Interaction of Activating Protein and Surfactants with Human Liver Hexosaminidase A and G_{M2} Ganglioside

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The effects of surfactants on the human liver hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside were assessed. Some non-ionic surfactants, including Triton X-100 and Cutscum, and some anionic surfactants, including sodium taurocholate, sodium dodecyl sulphate, phosphatidylinositol and *N*-dodecylsarcosinate, were able to replace the hexosaminidase A-activator protein [Hechtman (1977) *Can. J. Biochem.* **55**, 315–324; Hechtman & Leblanc (1977) *Biochem. J.* **167**, 693–701] and also stimulated the enzymic hydrolysis of substrate in the presence of saturating concentrations of activator. Other non-ionic surfactants, such as Tween 80, Brij 35 and Nonidet P40, and anionic surfactants, such as phosphatidylethanolamine, did not enhance enzymic hydrolysis of G_{M2} ganglioside and inhibited hydrolysis in the presence of activator. The concentration of surfactants at which micelles form was determined by measurements of the minimum surface-tension values of reaction mixtures containing a series of concentrations of surfactant. In the case of Triton X-100, Cutscum, sodium taurocholate, *N*-dodecylsarcosinate and other surfactants the concentration range at which stimulation of enzymic activity occurs correlates well with the critical micellar concentration. None of the surfactants tested affected the rate of hexosaminidase A-catalysed hydrolysis of 4-methylumbelliferyl *N*-acetyl- β -D-glucopyranoside. Both activator and surfactants that stimulate hydrolysis of G_{M2} ganglioside decrease the K_m for G_{M2} ganglioside. Inhibitory surfactants are competitive with the activator protein. Evidence for a direct interaction between surfactants and G_{M2} ganglioside was obtained by comparing gel-filtration profiles of ³H-labelled G_{M2} ganglioside in the presence and absence of surfactants. The results are discussed in terms of a model wherein a mixed micelle of surfactant or activator and G_{M2} ganglioside is the preferred substrate for enzymic hydrolysis.

The enzymic hydrolysis of sphingolipid substrates has been shown to require either bile detergents (Norden & O'Brien, 1973; Gatt, 1963; Brady *et al.*, 1965) or protein activators (Ho & O'Brien, 1971; Hechtman, 1977; Li *et al.*, 1974; Fischer & Jatzkewitz, 1975). Protein activators for hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside have been isolated from human liver (Li *et al.*, 1974; Hechtman & LeBlanc, 1977) and shown to be replaceable by sodium taurocholate. Human fibroblast hexosaminidase A (EC 3.2.1.52)-catalysed hydrolysis of G_{M2} ganglioside appears to be uniquely stimulated by sodium taurocholate and is unresponsive both to activator protein and to a large

number of commercially available surfactants (O'Brien *et al.*, 1977).

The mechanism of activation of sphingolipid hydrolases by surfactants, however, is not well understood. Some of these enzymes, such as human liver β -glucocerebrosidase (Peters *et al.*, 1976) and rat liver sphingomyelinase (Kanfer *et al.*, 1966) are extractable from membranous subcellular fractions only in the presence of surfactants. Many sphingolipid substrates as well are soluble in aqueous solutions only in the presence of surfactants. Thus surfactant effects may result from direct interaction with either enzyme, substrate or both.

Studies on the mechanism of activator proteins have also produced evidence for direct interaction of activator with enzyme (Ho, 1975; Peters *et al.*, 1977) as well as direct interaction of activator with substrate (Fischer & Jatzkewitz, 1977).

Abbreviations used: G_{M2} ganglioside, *N*-acetylgalactosaminyl-(*N*-acetylneuraminy)galactosylglucosylceramide; 4-MU-Glc-NAC, 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside.

We have examined the interaction of a number of surfactants with the hexosaminidase A- G_{M2} ganglioside enzyme-substrate system. Of 14 surfactants tested, all affected the rate of substrate hydrolysis. Both stimulation and inhibition occurred. The interaction of surfactants with the enzymic reaction was investigated with respect to: (1) concentration-dependent effects of surfactants; (2) effects of surfactants on hexosaminidase A-catalysed hydrolysis of the hydrophilic substrate 4-MU-Glc-NAc; (3) competitive effects between different surfactants; (4) the relationship of surfactant stimulation to formation of mixed micelles; (5) effects of surfactants on the kinetic parameters of hexosaminidase A-catalysed reactions; (6) the direct interaction of surfactants with G_{M2} ganglioside substrate.

Methods and Materials

Sphingolipid substrates

G_{M2} ganglioside was obtained from the brain of a patient who had died from Sandhoff disease. The ganglioside fraction was prepared by Folch extraction and dialysis (Folch *et al.*, 1957) and purified by preparative t.l.c. by the method of Wolfe (1972). Purified substrate (50mg) was used to prepare ^3H -labelled G_{M2} ganglioside specifically labelled in the *N*-acetylgalactosaminyl moiety by the procedure of O'Brien *et al.* (1977). The specific radioactivity of the labelled substrate was 2.28×10^7 c.p.m./ μmol . Radiochemical purity was estimated to be 95–98%.

Reagents

N-Dodecylsarcosinate, Triton X-100 [α -(4-(1,1,3,3-tetramethylbutyl)phenyl)- ω -hydroxypoly(oxyethane-1,2-diyl)], sodium cholate, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, Tween 80 [sorbitan mono-octadec-9-enoate poly (oxyethane-1,2-diyl)] and Triton CF-54 were purchased from Sigma. Sodium taurocholate and sodium deoxycholate were from Calbiochem. Sodium dodecyl sulphate, Cutscum (iso-octylphenoxypolyoxyethanol) and Brij 35 [poly(oxyethylene)-dodecyl ether] were from Fisher (Montreal, Que., Canada). Hexadecyltrimethylammonium bromide was from Eastman (Rochester, NY, U.S.A.). The peroxide concentration of the surfactant preparations was found to be less than 1 part in 3333 by the phenylenediamine spot test.

4-Methylumbelliferone and 4-MU-Glc-NAc were from Koch-Light (Edmonton, Alb., Canada) and Sephadex G-200 was purchased from Pharmacia (Uppsala, Sweden).

Hexosaminidase A

Hexosaminidase activity was measured by the fluorimetric procedure of Leaback & Walker (1961). One unit of enzyme activity is that amount of enzyme that catalyses the hydrolysis of 1 nmol of 4-MU-Glc-NAc/min at 37°C in 0.1 M-sodium citrate/sodium phosphate buffer, pH 4.4.

Hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside

The procedure used to quantify the hexosaminidase A activator has been described previously (Hechtman, 1977). Details of activator purification have also been published (Hechtman & LeBlanc, 1977). The activator preparation used in these investigations had a specific activity of 2200 units/mg compared with 24 100 units/mg for the purified activator. Measurement of activator or surfactant stimulation of hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside requires use of a partially purified enzyme preparation from which endogenous activator has been removed by chromatography of the enzyme on Sephadex G-100 (Hechtman, 1977). The specific activity of the human liver hexosaminidase A preparation was 6100 units/mg (1900-fold purified compared with initial liver extract).

A standard incubation mixture for G_{M2} ganglioside hydrolysis includes: hexosaminidase A, 600 units; ^3H -labelled G_{M2} ganglioside, 2 nmol, 10000 c.p.m.; sodium citrate 10 μmol , pH 4.0; NaN_3 , 2 μmol in a volume of 200 μl . Incubations were for 16 h. The method of Tallman & Brady (1972) was used to quantify the release of *N*-acetylgalactosamine. In the absence of activator or surfactants hexosaminidase A catalysed cleavage of 40–50 pmol of G_{M2} ganglioside/16 h. The release of *N*-acetylgalactosamine is linear for up to 40 h (measurements were taken every 10 h).

Determination of critical micellar concentrations of surfactants

Critical micellar concentrations of surfactants were determined from the surface tensions of solutions under conditions nearly identical with those of the reaction mixtures used to evaluate the effect of the surfactant on enzymic hydrolysis of G_{M2} ganglioside. Solutions of surfactants contained 50 mM-sodium citrate, pH 4.0, and 10 mM- NaN_3 . During surface-tension measurements the surfactant solutions were maintained at $37 \pm 1.5^\circ\text{C}$ by use of a heat lamp. The critical micellar concentration was defined as the concentration at which the lowest surface tension was observed with a Cenco-Joli balance to record spring tension at which ring detachment occurred (Harkins & Jordan, 1930). Spring-tension values corresponding to zero surface tension were recorded between each measurement and corres-

ponded to $188 \pm 12 \mu\text{N/cm}$. After recording the surface tension of a series of concentrations of each surfactant the surface tension of the buffer solution was measured. This solution had a surface-tension value of $502 \pm 6 \mu\text{N/cm}$ thus providing a stable baseline. Surface-tension values reported for surfactant solutions represent the average for three measurements with standard error not exceeding $12 \mu\text{N/cm}$.

Results

Critical micelle concentrations of surfactants

Values for critical micellar concentrations of many surfactants have recently been collated (Mukerjee & Mysels, 1971). Critical micellar concentrations for many surfactants commonly in use in enzyme studies, however, are not obtainable. Published values (Mukerjee & Mysels, 1971) were obtained by using a variety of conditions, which may not be relevant to the reaction conditions in which these reagents are employed in enzymological studies. Thus, critical micellar concentrations of ionic surfactants are minimally affected by temperature, but strongly influenced by the concentration of counter ions. For non-ionic surfactants the effects of temperature on the critical micellar concentration are likely to be greater than for ionic surfactants (Helenius & Simons, 1975).

Fig. 1 summarizes the surface-tension data from which critical micellar concentration values were obtained. The critical micellar concentration values are presented as concentration ranges in Table 1. Com-

parisons of the values obtained in this investigation with published critical micellar concentration values are difficult to make because of differences in temperature and ion concentration. Crook *et al.* (1963) observed that for a series of octylphenoxyethoxy-ethanol surfactants with chain lengths varying between 1 and 40, critical micellar concentration values at 25°C were between 0.15 and 0.5 mM. For

Fig. 1. Determination of critical micelle concentrations of surfactants

Surface-tension measurements were performed on solutions of 50 mM-sodium citrate, pH 4.0. 10 mM- NaN_3 containing surfactants at the indicated concentrations. The temperature was maintained at 37°C . Surfactants were sodium cholate ($\times$), sodium taurocholate ($\diamond$), Tween 80 ($\circ$), sodium dodecyl sulphate ($\blacktriangle$), Cutscum ($\square$), Triton X-100 ($\bullet$) and *N*-dodecyl sarcosinate ($\triangle$).

Table 1. Effect of surfactants on hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside

Surfactant	Optimal concn. (mg/ml)	Critical micellar concentration (mg/ml)	G_{M2} ganglioside hydrolysed (pmol/16 h)	G_{M2} ganglioside hydrolysed (pmol/16 h) (in the presence of activator)*	4-MU-Glc-NAc hydrolysis (% of control)
None			48	520	100
<i>N</i> -Dodecylsarcosinate	0.2–0.5	0.1–0.2	660	—	97
Sodium taurocholate	>5.0	3.5–5.0	>600	—	105
Cutscum	0.2–0.5	0.2–0.5	475	748	130
Triton X-100	0.2–0.5	0.1–0.2	495	750	107
Sodium taurodeoxycholate	0.5	—	415	—	115
Sodium cholate	0.2–0.5	0.2–0.5	265	—	98
Hexadecyltrimethyl-ammonium bromide	—	—	100	144	97
Brij 35	—	0.1–0.2	44	236	94
Phosphatidylethanolamine	—	—	65	195	98
Phosphatidylinositol	0.5	—	340	—	103
Tween 80	—	0.05–0.1	48	180	108
Triton CF-54	—	0.1–0.2	53	—	—
Sodium dodecyl sulphate	0.1	0.2–0.5	255	144	104

* Concentration of surfactants was 0.5 mg/ml.

Triton X-100, which belongs to this class of surfactants, assuming a mol.wt. of 600, our critical micellar concentration value is in the range 0.16–0.32 mM both at 25°C and at 37°C. An increase in surface-tension values at concentrations greater than the critical micellar concentration was noted (Fig. 1) for sodium cholate, sodium taurocholate and Tween 80. Appearance of minima in the surface-tension curves may be due to the presence of impurities (Weil & Stirton, 1956) or they may reflect a contribution of the micelle to the surface tension.

Inclusion of 10 μ M- G_{M2} ganglioside in the surfactant solution did not affect the concentration at which the lowest surface tension was achieved. Surface tension of G_{M2} ganglioside solutions increased as the concentration of this substrate was increased between 10 and 500 μ M.

Effect of surfactants on hexosaminidase A-catalysed reaction

A variety of surfactants can mimic the activator protein and stimulate the enzymic hydrolysis of G_{M2} ganglioside. Some of the characteristics of surfactant stimulation are shown in Figs. 2 and 3 and in Table 1. For the group of surfactants that increase the rate of hydrolysis of this substrate, the stimulation of enzyme activity is concentration-dependent, exhibiting a decrease in rate at super-optimal concentrations of surfactant. The activation curves for sodium taurocholate and activator protein fail to exhibit this decrease in stimulation at super-optimal concentrations. In the case of sodium taurocholate,

concentrations above 12 mg/ml interfere with the assay system by preventing co-precipitation of G_{M2} ganglioside with human serum albumin in the presence of trichloroacetic acid.

Fig. 3. *Effect of surfactants on activator protein-stimulated hydrolysis of G_{M2} ganglioside*

Reaction mixtures contained hexosaminidase A (3000 units/ml), activator protein (7 mg/ml) and Triton X-100 (●), Tween 80 (○) or Cutscum (△). Hexosaminidase A (3000 units/ml) and Cutscum and no activator (▲).

Fig. 2. *Stimulation of hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside*

(a) Reaction mixtures containing Triton X-100 (●), *N*-dodecyl sarcosinate (○), Tween 80 (△), sodium dodecyl sulphate (×) and phosphatidylinositol (□). All reaction mixtures contained 3 H-labelled 10 μ M- G_{M2} ganglioside, 50 mM-sodium citrate, pH 4.0, 10 mM- NaN_3 , 600 units of partially purified hexosaminidase A and indicated amounts of surfactants. (b) Reaction mixtures containing sodium taurocholate (●) and activator protein (▲) and other components as indicated in Fig. 2(a).

The activation curve for partially purified activator protein (Fig. 1b) shows that maximum velocity is obtained at an activator concentration of approx. 3 mg/ml. In a previous report (Hechtman & LeBlanc, 1977) we have shown that a highly purified preparation of activator will maximally stimulate G_{M2} ganglioside hydrolysis at a concentration of 0.2 mg/ml. The bile salt sodium taurocholate is thus unique among the surfactants tested in that its optimum concentration for stimulation of enzymic hydrolysis is approx. 10-fold greater on a milligram basis than for other activating surfactants.

Surfactants that stimulate enzymic hydrolysis of G_{M2} ganglioside increase the activity of hexosaminidase A in the presence of saturating concentrations of activator protein. Thus, as shown in Fig. 3, in the presence of activator protein the enzyme catalyses the hydrolysis of 520 pmol of G_{M2} ganglioside/16 h, whereas in the presence of Cutscum (0.5 mg/ml) plus activator 740 pmol of G_{M2} ganglioside/16 h were cleaved.

Tween 80 and a variety of surfactants listed at the foot of Table 1 have no effect on enzyme-catalysed hydrolysis of G_{M2} ganglioside. All of the surfactants in this group, however, inhibit activator-stimulated hydrolysis of the substrate.

The inhibitory effects of Tween 80 appear to be competitive with the activator. Fig. 4 shows Lineweaver-Burke plots for the activator-stimulated hydrolysis of G_{M2} ganglioside in the presence of two concentrations of Tween 80. The presence of the

inhibitory surfactant does not alter the V_{max} of the reaction, but does increase the K_m . This observation suggests that surfactants and activator are competing for the same site in the rate-limiting step of the activation reaction.

Each of the surfactants listed in Table 1 was tested for its effects on the hydrolysis of the synthetic substrate 4-MU-Glc-NAc. Over the entire concentration range (0.1–2.0 mg/ml) at which the surfactants were incubated with hexosaminidase A no significant change in the activity of the enzyme toward the synthetic substrate was seen relative to enzyme activity in the absence of surfactant.

The failure of any of the surfactants to alter the rate of hydrolysis of the synthetic substrate suggests the possibility that primary surfactant effects are due to interactions with the substrate. The behaviour of surfactants in aqueous solutions has been reviewed (Helenius & Simons, 1975). At low concentrations surfactants exist in monomeric form. Above the critical micellar concentration monomers will aggregate to form micelles, the size of which will depend on the structural features of the surfactant monomer. Two different amphiphilic monomers may aggregate to form a mixed micelle.

If surfactants stimulate enzyme hydrolysis of G_{M2} ganglioside by forming mixed micelles with the amphiphilic substrate, then the surfactant concentrations at which activation of the enzyme reaction occurs should correlate with the concentrations at which micelle formation occurs. These correlations are shown in Table 1.

Thus for sodium cholate, sodium taurocholate, Cutscum, Triton X-100, *N*-dodecylsarcosinate and sodium dodecyl sulphate the critical micellar concentration measurement and the optimum concentrations at which stimulation of enzyme hydrolysis occurs agree within a 2-fold range. Similarly the concentration-dependent inhibition of the activator-stimulated hydrolysis of G_{M2} ganglioside by Tween 80 begins to occur at the surfactant concentration range (0.05–0.1 mg/ml), which corresponds to the critical micellar concentration.

We have further explored the possibility that surfactant activation of G_{M2} ganglioside hydrolysis occurs through the formation of a mixed micelle of detergent and substrate. Sphingomyelin and Triton X-100 form a mixed micelle the characteristics of which have been reported (Yedgar *et al.*, 1974). The inclusion of sphingomyelin in a Triton X-100-containing reaction mixture at a 35-fold molar excess relative to G_{M2} ganglioside results in a complete loss of stimulation due to Triton X-100 as shown in Fig. 5(a). This loss of activity would be expected if sphingomyelin displaced G_{M2} ganglioside from the mixed micelle. Sphingomyelin at a 7-fold molar excess relative to 4-MU-Glc-NAc has no effect on hexosaminidase A-catalysed cleavage of the synthetic substrate

Fig. 4. Competitive inhibition of activator by Tween 80. Symbols: ●, reaction mixtures containing activator, hexosaminidase A (600 units), 3H -labelled G_{M2} ganglioside (2 nmol), sodium citrate, pH 4.0 (50 mM) and $NaNO_3$ (10 mM); ▲, reaction mixtures containing components indicated above plus 0.25 mg of Tween 80/ml; ○, plus 0.5 mg of Tween 80/ml.

Fig. 5. Effect of sphingomyelin on activator and Triton X-100 stimulation of hexosaminidase A

(a) G_{M2} ganglioside-hydrolysis stimulation by Triton X-100 (●) and in the presence of 350 μM-sphingomyelin (○). (b) G_{M2} ganglioside hydrolysis stimulated by activator (●) and in presence of 350 μM-sphingomyelin (○).

in the presence of 0.5 mg of Triton X-100/ml. The activator-stimulated reaction, however, is unaffected by excess sphingomyelin, as shown in Fig. 5(b). Activator binding of substrate may thus be more specific than the association that occurs during formation of the mixed micelle of surfactant and G_{M2} ganglioside.

Direct interaction of surfactants and G_{M2} ganglioside

Gel filtration of ³H-labelled G_{M2} ganglioside on Sephadex G-200 columns with initial ganglioside concentrations between 2 μM and 1 mM resulted in elution of the label in the void volume. Since the molecular weight of the G_{M2} ganglioside is 1400, the elution of the substrate in the high-molecular-weight fraction is presumably due to the formation of a ganglioside micelle of molecular size greater than 200 000, the exclusion limit of Sephadex G-200. The occurrence of this void-volume peak at initial substrate concentrations of 2 μM indicates that under conditions used for elution (deionized water at 25°C) the critical micellar concentration of G_{M2} ganglioside is less than 2 μM. When 10 μM-G_{M2} ganglioside was mixed with 1 mg of either Triton X-100 or Tween 80 per ml, there was a shift of the ³H-labelled G_{M2} ganglioside peak from the void volume to a *K_{av}* of 0.22, as shown in Fig. 6. At 1 mM substrate concentration, G_{M2} ganglioside was eluted in the void volume, but its *K_{av}* was unaffected by Triton X-100. Use of Triton X-100 at 0.4 mg/ml, a sub-optimal concentration for activation of enzymic hydrolysis, resulted in distribution of the substrate radioactivity between both the void volume and the peak at *K_{av}* = 0.22, although the lower-molecular-weight fraction emerged as a broader peak than in the presence of the higher Triton X-100 concentration.

Fig. 6. Elution of ³H-labelled G_{M2} ganglioside from Sephadex G-200

Sephadex G-200 was swollen and packed into columns (32 cm × 1.7 cm) of 70 ml bed volume. Samples applied to the column contained 2 mg of Blue Dextran, 0.5 mg of uridine, 5 nmol of ³H-labelled G_{M2} ganglioside, 36 mg of sucrose and various amounts of surfactants in a volume of 0.5 ml. Symbols: ●, no surfactants; ○, 2 mg of Triton X-100/ml; □, 0.4 mg of Triton X-100/ml. Fractions were 0.5 ml.

Co-chromatography of G_{M2} ganglioside with activator did not produce a shift in the size distribution of the substrate. Binding of activator to the substrate peak was not monitored.

Kinetics of G_{M2} ganglioside hydrolysis

A Lineweaver–Burke plot of rate of hydrolysis of G_{M2} ganglioside versus substrate concentration, illustrated in Fig. 7(b), showed no discontinuity of the concentration range employed (10–500 μM). Discontinuities in substrate-versus-velocity plots for sphingolipid hydrolysis have been interpreted by Gatt *et al.* (1972) as evidence for the occurrence of a phase transition between monomeric and micellar forms of the substrate in the concentration range tested and for preference for the enzyme for one form to the other. The absence of such a discontinuity indicates that the critical micellar concentration of the ganglioside substrate does not occur between 10 and 500 μM and is consistent with the behaviour of the substrate as a micelle on gel-filtration columns at concentrations greater than 10 μM.

The effect of either Triton X-100 or activator on the reaction kinetics is on the *K_m*, which is 333 μM in the absence of activator or detergent, 166 μM in the presence of 0.5 mg of Triton X-100/ml and 56 μM in the presence of saturating concentrations of acti-

Fig. 7. Effect of Triton X-100 on hexosaminidase A-catalysed reactions

(a) Substrate is 4-methylumbelliferyl-*N*-acetyl- β -D-glucopyranoside. Symbols: ●, velocity determined in the presence of 0.5 mg of Triton X-100/ml; ○, in the presence of 0.5 mg of Triton X-100/ml and 200 μM - $\text{G}_{\text{M}2}$ ganglioside. The broken line is a theoretical plot of expected Lineweaver-Burke plot using 200 μM - $\text{G}_{\text{M}2}$ ganglioside as a competitive inhibitor assuming a K_i of 0.154 mM in the presence of 0.5 mg of Triton X-100/ml. (b) Substrate is ^3H -labelled $\text{G}_{\text{M}2}$ ganglioside incubated with hexosaminidase A (●), hexosaminidase A plus 0.5 mg of Triton X-100/ml (○) and hexosaminidase A plus activator (Δ).

vator. A comparison of the V_{max} for $\text{G}_{\text{M}2}$ ganglioside hydrolysis and 4-MU-Glc-NAc hydrolysis (Fig. 7a) indicates a value of 1.38 μmol of $\text{G}_{\text{M}2}$ ganglioside hydrolysed/mol of 4-MU-Glc-NAc hydrolysed.

The broken line in Fig. 7(a) is a theoretical plot for competitive inhibition of hydrolysis of 4-MU-Glc-NAc by $\text{G}_{\text{M}2}$ ganglioside. This plot was obtained by making the single assumption that the two substrates bind at the same active site on hexosaminidase A. If this assumption is correct, then if both reactions are assayed in the presence of a surfactant concentration that is optimal for binding of $\text{G}_{\text{M}2}$ ganglioside (0.5 mg of Triton X-100/ml) the K_m for $\text{G}_{\text{M}2}$ ganglioside should be identical with the K_i for $\text{G}_{\text{M}2}$ ganglioside as a competitive inhibitor of 4-MU-Glc-NAc hydrolysis. The apparent K_m for hydrolysis of 4-MU-Glc-NAc in the presence of $\text{G}_{\text{M}2}$ ganglioside would thus be equal to $K_m(1 + [I]/K_i)$, where $K_m = 1.54 \text{ mM}$ -4-MU-Glc-NAc, $K_i = 0.166 \text{ mM}$ - $\text{G}_{\text{M}2}$ ganglioside and $[I] = 0.2 \text{ mM}$ in this experiment. The results of this attempt to predict competitive inhibition are shown in Fig. 7(a) (open circles). In the presence of an optimal concentration of surfactant no competitive inhibition of 4-MU-Glc-NAc occurs. The Lineweaver-Burke plots for hexosaminidase A-catalysed hydrolysis of 4-MU-Glc-NAc in both the presence and absence of Triton X-100 are identical (results not shown). One interpretation of these data is that hexosaminidase A has two distinct sites for the two substrates. This use of kinetic data to determine the number of distinct active sites has been formally treated by Ahmed & Scholefield (1962).

An alternative procedure for demonstrating the existence of two kinetically distinct active sites is the

Fig. 8. Inhibition of ^3H -labelled $\text{G}_{\text{M}2}$ ganglioside hydrolysis by potassium metabisulphite

Symbols: ●, hydrolysis of 4-MU-Glc-NAc; Δ, hydrolysis of ^3H -labelled $\text{G}_{\text{M}2}$ ganglioside stimulated by activator; ○, hydrolysis of ^3H -labelled $\text{G}_{\text{M}2}$ ganglioside stimulated by Triton X-100.

demonstration that the two reactions are differentially inhibited by a series of non-specific enzyme inhibitors. The comparative effects of some inhibitors on the two reactions is shown in Fig. 8 and Table 2. The reducing agent potassium metabisulphite strongly inhibits hydrolysis of $\text{G}_{\text{M}2}$ ganglioside, but has little or no effect on hydrolysis of 4-MU-Glc-NAc. Inhibition of $\text{G}_{\text{M}2}$ ganglioside hydrolysis by metabisulphite ions occurs both in activator

Table 2. Inhibition of hydrolysis of G_{M2} ganglioside and 4-MU-Glc-NAc

Compound	Concn. (mM)	G_{M2} ganglioside hydrolysis (% of control)*	4-MU-Glc-NAc hydrolysis (% of control)*
HgCl ₂	5	0	0
MgCl ₂	5	103	99
BaCl ₂	5	104	97
ZnCl ₂	5	100	90
ZnCl ₂	10	124	62
ZnCl ₂	20	137	52
EDTA	10	107	103
2-Mercaptoethanol	50	91	98
Dithiothreitol	50	29	0
Sodium acetate	5	48	47

* Enzyme activities were determined in the presence of 0.5 mg of Triton X-100/ml.

and Triton X-100-stimulated reactions, thus ruling out that the inhibition is due to interaction of meta-bisulphite with the activator protein.

Table 2 compares the response of the two reactions to a series of potential inhibitors. The results show that both reactions are equally insensitive to the mild reducing agent 2-mercaptoethanol. However, hydrolysis of the synthetic substrate is completely inhibited at a concentration of 50 mM of the more potent reducing agent dithiothreitol. Among a group of metal ions tested only Zn^{2+} had a markedly different effect on hydrolysis of the two substrates. The effects of Zn^{2+} could not be reversed by EDTA.

Discussion

We conclude that stimulation of G_{M2} ganglioside hydrolysis by surfactants, and probably by activator protein as well, occurs by formation of a mixed micelle containing both surfactant and substrate. If the geometry of the mixed micelle is such that the *N*-acetylgalactosaminyl linkage is made accessible to the active site of the enzyme, then the mixed micelle will be a preferred substrate. If the terminal sugar linkage is buried in the interior of the micelle then the surfactant will behave as a competitive inhibitor by sequestering the ganglioside substrate in metabolically inert micelles.

A number of observations support this hypothesis. (1) None of the surfactants stimulated or inhibited enzymic hydrolysis of G_{M2} ganglioside at surfactant concentrations below the critical micellar concentration, indicating that the active form of the surfactant is the micelle rather than the monomer. (2) The inhibition of Triton X-100-stimulated hydrolysis by sphingomyelin is most economically explained in terms of competition of the two sphingolipid molecules for surfactant. The two sphingolipids are structurally dissimilar and sphingomyelin

does not compete with the 4-MU-Glc-NAc substrate. (3) Formation of mixed micelles between surfactants and G_{M2} ganglioside can be observed directly by gel filtration. Our finding that the ganglioside behaves like a micelle at a concentration of $2 \mu M$ is in disagreement with older critical micellar concentration values for gangliosides (Gammack, 1963), but in agreement with the recently published data of Formisano *et al.* (1979). The downward revision of the critical micellar concentrations for gangliosides to $10^{-8} M$ indicates that ganglioside monomers would not prevail at the substrate concentrations used to monitor surfactant effects. The molecular weight of the substrate micelle is in excess of 200 000. In the presence of Triton X-100, an activating surfactant, or Tween 80, an inhibiting detergent, a lower-molecular-weight mixed micelle is formed.

The failure to demonstrate competitive inhibition of 4-MU-Glc-NAc hydrolysis by G_{M2} ganglioside leads us to propose the existence of two kinetically distinct sites on hexosaminidase A. An alternative proposal is that two distinct enzymes are required for the two reactions (Bach & Suzuki, 1975) and that both enzymes are present in the partially purified enzyme preparation used in these experiments. It is unlikely that two distinct enzymes are required. In a previous report (Hechtman, 1977) we have shown that enrichment of specific activity of hexosaminidase A by a factor of 1000 did not change the value for G_{M2} hydrolysis/synthetic substrate hydrolysis.

The proposal of two distinct sites on hexosaminidase A is not inconsistent with observations on the inheritance of variant genotypes that affect the activity of hexosaminidase A. Thus O'Brien *et al.* (1978) have identified pedigrees in which allelic mutant genes confer loss of hexosaminidase A activity to either 4-MU-Glc-NAc or to G_{M2} ganglioside, but not to both substrates.

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