Enzymic Hydrolysis of Sphingomyelin in the Presence of Bile Salts

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(Received 3 July 1979)

Sphingomyelin in mixed dispersion with bile salts was hydrolysed by the solubilized sphingomyelinase of rat brain lysosomes. In parallel studies, physical properties of these dispersions were determined. The kinetic curves that described the rate of hydrolysis as a function of increasing concentrations of bile salt were multiphasic. A region of very low activity was followed by an ascending portion, a peak, a descending portion, a trough and a second ascending portion. The positions of the initiation points, peaks and troughs were found to be a function of the respective ratios of the bile salt to sphingomyelin for the detergent sodium taurodeoxycholate, but of the absolute concentration of the detergent for sodium taurocholate. Turbidity studies suggested that hydrolysis of sphingomyelin begins at a bile salt concentration that solubilizes the lipid and incorporates it into a mixed micelle with the detergent. Ultracentrifugation studies suggested that the sizes of the mixed aggregates of detergent and lipid were a function of the ratio of taurodeoxycholate to sphingomyelin, but of the absolute concentration of the bile salt, for sodium taurocholate.

Bile salts have been used extensively to disperse lipids for the purpose of interacting them with enzymes (for a summary of procedures see Glew & Peters, 1977). Numerous studies were done on the physicochemical properties of these detergents and models were proposed for the structure and shape of mixed dispersions of bile salts and phospholipids (Small, 1971). Nevertheless, their use in enzymology of the complex lipids still lacks a sound theoretical basis and procedures are set up on a trial-and-error basis. Frequently, the degree of purity or type of commercial preparation determines the rate of the enzymic reaction, or even if it proceeds at all (Wenger *et al.*, 1975; Tanaka & Suzuki, 1975, 1977; Suzuki, 1977).

Hydrolysis of sphingomyelin by the lysosomal sphingomyelinase of rat brain is facilitated by dispersing this lipid either in a non-ionic detergent, such as Triton X-100, or a bile salt (Barenholz *et al.*, 1966). In previous papers we investigated the hydrolysis of sphingomyelin as affected by Triton X-100. A model was proposed for the structure of mixed micelles of this detergent and sphingomyelin (Yedgar *et al.*, 1974a,b) and the complex kinetics of enzymic hydrolysis was clarified by correlating it to the physical properties of the mixed dispersions (Yedgar & Gatt, 1977). In the present paper the same enzymic activity was studied in the presence of the trihydroxy bile salt taurocholate or the dihydroxy

salt taurodeoxycholate. The kinetics of enzymic hydrolysis were related to the physical properties of the mixed dispersions of sphingomyelin and the respective bile salt.

Experimental Procedure

Materials

The preparation of bovine brain sphingomyelin and the ^3H -labelled lipid were previously described (Barenholz *et al.*, 1966; Gatt *et al.*, 1973). Sodium taurochenodeoxycholate was purchased from Calbiochem. Synthetic sodium taurocholate and taurodeoxycholate were gifts from Dr. L. Karbisch and Dr. B. Borgstroem. The solubilized sphingomyelinase of the rat brain lysosomes was previously described (Gatt & Gottesdiner, 1976).

Methods

Bile acid in methanol solution and sphingomyelin in chloroform/methanol (2:1, v/v) were mixed to the desired concentrations and evaporated to dryness under N_2 . Dimethylglutarate buffer (0.195 ml of 0.1 M at pH 6.0) was added and the mixture was preincubated for 15 min in a shaking bath at 37°C. The enzyme preparation was diluted with the same buffer so that 5 μl contained the desired quantity (from 10 to 100 μg) and added to the reaction mixture. After 1 h the reaction was terminated and

the initial velocity of sphingomyelin hydrolysis was measured by assaying the [^3H]ceramide product as previously described (Barenholz *et al.*, 1966; Gatt & Barenholz, 1969). The turbidity of aqueous dispersions of sphingomyelin and bile salt, mixed as described above, was determined at constant temperature and with continuous stirring by measuring the absorbance at 360 nm with a Zeiss spectrophotometer. Time-dependence of absorbance was observed through 30 min. Sedimentation-velocity studies were carried out at $20 \pm 0.05^\circ\text{C}$ in a Beckman Spinco model E analytical ultracentrifuge, equipped with a schlieren optical system fitted with a phase plate and an RTIC temperature-control unit. The An-D rotor, operated at 42040 rev./min was employed; a double sector capillary-type synthetic boundary cell with a 12 mm optical path was used.

Results

Figs. 1–3 show the dependence of the rate of hydrolysis of sphingomyelin by the solubilized sphingomyelinase of rat brain lysosomes on increasing concentrations of di- or tri-hydroxy bile salts. Fig. 1 shows the effect of sodium taurocholate (a trihydroxy bile salt) and of two dihydroxy bile salts, sodium taurodeoxycholate and taurochenodeoxycholate. The curves, which describe the enzymic hydrolysis of sphingomyelin as a function of increasing concentrations of the bile salts, show four

distinct regions. A region of very low enzymic activity was followed by increasing rates of hydrolysis up to a peak. With more bile salt the reaction rates decreased, reaching a trough, and then increased again. The two respective dihydroxy bile salts yielded practically superimposable curves,

Fig. 2. Hydrolysis of [^3H]sphingomyelin as a function of increasing concentrations of sodium taurodeoxycholate. Conditions were similar to those of Fig. 1 except that increasing concentrations of sodium taurodeoxycholate and several fixed concentrations of [^3H]sphingomyelin were used. These concentrations, in mM, are shown on the respective curves. The letters on the curves are explained in the text.

Fig. 1. Hydrolysis of [^3H]sphingomyelin as a function of increasing concentrations of three bile salts

Incubation mixtures in volumes of 0.2 ml contained $20\mu\text{mol}$ of dimethyl glutarate buffer, pH 6.0, $0.2\mu\text{mol}$ of [^3H]sphingomyelin, $10\mu\text{g}$ of rat brain sphingomyelinase and increasing concentrations of sodium taurocholate ($\square$), taurodeoxycholate ($\bullet$) or taurochenodeoxycholate ($\circ$). After 1 h at 37°C [^3H]ceramide was extracted and counted for radioactivity.

Fig. 3. Hydrolysis of [^3H]sphingomyelin as a function of increasing concentrations of sodium taurocholate and several fixed concentrations of [^3H]sphingomyelin shown by the adjacent numbers (expressed in mM). Conditions were similar to those of Fig. 2.

whereas the trihydroxy salt (taurocholate) resulted in a curve of very similar pattern, but where the transitions between the above regions (the 'turning points') occurred at greater concentrations of the detergent.

In the experiments described in Figs. 2 and 3 several fixed concentrations of sphingomyelin and various concentrations of a dihydroxy (Fig. 2) and the trihydroxy bile salt (Fig. 3) were used. Fig. 2 shows kinetic curves describing the effect of increasing concentrations of sodium taurodeoxycholate on the hydrolysis of sphingomyelin at four fixed concentrations of this substrate (0.5, 1, 2 and 6 mM respectively). Each of these curves had the same pattern as those shown in Fig. 1, but the turning points between the various regions of the respective curves were not fixed values but varied in relation to the molar ratio of taurodeoxycholate to sphingomyelin. Thus, the initiation of sphingomyelin hydrolysis (point a, Fig. 2) occurred when the molar ratio of bile salt to sphingomyelin equalled about 0.3. The peaks (point b) and troughs (point c) were obtained when these ratios were about 0.8–1 and 2–2.5 respectively; and the second ascending region of the curves (beyond the trough) plateaued out at a bile salt to sphingomyelin ratio of about 4 (point b). The plateau regions, although obtained at all curves, are shown here for two concentrations.

Fig. 3 presents results of reactions done in the presence of increasing concentrations of sodium taurocholate and three fixed concentrations of sphingomyelin. Dissimilar to the data of the previous Figure, the turning points of kinetic curves occurred at fixed concentrations of the trihydroxy bile salt, rather than fixed ratios of detergent to lipid. Thus at all concentrations of sphingomyelin the initiation values of enzymic activity, the peaks and troughs occurred at about 0.5, and 2 and 4 mM respectively. It seems, however, that with higher sphingomyelin concentration the trough range is wider, and it is difficult to conclude from this Figure whether the plateau regions occurred at the same sodium taurocholate concentration.

The complex kinetics shown in Figs. 1–3 might be a consequence of an effect of the detergent on dispersion of the substrate, the physical state of the enzyme or both of these components. The possibility that the bile salt affects the enzyme was tested in the experiment shown in Fig. 4. The dependence of the enzymic activity on the concentration of taurodeoxycholate was studied at a fixed concentration of sphingomyelin and several concentrations of the enzyme. Except for the expected increase of total activity with increasing concentrations of the enzyme, the shapes of the curves were practically unaffected by varying the concentration of the enzyme. The initiation and peak activities occurred at the same respective detergent

Fig. 4. Hydrolysis of [^3H]sphingomyelin as a function of increasing concentrations of sodium taurodeoxycholate. Conditions were similar to those of Fig. 2 except that three concentrations of enzyme (2, 10 and 20 μg) and 0.1 μmol of [^3H]sphingomyelin were used.

concentrations even over a 10-fold range of enzyme concentrations, suggesting that the bile salts affect primarily the dispersion state of substrate, rather than the enzyme itself. The data of Fig. 4 do not permit a clear-cut conclusion as to whether the trough values were shifted to higher concentrations of detergent when enzyme concentrations were increased.

Parallel to the enzymic studies, physical properties of mixed dispersions of bile salts and sphingomyelin were investigated. Solubilization of sphingomyelin by a bile salt was followed by measuring changes in absorbances of dispersions of this lipid in the presence of increasing concentrations of the detergent. Fig. 5 shows that the turbid dispersions of sphingomyelin cleared on addition of the bile salt over a very narrow range of this detergent. Each of these dispersions cleared when the molar ratio of taurodeoxycholate to sphingomyelin reached a value of about 0.3, which equals that ratio at which enzymic hydrolysis of sphingomyelin begins (Fig. 2). The findings of Figs. 5 and 2 suggest that as long as the ratio of taurodeoxycholate to sphingomyelin is less than 0.3, the lipid molecules are aggregated as large multibilayers; in this state, the substrate molecules are not available for interaction with the enzyme. Above this ratio, the lipid is solubilized, forms mixed micelles with the detergent and is hydrolysed by the enzyme.

Analytical ultracentrifugation was used to study the size and composition of the mixed micelles of bile salt and sphingomyelin. This lipid was dispersed with taurocholate or taurodeoxycholate in dimethylglutarate buffer, pH 6.0. A solution of this buffer, or alternatively a solution of bile salt in buffer, was

Fig. 5. Turbidity of mixed dispersions of sphingomyelin and sodium taurodeoxycholate as a function of increasing concentrations of the bile salt, at four constant concentrations of sphingomyelin (1, 2, 5.2 and 10.4 mM)

Mixed dispersions of [^3H]sphingomyelin and sodium taurodeoxycholate were prepared and their absorbances were measured at 360 nm as described in the text.

introduced in the reference cell. Various ratios of bile salt to lipid were used in these experiments. Some of these are presented in Figs. 6 and 7.

Fig. 6 shows schlieren patterns of a dispersion of 4 mM-sodium taurocholate/4 mM-sphingomyelin, with buffer only (Fig. 6a) or buffer plus 4 mM bile salt (Fig. 6b) as reference. Fig. 6(a) shows a non-symmetrical peak preceded by a 'shoulder'. Inclusion of the detergent in the reference cells (Fig. 6b) eliminated the 'shoulder' and resulted in symmetrical peaks. Comparing several detergent-to-lipid ratios showed that the 'shoulder' was more pronounced in those dispersions where the concentration of the detergent exceeded the lipid. The data of Fig. 6 suggest that mixtures of sphingomyelin and taurocholate contain a population of mixed micelles (presented by the main schlieren peak), whereas the 'shoulder' represents a population of micelles consisting of pure detergent or mixed micelles highly enriched with detergent. Attempts to characterize micelles of pure taurocholate by centrifuging its solutions in buffer (with the buffer as reference) were unsuccessful. Although a very shallow and broad peak was seen, accurate measurement of sedimentation coefficients was not possible. This suggested that the solution of taurocholate may contain a heterogeneous population of micellar aggregates of various sizes (see also Small, 1971).

Fig. 6. Schlieren patterns of the mixed dispersions of sphingomyelin and sodium taurocholate

For explanation see the text. A mixed dispersion consisting of 4 mM-sphingomyelin and 4 mM-taurocholate was centrifuged at 42040 rev./min using a reference of dimethylglutarate buffer (a) or 4 mM bile salt in this buffer (b). Photographs were taken at 8 min intervals.

Fig. 7. Schlieren patterns of two mixed dispersions of sphingomyelin and bile salt

Mixed dispersions consisted of 2 mM-sphingomyelin and (a) 2 mM-taurocholate, (b) 2 mM-taurodeoxycholate or (c) 8 mM-taurodeoxycholate; dimethylglutarate buffer was used as reference.

Fig. 7 shows schlieren patterns at two molar ratios (2/2 and 8/2) of sodium taurodeoxycholate and sphingomyelin, with buffer as reference. For comparison, a schlieren pattern of a mixed dispersion of taurocholate and sphingomyelin at a molar ratio of 2/2 is also included (Fig. 7a). The peaks of the taurodeoxycholate/sphingomyelin mixtures were symmetrical throughout. This was so even in the experiment shown in Fig. 7(c), where the detergent exceeded the lipid by 4-fold. This contrasts with the pattern obtained for taurocholate, which again shows an asymmetrical peak and a 'shoulder' (Fig. 7a). The symmetrical shapes of the patterns of Figs. 7(b) and 7(c) suggest the presence of homogeneous mixed dispersions of taurodeoxycholate and sphingomyelin. Micelles of free bile salt probably exist in the system, but do not form a population that could be detected in the schlieren pattern.

Fig. 8. Sedimentation coefficients of mixed dispersions of sphingomyelin and bile salts as a function of the concentration of taurocholate (a) or the molar ratio of taurodeoxycholate to sphingomyelin (b)

The numbers adjacent to the symbols indicate the concentrations (mM) of the bile salt (numerator) to sphingomyelin (denominator). A different symbol is assigned for each molar ratio (R) of the bile salt to the phospholipid. In Fig. 8(a): O, R = 3; □, R = 6; △, R = 12. In Fig. 8(b): □, R = 1; O, R = 2; △, R = 4.

Discussion

The multiphasic curves shown in Figs. 1–3 suggest a rather complex effect of the bile salts on the parameters that affect the hydrolysis of sphingomyelin by the solubilized sphingomyelinase of rat brain lysosomes. The data of Figs. 2 and 5 show that hydrolysis occurs only when the lipid is solubilized and forms mixed micelles with bile salt. When more bile salt is added, the number of micelles increases, their size decreases (see Figs. 7 and 8) and they become enriched in detergent. The biphasic portions of the kinetic curves, before and beyond the respective peaks, suggest two opposing effects. With increasing bile salt, interaction between enzyme and sphingomyelin molecules is facilitated because of the greater number of mixed micelles. This is opposed by enrichment of the micelles with bile salt, which may interfere with the interaction of the enzyme with the micellar surface due to steric hindrance, surface dilution of the substrate or electrostatic repulsion (caused by the negative charge of the sulphate group). The balance between these opposing factors results in the first ascending and the descending part of the observed kinetic curves. The decrease in micellar size is not a linear function of detergent concentration, and micelles already rich in bile salts are less affected by further addition of the detergent. In the region of the first ascending portion of Figs. 1–3 the micellar size decreases rapidly and this factor predominates. Beyond the peak, the increased concentration of bile salt molecules in the micellar surface becomes the predominant factor, resulting in lower reaction rates. The variations in the reaction rates, from zero bile salt to the trough values, might thus be ascribed to the effect of the bile salts on the physical state of lipid dispersion. Beyond the trough where a second ascending region was observed, a critical change in the nature of the aggregation was not observed. With increasing bile salt concentrations the micellar size further decreased slowly and gradually. The 'shoulder' that appears in the schlieren pattern of taurocholate/sphingomyelin mixtures (Figs. 6a and 6b) suggests the presence of micelles containing only bile salts or that are highly enriched in molecules of these detergents. The gradual increase of the 'shoulder', observed at higher ratios of detergent to lipid, suggests that the concentration of micelles of free detergent depends on the total concentration of taurocholate. Although separate bile salt micelles could not be detected for taurodeoxycholate, they are also most probably present in the mixtures. Since the increase in the reaction rates beyond the trough (point c–d) is not explained by the changes in the size of the mixed micelles, we assume that this may be a consequence of an effect of the detergent on the enzyme rather than the substrate dispersion. Thus,

The relation of the size of the sphingomyelin/bile salt mixed micelle to its components was studied by plotting the calculated values of the sedimentation coefficients against the following three parameters: (a) concentration of the phospholipid (sphingomyelin); (b) concentration of the detergent (sodium taurocholate or sodium taurodeoxycholate); (c) the molar ratio of the detergent to the phospholipid. When the sedimentation coefficient of the sodium taurocholate/sphingomyelin mixed aggregates were plotted against each of these three parameters, the only correlation that was observed was that with the absolute concentration of the bile salt, as shown in Fig. 8(a) (one can easily derive from this Figure a plot of [S] against [sphingomyelin] or against the molar ratio of the detergent to the phospholipid). It appears that above a certain concentration of the taurocholate [which is in the range of its critical micellar concentration (Small, 1971)] the sedimentation coefficient of the sodium taurocholate/sphingomyelin mixed micelle is determined predominantly by the absolute concentration of the trihydroxy bile salt. Thus, at 12 mM-sodium taurocholate even a 4-fold change in the sphingomyelin concentration (and a 3-fold change of the molar ratio, from 12/1 to 12/4) did not significantly change the sedimentation coefficient. On the other hand, when sphingomyelin was dispersed with the dihydroxy bile salt (sodium taurodeoxycholate) the sedimentation coefficients were best correlated with the molar ratio of taurodeoxycholate to sphingomyelin, as shown in Fig. 8(b).

the excess bile salt may transform the enzyme to a form that is not inhibited by the detergent. This may be related to a positive co-operative binding of detergent to proteins (Makino *et al.*, 1973). Alternatively, the effect may be related to the fact that the enzyme preparation is a multimolecular aggregate whose mol. wt. is about 300000 (Gatt & Gottesdiner, 1976). The excess of free detergent may decompose this aggregate to smaller units that are not inhibited by excess detergent (Helenius & Simmons, 1975; Lisman *et al.*, 1971).

When comparing the effects of the di- and tri-hydroxy bile salts, a marked difference was observed. With dihydroxy bile salt the inversions in the enzymic activity were related to the molar ratio of detergent to lipid (Fig. 2), whereas with the trihydroxy bile salt the absolute concentration of the detergent was the dominant factor. This correlates well with the ultracentrifugation data (Figs. 7 and 8), which show that the sizes of the mixed micelles, as indicated by the sedimentation coefficients, are determined predominantly by the molar ratio of bile salt to sphingomyelin for taurodeoxycholate, but by the absolute concentration of the bile salt when taurocholate was used in mixed dispersion with sphingomyelin.

We thank Dr. L. Krabisch and Dr. B. Borgstroem for a gift of synthetic taurocholate and taurodeoxycholate. The technical assistance of R. Hertz and B. Morag is acknowledged. We thank Dr. C. Huang for stimulating discussions and advice in the ultracentrifugation studies. This work was supported in part by N.I.H. grant NSO2967.

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