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Effect of Triton X-100 on the Hydrolysis of Sphingomyelin by Sphingomyelinase of Rat Brain

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ABSTRACT: Mixed dispersions of the nonionic detergent Triton X-100 and sphingomyelin were used as substrate for sphingomyelinase of rat brain. The dependence of the rate of hydrolysis on the concentration of sphingomyelin was measured in two ways: at a fixed concentration of Triton X-100 or at varying concentrations of this detergent, while maintaining a fixed molar ratio of Triton X-100 to sphingomyelin. In either case, the v vs. S curves deviated from the hyperbolic shape predicted by the Michaelis–Menten kinetic theory. These deviations are discussed and interpreted on the basis of the physicochemical properties of the mixed dispersions of detergent and lipid studied in previous papers.

In previous papers (Cooper et al., 1974; Yedgar et al., 1974, 1975), the physico-chemical properties of aqueous dispersions of sphingomyelin (SM)[1] and the nonionic detergent Triton X-100 (TR) were investigated. Below about 0.2 mM Triton, sphingomyelin was dispersed as bilayered liposomes. At greater concentrations of this detergent, the lipid was solubilized and mixed micelles of Triton X-100 and sphingomyelin were formed. Once formed, the nature of these mixed micelles depended on the molar ratio of detergent to lipid. When the ratio of TR to SM was between 0.5 and 4.0, the system was homogeneous and monodispersed. In this region the aggregation number of sphingomyelin, i.e., the number of SM molecules in the mixed micelle, varied from about 50 to 440, as the TR to SM ratio varied from about 4 to 0.5, respectively.

At the same time the aggregation number of TR remained practically constant, about 200, irrespective of the TR to SM ratio. This suggests that the concentration of the mixed micelles is a linear function of the detergent concentration only. The micellar concentration can therefore be obtained by dividing the molar concentration of TR by 200, its aggregation number.

In this paper the effect of Triton X-100 on the enzymatic hydrolysis of sphingomyelin by sphingomyelinase of rat brain was investigated. Deviations from the hyperbolic shapes of v vs. S curves are interpreted on the basis of the physicochemical properties of the mixed dispersions of the detergent and the lipid elaborated in the above publications.

Materials and Methods

Sphingomyelin was prepared from bovine brain by extraction with mixtures of chloroform and methanol, followed by chromatography on alumina and silicic acid. The purity of the preparation was tested on thin-layer plates of silica gel. Tritium-labeled sphingomyelin of spinal cord was prepared by catalytic hydrogenation with tritium gas in the presence of palladium on charcoal (Gatt et al., 1973) and was diluted with nonradioactive SM[1] of bovine brain. Triton X-100 was purchased from BDH and one lot (No. 30532) was used throughout. Tritium-labeled Triton X-100 was a generous gift of Rohm and Haas; it was further purified and the purity of the two preparations was checked chromatographically in two solvent systems as described (Yedgar et al., 1975).

Sphingomyelinase of rat brain was an extract of lysosomes of brains of 14 day old rats (Gatt and Gottesdiner, 1976). Its specific activity was about 1000 nmol of sphingomyelin hydrolyzed by 1 mg in 1 h.

The rate of enzymatic hydrolysis of sphingomyelin was followed by the method of Barenholz et al. (1966) as modified for assaying the hydrolysis of lecithin (Gatt, 1968). The assay mixtures were prepared by method B of Yedgar et al. (1975). Incubation time was 1 h at 37 °C. The results reported here are the average of two or more determinations.

Results

Detergents have been used extensively to facilitate enzymatic utilization of lipid substrates (reviewed by Gatt, 1973). Two approaches have been used to study the effect of a detergent on v vs. S curves. In the first, the concentration of the substrate was varied, but that of the detergent was maintained at a fixed concentration. In the second, the concentrations of both substrate and detergent were varied simultaneously while maintaining a fixed ratio between these two components.

The experiment of Figure 1 shows v vs. S curves using the first approach. The concentration of sphingomyelin was varied and that of Triton X-100 was constant; this was repeated at several fixed concentrations of the detergent. All curves were biphasic, exhibiting a hyperbolic region at low substrate concentrations, a maximal value ("inversion point") followed by

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[1] Abbreviations used are: SM, sphingomyelin; TR, Triton X-100.

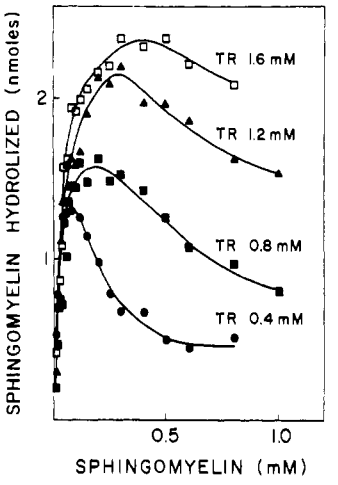

FIGURE 1: v vs. S curves at four concentrations of Triton X-100. Standard assay conditions were used, in the presence of 2 μg of enzyme. The numbers on the curves represent the respective concentrations of Triton X-100.

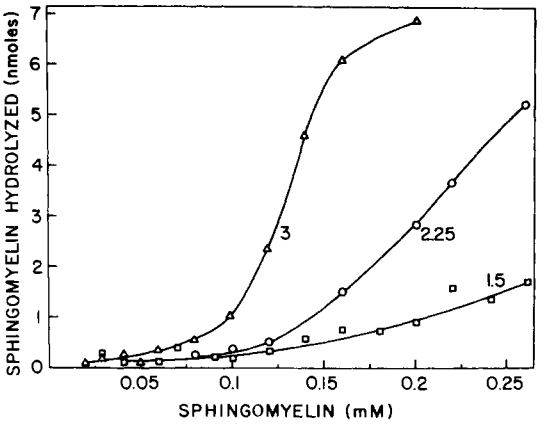

FIGURE 2: v vs. S curves at several fixed molar ratios of Triton X-100 to sphingomyelin (8 μg of protein was used). The numbers on the curves represent the respective molar ratios of Triton X-100 to sphingomyelin.

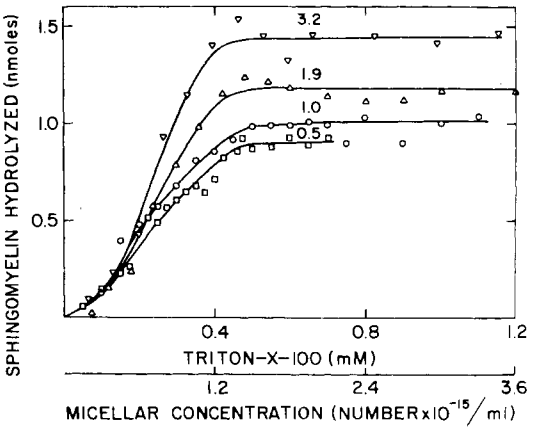

FIGURE 3: Hydrolysis of sphingomyelin as a function of the concentration of Triton X-100 or of the mixed micelles of detergent and lipid. The numbers on the curves represent molar ratios of Triton X-100 to sphingomyelin.

an inverted, descending region. The inversion points (i.e., those substrate concentrations where the curves reached their peaks) of the various curves of Figure 1 depended on and were directly proportional to the concentration of the detergent. Each occurred at an SM concentration which is about one-fourth of the molar concentration of the Triton X-100.

The second approach is based on the observation that the nature of the SM–TR aggregate, i.e., the nature of the substrate, is determined by the ratio of detergent to lipid (Cooper et al., 1974; Yedgar et al., 1974). Figure 2 shows results of an experiment using this approach, i.e., it presents v vs. S curves in which fixed molar ratios of TR and SM were maintained throughout the entire concentration range. The three curves shown in this figure were obtained using the following TR to SM ratios: 1.5, 2.25, and 3.0, respectively. At all these ratios, the micellar system is homogeneous, namely, a single species of mixed TR–SM micelles, whose composition depends on the respective ratio, is present in the solution (Yedgar et al., 1974). As seen in the figure, the reaction rates increased with increasing TR to SM ratio, but all v vs. S curves deviated from the hyperbolic shapes expected according to the kinetic theory of Michaelis and Menten. At low substrate concentrations each curve was part of a nonsymmetric sigmoid, whose shape depended on the TR to SM ratio. This was followed by an ascending, seemingly hyperbolic region.

Because of the nature of SM, an "insoluble, swelling, amphiphilic lipid" (Small, 1970), we assumed that the true substrate for the enzyme was not a molecule of sphingomyelin in solution but a mixed micelle of detergent and lipid. It therefore seemed appropriate to plot the reaction rate as a function of the concentration of these micelles rather than the total concentration of the SM. It has been shown (Cooper et al., 1974; also see introductory section to this paper) that the micellar concentration is a function of the TR concentration and may be calculated by dividing the latter by about 200, which is the number of Triton X-100 molecules in each mixed micelle. For an accurate calculation of the micellar concentration, the concentration of the molecular (monomeric) form of the detergent should have been deducted. The latter is about 0.25 mM, for pure Triton X-100 (Yedgar et al., 1975). However, the probability was considered that, in the presence of sphingomyelin, an "insoluble" lipid, tne concentration of the molecular form of TR will be less than this value (Shinoda et al., 1963; Elworthy et al., 1968). The following experiment was designed to ascertain this possibility. Two milliliters of a solution containing 0.6 mM tritium-labeled TR and 0.2 mM SM (unlabeled) was prepared as described in Methods. After 1 h at 37 °C the mixture was concentrated to $\frac{1}{10}$ of its volume through a UM2 Diaflo membrane, which retains the micelles but not the molecular form of the detergent. At least 90% of the radioactive detergent was retained by the filter and the rest was recovered in the filtrate. This suggested that the concentration of the molecular form of Triton X-100, in mixture with SM, is 0.06 mM or less. This low value was disregarded in the calculation of the concentration of the mixed micelles.

The concentration of the mixed micelles of TR and SM may also be derived by dividing the sum of the concentration of TR and SM by the molecular weight of the mixed micelle, as determined by ultracentrifugation (Yedgar et al., 1974). For this purpose, mixtures containing a fixed concentration of SM and varying concentrations of TR were sedimented in an analytical untracentrifuge. The molecular weights of the mixed micelles were derived (Yedgar et al., 1974) and used to calculate the micellar concentrations. The values obtained by this method equaled those calculated by dividing the concentration of Triton X-100 by 200. This supports the conclusion that the concentration of the mixed SM–TR micelles is a linear function of that of the detergent only. As will be further expounded in the Discussion, the sigmoidal shapes of the curves of Figures 2 and 3 are related to a transition in the aggregation state of

the substrate from liposomes to mixed micelles (Yedgar et al., 1975). For this reason no attempts were made to derive kinetic constants from these figures.

To further evaluate the plateau regions, bireciprocal curves were derived from the convex part of each v vs. S curve. The resulting $1/v$ vs. $1/S$ curves were not linear and the calculated V_{max} values were greater than the maximal rates of the v vs. S curves. This showed that the plateau region starts at a point of discontinuity in the curve. Preliminary experiments suggest that the discontinuity in the v vs. S curves might be due to adsorption of the enzyme onto the mixed micellar substrate.

Discussion

Numerous investigators have demonstrated that the nonionic detergent, Triton X-100, increases the rates of enzymatic hydrolysis of "insoluble, swelling" amphiphilic lipids (reviewed by Gatt, 1973). This paper describes the effect of this detergent on the rates of hydrolysis of sphingomyelin by sphingomyelinase solubilized from rat brain. Dissimilar to the approach of other investigators (e.g., Dennis, 1973), this work analyzed the enzymatic hydrolysis of SM at low concentrations of both detergent and substrate. At these concentrations, changes occur in the aggregation states of the substrate, because of solubilization by the detergent. This paper correlates the enzymatic data with the physico-chemical properties of mixtures of Triton X-100 and sphingomyelin, investigated in former papers (Yedgar et al., 1974, 1975).

Figures 2 and 3 show extreme deviations from the hyperbolic shape expected from the Michaelis-Menten model of E-S interaction. The curves have three separate regions: (a) a region of low activity; (b) an ascending portion; (c) a part of zero-order kinetics, where the v vs. S curve parallels the abscissa, i.e., adding more substrate does not further increase the reaction rate in this region. The region of low activity is characterized by liposomal, rather than micellar aggregates of the substrate. It has been shown that liposomes are a poorer substrate than the mixed micelles (Gatt et al., 1973). When the TR concentration is increased to more than 0.2 mM, mixed micelles are formed (Yedgar et al., 1975), resulting in curves which ascend in a seemingly hyperbolic manner (cf. Figures 2 and 3). The rate of hydrolysis in the ascending region depended on the TR to SM ratios. It increased with increasing ratio, irrespective of the parameter to which the rate was related, namely the concentrations of Sm (Figure 2), TR, or the mixed micelles (Figure 3). Thus, (in Figure 3) at a TR to SM ratio of 3, the rate of hydrolysis was about double that obtained at a TR to SM ratio of 0.5. A lower Triton to SM ratio, at a fixed concentration of the mixed micelles, represents a greater number of sphingomyelin molecules in each micelle. The fact that, in spite of this greater abundance of SM in the micelle, lesser reaction rates are observed suggests that the enzyme-substrate interaction is effected by the surface density of the substrate (i.e., the number of SM molecules per unit surface area of the micelle). The substrate probably becomes more prone to interaction with the enzyme as its surface density approaches an optimal value. This optimum is less than that of the surface density of the SM-rich mixed micelles and much smaller than that of SM in a bilayered liposome. For this reason, SM liposomes without detergents result in low enzymic reaction rates. As the SM is solubilized by the micelle of Triton X-100, its surface density decreases and the rate of enzymatic hydrolysis increases. This behavior resembles that of phospholipids at an air-water interface, interacted with phospholipases. Thus, the maximal rate of hydrolysis of a monolayer of lecithin by pancreatic phospholipase A occurs at an optimal, rather than maximal surface density (Zografi et al., 1971). In the present case, the enzyme which cleaves off the hydrophilic head group from the hydrophobic part ought to reach the corresponding site on the SM molecule. It is therefore likely that the Triton exhibits its effect because of the open and highly hydrated nature of its micelle, which contains about 40 molecules of water per 1 molecule of TR (Kushner and Hubbard, 1954; Yedgar et al., 1974). In liposomes or in those mixed micelles of low TR to SM ratio, the SM molecules are packed in a condensed and rigid structure which permits but poor accessibility of the enzyme to the SM molecule. At increasing TR to SM ratios, mixed micelles are obtained which are "open" and hydrated and, therefore, more accessible to the enzyme (see Model and Discussion of Yedgar et al., 1974).

The v vs. S curves at several fixed concentrations of detergent (Figure 1) also deviated from the hyperbolic shapes, but differed markedly from those of Figures 2 and 3. The curves were biphasic, having an ascending, seemingly hyperbolic region, a peak, and a descending part. The irregularity of these curves can be interpreted on the basis of two previously described observations: (a) the maximal ratio of TR to SM in the mixed micelles is 4; at greater ratios, the excess detergent separates as pure TR micelles; (b) as the ratio of TR to SM decreases, so does the utilization of the substrate by the enzyme. Correspondingly, the curves of Figure 1 have two regions. At the ascending region there is an excess of micelles of pure TR in the presence of mixed micelles which have a TR to SM ratio of 4. Addition of SM will increase the latter species at the expense of pure TR micelles. This region is therefore a hyperbolic curve of rate vs. increasing concentrations of the mixed micelles. Calculation of the data of Figure 1 shows that the peak of each curve is reached when the ratio between TR and SM is 4. At this point pure TR micelles are no longer available, having been fully converted to mixed TR-SM micelles. Additional SM will incorporate into the already existent mixed micelles, thereby increasing their aggregation number but not their concentration. As has been discussed, these SM-rich micelles are a poorer substrate. Adding SM therefore lowers the reaction rates as evident in the descending region of the curves of Figure 1.

Biphasic shapes of v vs. S curves, similar to those of Figure 1, are usually interpreted as due to inhibition by excess substrate. It should therefore be emphasized that in the present case these biphasic shapes are related to changes in the availability of the substrate, caused by variations in its distribution in the mixed micellar system.

The enzyme used in this study was purified about 100-fold. Attempts to further purify it were not successful to date because of decreased stability of purer preparation. Several preparations of enzymes which differed in the degree of purity and specific activity were used in different stages of this work, but the degree of purity had no significant effect on the shapes of the kinetic curves. This study was therefore confined to the correlation between the enzymatic activity and changes in the physical state of the substrate induced by the detergent. Study of possible effects of the detergent on the enzymatic protein will have to be postponed until large quantities of pure enzyme become available.

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Carboxypeptidase Inhibitor from Potatoes. Interaction with Derivatives of Carboxypeptidase A†

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ABSTRACT: The mechanism of action of a carboxypeptidase inhibitor from potatoes has been probed by studying its interaction with derivatives of carboxypeptidase A containing modified residues at the active site. Arsanilazocarboxypeptidase A, a derivative containing a chromophore attached to tyrosine 248, exhibits a circular dichroism spectrum which is sensitive to the presence of ligands at the active site (Kagan, H. M., and Vallee, B. L. (1969), *Biochemistry 8,* 4223). Since the spectral change attending binding of the carboxypeptidase inhibitor to arsanilazocarboxypeptidase A is similar to that produced by small substrates and inhibitors, the enzyme-inhibitor interaction also involves the enzyme active site. Catalytic activity is not required for inhibitor binding. Complexes of the inhibitor with apocarboxypeptidase A and carboxypeptidase A which was inactivated by treatment with the affinity label, *N*-bromoacetyl-*N*-methyl-L-phenylalanine, are demonstrated by gel filtration experiments. Moreover, competitive binding studies reveal that the latter derivative, in which the binding pocket is presumably blocked by reagent, binds inhibitor nearly as strongly as does the native enzyme, the differences in free energy of association being only 0.4 kcal/mol of a total binding energy of −11 kcal/mol. A model is proposed to account for both the tight binding of inhibitor to the *N*-bromoacetyl-*N*-methyl-L-phenylalanine derivative and the involvement of the active site of arsanilazocarboxypeptidase A. It is suggested that the inhibitor fits into a shallow depression at the active site of the enzyme but does not penetrate into the binding pocket.

In contrast to the widespread distribution of inhibitors of the "serine" proteases, inhibitors of carboxypeptidases have only been found in potatoes (Ryan, 1971) and *Ascaris lumbricoides* (Homandberg and Peanasky, 1974). The inhibitor from potatoes is a mixture of polypeptides of 38 and 39 amino acid residues. It exhibits specificity toward both carboxypeptidases A and B with inhibition constants, K_I, of 4.2×10^{-9} and 1×10^{-8} M, respectively. An earlier report (Hass et al., 1976) describes the effects of chemical modifications of the inhibitor on binding to carboxypeptidases A and B, providing evidence that these enzymes utilize the same inhibitor binding site which has been found to be the carboxyl-terminal region.

This report describes the binding of carboxypeptidase inhibitor to derivatives of carboxypeptidase A containing modified active site structures and specifically addresses the following questions: (1) Does the binding of carboxypeptidase inhibitor occur at or near the enzyme active site? (2) Is catalytic activity required for binding the carboxypeptidase inhibitor? and (3) Does interaction of carboxypeptidase inhibitor in the enzyme binding pocket contribute to the stability of the complex? These studies clarify the mechanism of action of carboxypeptidase inhibitor and provide a basis of comparison with well-documented "serine" protease-proteinase inhibitor systems.

Experimental Section

Materials. The carboxypeptidase inhibitor was prepared according to Ryan et al. (1974) with the minor modification of substituting Sephadex G-50F (equilibrated with water) and SE-cellulose (Gallard Schlesinger) for Sephadex G-75 and phosphocellulose, respectively.

A radioactive derivative was prepared by treating inhibitor (4 mg) with 10.2 mg of [^{14}C]acetic anhydride (0.1 mCi; New England Nuclear) for 30 min in an ice-salt mixture (Riordan

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