Effect of bile salts on the hydrolysis of gangliosides, glycoproteins and neuraminyl-lactose by the neuraminidase of *Clostridium perfringens*

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Studies were done on the effect of bile salts on the rates of hydrolysis of the *N*-acetylneuraminyl linkages of several sialic acid-containing compounds by the neuraminidase of *Clostridium perfringens*. When G_{M3} -ganglioside, two glycolipids (glycophorin and orosomucoid) and neuraminyl-lactose were used as substrates, hydrolysis was obtained even in the absence of bile salts, but addition of this detergent, below its critical micellar concentration, increased the reaction rates; above the critical micellar concentration of the detergent rates decreased again. When a second ganglioside, G_{M1} , was used as substrate, the requirement for bile salts was absolute; hydrolysis was not observed at all without this detergent. With increasing concentrations of bile salt and in the presence of high concentrations of enzyme, rates of hydrolysis increased, reaching maximal values at fixed ratios of bile salt to G_{M1} -ganglioside. Physical measurements showed that mixtures of bile salt and G_{M1} -ganglioside form mixed micelles that have a higher critical micellar concentration, a lower molecular weight and greater axial ratio than the corresponding micelles of pure G_{M1} -ganglioside.

Bacterial neuraminidases release the sialic acid residues of glycolipids, glycoproteins and low-molecular-weight compounds such as neuraminyl lactose. Hydrolysis of mixed brain gangliosides by these enzymes yield the monosialogangliosides, G_{M1} [galactosyl-*N*-acetyl-galactosaminyl-galactosyl(*N*-acetylneuraminyl)glucosylceramide] and G_{M2} [*N*-acetyl-galactosylaminyl-galactosyl(*N*-acetylneuraminyl)glucosylceramide], whose sialyl linkages resist hydrolysis (Burton, 1963). However, even these linkages can be split by the enzyme of *Clostridium perfringens* (but not *Vibrio cholerae*) if bile salts are included in the incubation mixture (Wenger & Wardel, 1972, 1973). This work aimed to define if this effect results from the action of the detergent on the substrate, enzyme or both. For this purpose we compared the effect of bile salts on the hydrolysis by the clostridial neuraminidase of the sialyl linkages of two gangliosides [G_{M3} (*N*-acetylneuraminylgalactosylglucosylceramide) and G_{M1}] two glycoproteins (glycophorin and orosomucoid) and two oligosaccharides (neuraminyl-lactose and the lipid-free carbohydrate residue of G_{M1} -ganglioside). The data suggested a dual effect of the bile salts on the dispersion state of the substrate as well as enzyme. The first effect operates mostly in the case

of G_{M1} -ganglioside, whereas enhanced hydrolysis due to enzyme activation was observed for the other substrates. The conclusions on the effect of bile salts on the hydrolysis of the sialyl residue of G_{M1} -ganglioside were further substantiated by determining several physical parameters on mixed micelles of this compound and di- or tri-hydroxy bile salts.

Materials and methods

Materials

G_{M1} -Ganglioside was a gift from Professor L. Svennerholm (University of Goteborg, Goteborg, Sweden); G_{M3} -ganglioside was a gift from Dr. G. Schwartzmann and Professor H. Wiegandt (University of Marburg, Marburg, Germany). The lipid-free carbohydrate of G_{M1} -ganglioside was a gift from Professor H. Wiegandt; glycophorin and α -acid glycoprotein (orosomucoid) were gifts from Dr. I. Kahane (Hebrew University-Hadassah Medical School, Jerusalem); synthetic and ^3H -labelled sodium taurodeoxycholate were gifts from Dr. L. Krabich and Professor B. Borgstrom (University of Lund, Lund, Sweden); sodium taurocholate was purchased from Koch-Light;

neuraminyl-lactose and *Cl. perfringens* neuraminidase (type VI) were from Sigma. The purity of G_{M1} -ganglioside and sodium taurodeoxycholate was tested by t.l.c. on silica gel G and found to be about 98–99%.

Methods

Micellar dispersions of G_{M1} -ganglioside, by themselves or in mixtures with sodium taurodeoxycholate, were prepared by evaporating methanol solutions of these compounds under N_2 and dispersing the residue in 0.1 M-sodium acetate, pH 5.6, for 15 min at 37°C.

Sedimentation velocity coefficients (s) were determined as previously described (Yedgar *et al.*, 1974), diffusion coefficients (D) and the Stokes' radii of the

micelles were determined with the aid of photon-correlation spectroscopy, all at $20 \pm 0.5^\circ\text{C}$ (Cooper *et al.*, 1974) or analytical ultracentrifugation (Huang, 1969). The apparent partial specific volumes were measured at $20 \pm 0.01^\circ\text{C}$ in a Paar density meter (Barenholz *et al.*, 1977). These parameters permitted the calculation of molecular weights. $s_{20,w}^0$ and $D_{20,w}$ values were determined by using five or six different concentrations; standard-deviation values were $\pm 4\%$ and $\pm 2.5\%$ respectively. The sampling time for each diffusion measurement, by photon-correlation spectroscopy, was 1 min. Each value of D represents an average of 12–20 experiments, the standard deviation for each D value being less than 1%. Standard-deviation values of $D_{20,w}$, by using the analytical centrifuge (Huang, 1969), were $\pm 5\%$. The agreement with the data

Fig. 1. Effect of sodium taurodeoxycholate on the hydrolysis of G_{M3} -ganglioside (a), glycoporphin (b), orosomucoid (c) and neuraminyl-lactose (d)

Standard conditions were used throughout. (a) $\circ$, 0.5 mM- G_{M3} -ganglioside; $\bullet$, 1.5 mM- G_{M3} -ganglioside; 0.2 m-unit of enzyme; (b) $\circ$, 0.24 mM-glycoporphin; $\bullet$, 1 mM-glycoporphin; 0.6 m-unit of enzyme; (c) $\circ$, 0.24 mM-orosomucoid; $\bullet$, 1 mM-orosomucoid; 0.2 m-unit of enzyme; (d) $\circ$, 0.5 mM-neuraminyl-lactose; $\bullet$, 1.5 mM-neuraminyl-lactose; 0.2 m-unit of enzyme.

determined by photon-correlation spectroscopy was better than 95%. The density measurements were performed at $20 \pm 0.005^\circ\text{C}$ and the standard deviation on the slope and the intercept of the curve describing the density as a function of concentration was better than $\pm 2.0\%$, with a correlation coefficient higher than 0.9970. The density values obtained for water agreed well with the values in the International Critical Tables.

Enzyme assays

Chloroform/methanol (2:1, v/v) solutions of gangliosides and bile salts were evaporated, 0.05 ml of 0.2 M-sodium acetate, pH 5.6, was added followed by water and enzyme to a volume of 0.1 ml. When non-ganglioside substrates were used, they were dispersed in water, buffer was added, followed by an aqueous solution of the bile salt and enzyme. The reaction was terminated and the *N*-acetylneuraminic acid released was estimated by the procedure of Warren (1959), with only 2 ml of cyclohexanone.

Results

Hydrolysis of the sialyl linkages of glycolipids, glycoproteins and oligosaccharides

Preliminary experiments confirmed the observations of Wenger & Wardel (1972, 1973) that G_{M1} -ganglioside is hydrolysed by the neuraminidase of *Cl. perfringens* in the presence of bile salts. In the absence of this detergent, about 1.5% of the *N*-acetylneuraminic acid was split, this being due to the presence of contaminating glycolipids susceptible to neuraminidase attack. Increasing concentrations of bile salt showed dissimilar effects on the rate of hydrolysis of the six compounds used as substrates. Three separate effects were recorded. 1. The lipid-free carbohydrate residue of G_{M1} -ganglioside was not hydrolysed at all. 2. The rates of hydrolysis of G_{M3} -ganglioside, glycophorin, orosomucoid and neuraminyllactose increased in the presence of molecular solutions of bile salts. Peak activities were obtained in the range of the critical micellar concentration of the detergent; a further increase in bile salt content decreased the reaction rates (Fig. 1). 3. The curves that described the rate of hydrolysis of G_{M1} -ganglioside as a function of increasing concentrations of bile salts were also biphasic, but in this case peak activities were observed at a fixed ratio of detergent to ganglioside rather than fixed concentration of the bile salt. This molar ratio was about 1.6–2.0 with sodium taurodeoxycholate (Fig. 2) and about 5–8 with sodium taurocholate (result not shown in Figure). It was of interest that G_{M1} -ganglioside was hydrolysed only when present as a mixed micelle with the bile

Fig. 2. Dependence of G_{M1} -ganglioside hydrolysis on sodium taurodeoxycholate

Standard experimental conditions were employed, using three fixed concentrations of G_{M1} -ganglioside [0.5 mM ($\square$), 1.25 mM ($\circ$) and 2.0 mM ($\triangle$)] and increasing concentrations of sodium taurodeoxycholate. Enzyme concentration was 32.5 m-units.

salt. This is shown in the insert to Fig. 3, where hydrolysis was negligible below the critical micellar concentration of G_{M1} -ganglioside (see Fig. 5 for critical micellar concentration determination of G_{M1} -ganglioside, alone or in the presence of bile salt).

Compositional characterization of sodium taurodeoxycholate/ G_{M1} -ganglioside mixtures

Though both the bile salts and G_{M1} -ganglioside are classified as 'soluble amphiphilic lipids' (Small, 1970) and are negatively charged, they differ considerably in a variety of physical properties, such as critical micellar concentration, the size of their micelles and the respective sizes of their polar head groups. Experiments were undertaken to determine the distribution of a bile salt between the aqueous medium and the mixed micelle of detergent and ganglioside. For this purpose mixtures of G_{M1} -ganglioside and ^3H -labelled sodium taurodeoxycholate were prepared and filtered through small columns of Sephadex G-25. The elution pattern of the bile salt was determined from the radioactivity content and that of G_{M1} -ganglioside from the *N*-acetylneuraminic acid content. Dispersions of pure G_{M1} -ganglioside were eluted entirely at the void volume (V_0), whereas those of pure sodium taurodeoxycholate were eluted close to V_t . Fig. 4 presents results of an experiment in which mixtures, contain-

Fig. 3. Dependence of G_{M1} -ganglioside hydrolysis on substrate concentration

Standard experimental conditions were employed, with increasing concentrations of G_{M1} -ganglioside in the presence of two fixed concentrations of sodium taurodeoxycholate (O, 0.5 mM; ●, 3.5 mM). The insert shows G_{M1} -ganglioside hydrolysis with a 20-fold expanded scale on the abscissa.

ing a fixed concentration of G_{M1} -ganglioside and various sodium taurodeoxycholate concentrations were filtered through Sephadex G-25 columns. The Figure shows that the molar fraction of sodium taurodeoxycholate incorporated into the mixed micelle is a linear function of the molar fraction of G_{M1} -ganglioside in the initial mixture. The curve of Fig. 4 is described by the following relationship: molar fraction of sodium taurodeoxycholate in the mixed micelle = $0.592 - (0.585 \times \text{the molar fraction of } G_{M1}\text{-ganglioside in the initial mixture})$. The intercept is obtained when G_{M1} -ganglioside extrapolates to zero, namely when the mixed micelles are saturated with sodium taurodeoxycholate. The slope of the curve is 0.585 and the correlation coefficient is 0.9770.

The above relationship permitted calculation of the molar fraction of sodium taurodeoxycholate in the mixed micelle at any initial ratio of these two components (see also Table 1). The concentration of G_{M1} -ganglioside and the critical micellar concentration of this compound in the presence of sodium taurodeoxycholate permits calculation of the partitioning of sodium taurodeoxycholate between the mixed micelles and the aqueous phase. The experiment shown in Fig. 4, as well as other similar

Fig. 4. Dependence of sodium taurodeoxycholate incorporated into mixed micelles of bile salt and G_{M1} -ganglioside on initial concentration of the bile salt

For experimental conditions see the text. In the entire experiment the concentration of G_{M1} -ganglioside was 0.25 mM; the initial volume, applied to the Sephadex column (5 cm $\times$ 1 cm), was 0.5 ml. The void volume was 2–3 ml. The ganglioside was eluted in a volume of 1.5–2.0 ml; these fractions were also counted for radioactivity in a scintillation spectrometer.

experiments, emphasized that only a part of the sodium taurodeoxycholate is associated with the ganglioside in the mixed micelle. The remainder of the sodium taurodeoxycholate resides in the aqueous phase, probably as monomers or small micelles of pure sodium taurodeoxycholate, which exchange rapidly to equilibrium with the detergent incorporated in the mixed micelle. This rapid exchange was evident when mixed micelles containing non-radioactive sodium taurodeoxycholate and buffer containing ^3H -labelled sodium taurodeoxycholate or, alternatively, mixed micelles containing ^3H -labelled sodium taurodeoxycholate and buffer containing non-radioactive sodium taurodeoxycholate were filtered through Sephadex columns.

Determination of critical micellar concentration of G_{M1} -ganglioside and sodium taurodeoxycholate

The critical micellar concentration of G_{M1} -ganglioside was determined by measuring the fluorescence intensity of the hydrophobic fluorophore perylene as a function of the concentration of this ganglioside. This provides a procedure of high sensitivity that uses very small quantities of the external fluorophore: 0.5–5 nmol of perylene was used and had no effect on the critical micellar concentration value. By using a previous procedure (Yedgar *et al.*, 1974b), the perylene was coated on to glass beads and added to an aqueous dispersion of G_{M1} -ganglioside; alternatively these two compounds were dissolved in chloroform/methanol, the solvent was evaporated and the residue was dispersed in

buffer. Practically identical critical micellar concentration values were obtained by using two, respective procedures, though the fluorescence intensities were greater in the second procedure. The critical micellar concentration was defined as that concentration of G_{M1} -ganglioside where the curve that describes the fluorescence as a function of G_{M1} -ganglioside concentration begins sloping upwards (Fig. 5).

The following values were recorded for the critical micellar concentrations, using 0.1 M-sodium acetate, pH 5.6 (Fig. 5): G_{M1} -ganglioside, 1.5–2.5 μM (curve A); sodium taurodeoxycholate, 1.4–1.8 mM (curve D). When the critical micellar concentration of G_{M1} -ganglioside was measured in the presence of bile salt, higher values were obtained. Thus, in the presence of 0.5 mM and 8 mM-sodium taurodeoxycholate, the respective critical micellar concentration values of G_{M1} -ganglioside were about 20 μM and 0.1 mM (Fig. 5, curves B and C respectively). The critical micellar concentration value obtained in the absence of bile salt is somewhat lower than that reported by Yohe *et al.* (1976) and Rauvala (1979), who used less sensitive procedures, but considerably greater than those reported by Formisano *et al.* (1979) and Mraz *et al.* (1980).

Structural parameters of mixed dispersions of sodium taurodeoxycholate and G_{M1} -ganglioside

Table 1 shows several physical parameters of pure G_{M1} -ganglioside and of two mixtures of sodium taurodeoxycholate and G_{M1} -ganglioside (3:1 and

Fig. 5. Estimation of critical micellar concentration of G_{M1} -ganglioside

For explanation see the text. A, G_{M1} -ganglioside; B, G_{M1} +0.5 mM-sodium taurodeoxycholate; C, G_{M1} -ganglioside + 8 mM-sodium taurodeoxycholate; D, sodium taurodeoxycholate.

show that inclusion of bile salts decreases the micellar size and induces a greater asymmetry. In micelles of pure G_{M1} -ganglioside, intermolecular attraction between neighbouring ganglioside molecules (D. Lichtenberg, Y. Barenholz and S. Gatt, unpublished work) results in a packing that is not amenable to interaction with the neuraminidase. Cestaro *et al.* (1980) reached a similar conclusion on the hydrolysis of di- and tri-sialogangliosides in mixed liposomes with phosphatidylcholine by bacterial neuraminidases. In the latter case, the phospholipids and with G_{M1} , the bile salts act as 'spacers', separating the ganglioside molecules and thereby decreasing interaction between them. Taurodeoxycholate by itself does not form a liquid crystalline phase and its micelles are small (Small, 1971). The ganglioside forms large micelles and, though classified as a 'soluble' lipid, exhibits properties that are close to the 'insoluble, swelling amphiphilic' lipids (Small, 1970). Mixtures of the two show similarities to those of mixed dispersions of sodium taurodeoxycholate and phosphatidylcholine, e.g. saturation is reached at 60 mol % of sodium taurodeoxycholate (Fig. 4), a value very similar to that observed by Borgstrom (1978) for sodium taurodeoxycholate/phosphatidylcholine mixtures.

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