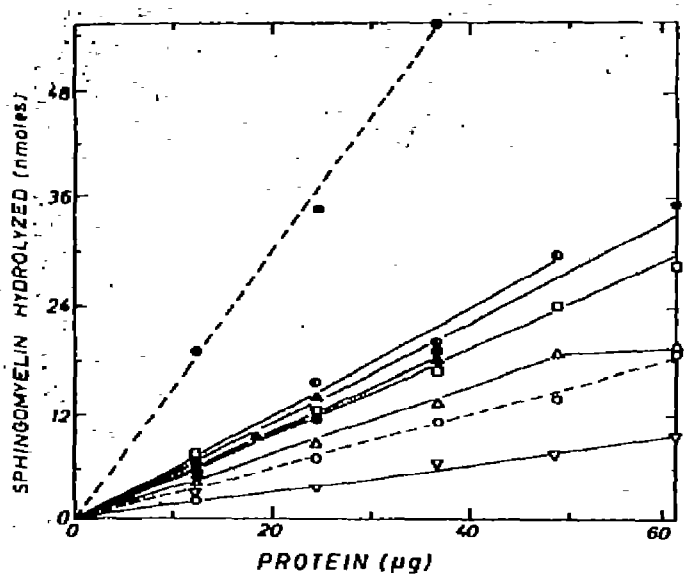

Fig. 1. Effect of concentration of the soluble enzyme on the reaction rates. Incubations and assays were done as described under Materials and methods. (o- -o- -o) no additive; (•- -•- -•) Triton X-100 (2.7)*; (o—o—o) methyl oleate (0.33); (▽—▽—▽) egg lysophosphatidylcholine (0.1); (△—△—△) octadecylamine (0.1); (□—□—□) dicetylphosphate (0.1); (•—•—•) phosphatidic acid (0.1); (▲—▲—▲) Triton X-100 (0.1).

* The numbers in parentheses represent the molar ratios of the additive to sphingomyelin.

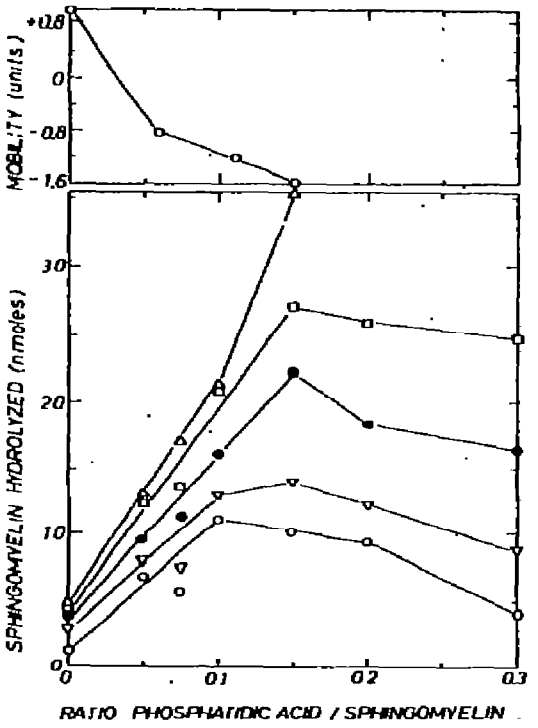

Fig. 2. Effect of the ratio of phosphatidic acid to sphingomyelin on the reaction rates and the electrophoretic mobility. Incubations, assays and the electrophoresis were done as described under Materials and methods. (o—o—o) 18.3 μg of the soluble enzyme; (▽—▽—▽) 24.4 μg; (•—•—•) 36.6; (□—□—□) 48.8 μg; (△—△—△) 73.2 μg.

The non-sonicated preparation consisted of big (about 1 μm) and multilayered liposomes. Using the small liposomes, the rates of hydrolysis by the soluble enzyme were at least three times greater than with the non-sonicated preparation. The curves describing the rate against substrate concentration (*V*/E) were linear up to at least 50 μg protein, using the sonicated liposomes, but deviated from linearity already at about 15 μg with the non-sonicated preparation. The nonionic detergent, Triton X-100 (1.5 mM) further increased the reaction rates of both preparations (table 1). This effect is probably due to the breaking up of the liposomal structure and the formation of smaller, mixed Triton-sphingomyelin micelles (manuscript in preparation). This assumption is supported by the fact that with Triton, the same reaction rates were obtained using either the small or the big liposomes (table 1).

3.2. *Effect of lipid additives*

The rates of hydrolysis of liposomes of pure, hydrogenated sphingomyelin were variable and inconsistent. Incorporation of small quantities of a lipid into the liposomal structure improved the reproducibility and increased the reaction rates. It also increased the stability of the liposomes, which could be stored for several weeks, at 4° without precipitation. Table 1 shows the effect of 13 lipid additives on the enzymatic reaction; except for lecithin, lysolecithin and monoolein, all increased the reaction rates. They were used at concentrations of about 10–20% that of the sphingomyelin, and were mixed with this compound prior to the sonic irradiation. Fig. 1 shows that the rates were directly proportional to enzyme concentration up to at least 60 μg. In separate experiments in which the liposomes consisted of phosphatidic acid–sphingomyelin, at a ratio of 0.15:1 the *V*/E curve was a straight line up to at least 150 μg protein. Triton X-100 increased the reaction rates when used at 20–30% of the sphingomyelin; electron microscopic and ultracen-

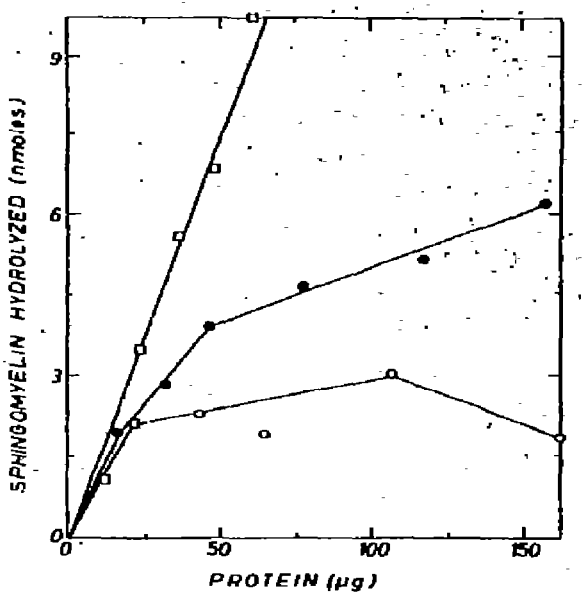

Fig. 3. Effect of the concentrations of three enzyme preparations on the reaction rates. Incubation, assays and enzyme preparation were done as described under Materials and methods. (o—o—o) Particles; (•—•—•) sonically disrupted particles; (□—□—□) soluble enzyme.

trifugal observations suggested that at this ratio the dispersion is still liposomal (manuscript in preparation). This detergent further stimulated the rates when increased to a Triton-sphingomyelin molar ratio of about 3:1. In this range liposomes were absent and the substrate was a mixed lipid–detergent micelle.

The lipid additives which resulted in elevated reaction rates were neutral (i.e., methyl esters of long chain fatty acids and cholesterol), anionic (i.e., phosphatidic acid and dicetylphosphate) or cationic (octadecylamine). The wide variety of these compounds precludes the possibility that they affected the enzyme–substrate interaction by conferring a charge to the surface of the liposomes. Fig. 2 shows that optimal reaction rates were obtained at a phosphatidic acid to sphingomyelin ratio of 0.15. The upper portion of the figure shows that the electrophoretic mobility decreased from 0.8 mobility units, using pure sphingomyelin to −1.6 units at the optimal, phosphatidic acid to sphingomyelin ratio. However, in separate experiments it was found that octadecylamine, at 10% of the sphingomyelin, increased the reaction rates by 40%, while concurrently increasing the mobility to +2.5 units. Methyl oleate, which more than doubled the reaction rate, did not affect at all the electrophoretic mobility. There therefore seems to be no relation between the reaction rates and the type of charge on the liposomal surface.

3.3. *Factors affecting liposome formation*

Several parameters which could affect the liposomal structure and size were examined, using a mixture of phosphatidic acid and sphingomyelin, at a molar ratio of 0.2. The time of sonication was varied between 2 and 5 min, and the tubes were either kept in ice or sonicated without cooling. In either case the time did not affect the reaction rates. However, sonic irradiation without cooling yielded somewhat more stable liposomes and reaction rates were up to 50% greater than with those prepared with external cooling. It should be emphasized that without external cooling the temperature of the dispersion may rise to about 70° during the sonic irradiation. When a sonicated dispersion of the mixed phosphatidic acid–sphingomyelin liposomes was centrifuged for 30 min at 100,000 *g*, no sediment was obtained at 1–5 mM sphingomyelin. When this experiment was done with liposomes of pure sphingomyelin, sonicated for 5 min without cooling, a sediment was not obtained at 1 mM sphingomyelin, but up to 80% sedimented when the initial concentration was 5 mM. Table 1 shows that phosphatic acid also increased the reaction rate with big liposomes of sphingomyelin. At 20% phosphatic acid the reaction rate with the big liposomes about equalled that using a sonicated preparation of sphingomyelin without any additive.

3.4. *Comparison of soluble and particulate enzymes*

Fig. 3 shows the rates obtained with three preparations of rat brain sphingomyelinase: The soluble enzyme, brain particles and sonically disrupted particles (see Methods). The *V*/E curve was linear up to at least 60 μg using the soluble enzyme but deviated from linearity already at 20 μg with either of the two particulate preparations.

4. Discussion

This work aimed to develop a method for the assay of an enzymic reaction, where the substrate is an "insoluble" lipid, in the absence of detergents. This

was achieved by using sphingomyelin liposomes as substrate for the hydrolytic enzyme, sphingomyelinase of rat brain. These liposomes were prepared by ultrasonic irradiation of tritium-labeled hydrogenated sphingomyelin. The liposomes were more stable and reproducible and the rates of hydrolysis were greater, when the substrate was mixed with small quantities of one of several lipids prior to the ultrasonic irradiation. These additives could be anionic, cationic or neutral lipids. This variability suggested that their effect is mostly on the size of the liposomes and probably on their fluidity and the correct spacing of the spingomyelin molecules. These liposomes were substrates for both soluble and particulate preparations of sphingomyelinase.

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