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Modulation of the activity of *Clostridium perfringens* neuraminidase by the molecular organization of gangliosides in monolayers

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Abstract

The activity of *Clostridium perfringens* neuraminidase against gangliosides GM_3, GD_{1a} and GM_1 was studied in lipid monolayers at the air-buffer solution interface. The enzyme activity assay against pure ganglioside monolayers is based on the markedly different molecular packing areas of the substrate gangliosides and the resulting product glycosphingolipids. This allows to control and monitor the surface pressure and the ganglioside intermolecular organization (cross-sectional packing areas and dipole potentials) in a continuous manner during the catalytic process. It was found that the rate and the extent of the enzymatic reaction depended markedly on the lateral surface pressure. In general, the activity of neuraminidase against GM_3 and GD_{1a} was higher at lower surface pressure. This corresponded to larger intermolecular spacings among the ganglioside molecules. Both the activity and the extent of the reaction against GM_3 were higher than toward GD_{1a}. GM_1 could not be degraded by the enzyme, irrespective of the surface pressure but the enzyme could interact with this ganglioside. A latency period, longer for GM_3 than for GD_{1a}, was observed prior to the onset of rapid degradation; this indicates that pre-catalytic steps are occurring at the interface before effective ganglioside degradation takes place. The latency period, the total amount of ganglioside degraded, and the velocity of the reaction varied with the surface pressure in different manners. Our data indicate that the different steps of the catalytic reaction occurring at the surface (i.e., substrate recognition and interfacial adsorption, catalysis, maximum extent of substrate conversion) are independently regulated by the molecular organization of the substrate gangliosides.

Key words: Neuraminidase; Ganglioside; Monolayer; Surface hydrolysis

1. Introduction

Neuraminidases (EC 3.2.1.18; *N*-acetylneuraminyl glycohydrolases, sialidases) are exoglycosidases responsible for the release of sialic acids from a variety of sialic acid-containing lipids and proteins, and are widely distributed in nature. These enzymes can be found in viruses, bacteria, protozoa and animal tissues either in soluble or membrane-bound forms. The substrate specificity of neuraminidases is complex and is affected by the type of glycosidic linkage of the sialic acid to the neighboring sugar, the nature of the neighboring residue, and the position of the sialic acid along the oligosaccharide chain [1]. Few sialidases are available in purified form in the quantities required for detailed studies of the molecular mechanism of their catalytic reaction. Gangliosides such as GM_3, GM_1, GD_3, GD_{1a}, GD_{1b}, and GT_{1b} are susceptible to degradation by neuraminidases from a variety of bacterial, viral and animal sources, including a recently described myelin-associated neuraminidase [2,3]. These gangliosides are converted to the asialo neutral GSLs (for GM_3 and GD3) or to GM_1 (for di and trisialogangliosides) which is a poor substrate for these enzymes.

The properties and specificity of neuraminidase with ganglioside substrates have been extensively studied from a biological point of view, and neuraminidases

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Abbreviations: Cer, ceramide (*N*-acylsphingosine); LacCer, Galβ1-4Glcβ1-1'Cer; NeuAc, *N*-acetylneuraminic (sialic) acid; GM_3, Gal(3-2αNeuAc)β1-4Glcβ1-1'Cer; GM_1, Galβ1-3GalNacβ1-4Gal(3-2α NeuAc)β1-4Glcβ1-1'Cer; GD_{1a}, NeuAc(α2-3)Galβ1-3GalNacβ1-4 Gal(3-2αNeuAc)β1-4Glc β1-1'Cer; Ndase, neuraminidase.

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have been widely employed to modify the ganglioside composition in biomembranes [4,5]. However, the molecular mechanisms underlying the catalytic reaction and the effect of the surface organization of the ganglioside substrates on the activity have been scarcely investigated. Changes of the membrane phase state or in the state of aggregation of gangliosides in the membrane affect the activity of *Clostridium perfringens* neuraminidase [6,7]. Also, the differential activity of *V. cholerae* neuraminidase against species of GD_{1a} having different long-chain base composition has been correlated to the state of dispersion of the ganglioside substrate in the membrane [7,8].

It is well known that lipid hydrolases are extremely sensitive to subtle variations of the intermolecular organization and dipolar interactions occurring at the membrane interface [9–12]. A major limitation of bulk systems (bilayer vesicles and natural membranes) for studying molecular mechanisms by which the surface organization regulates lipid degradation is that the variation of surface parameters brought about by the very enzymatic activity can not be known or controlled during the reaction. In this work we have studied the interaction and activity of *C. perfringens* neuraminidase in monolayers formed by individual gangliosides GD_{1a}, GM_3 and GM_1 in order to monitor continuously the lateral surface pressure, intermolecular packing and surface (dipole) potential of the ganglioside interface. The results indicate that the recognition of the ganglioside by neuraminidase, and the velocity and extent of degradation are modulated independently by the intermolecular organization.

2. Materials and methods

2.1. Materials

Gangliosides GM_3, GM_1 and GD_{1a} were purified from bovine brain as previously described [13]. They were over 99% pure according to HPTLC [14]. Neuraminidase (Ndase) type X from *C. perfringens* was from Sigma (St. Louis, MO, USA). We attempted to measure the activity of *V. cholerae* Ndase (type II from Sigma) but were not successful due to the low specific activity of the preparation available, in relation to the enzyme protein needed to reach the required concentration in the subphase for the assay against ganglioside monolayers. Therefore, only Ndase from *C. perfringens* was used in this work. On SDS-PAGE 97% of the protein ran as a single band of 66 kDa on 10–20% polyacrylamide minigels (Bio-Rad, Richmond, VA, USA). Organic solvents and salts used to prepare buffers were of the highest purity available. Absence of surface active impurities in the aqueous solutions and in the spreading solvents was routinely controlled as described before [13,15]. Water was double-distilled in an all-glass apparatus.

2.2. Equipment

The equipment used was a Monofilmmeter (Mayer Feintechnik, Gottingen, Germany) with a circular (Fromherz type) Teflon-coated trough especially designed for these experiments, having several compartments of different surface area and volume, connected by shallow and narrow slits (5-mm wide × 5-mm long × 1-mm deep). This permits studying the surface kinetics of the ganglioside degradation by Ndase injected in the subphase under conditions of constant surface area or constant surface pressure. The equipment is fitted with two barriers that can move independently or synchronously by electronic switching. The surface potential was measured with a Corning ionalyzer 250 millivoltmeter and a ^{241}Am air-ionizing (set at 5 mm above the surface) and calomel (inserted into the subphase) electrode pair. The signal from the millivoltmeter, that corresponding to the surface area (automatically determined by the Monofilmmeter according to the relative position of the two compression barriers) and the output from the surface pressure transducer (measured automatically by the Monofilmmeter with a platinized Pt foil (12.5-mm wide × 20-mm long × 0.025-mm thick) were fed into a double channel X-Y-Y recorder (Yokogawa, Japan). The surface pressure can be maintained constant over any of the trough compartments by an electronic compensation circuit, operating as a surface barostat, that controls the movement of one of the compression barriers. The trough temperature was controlled with a Haake F3C thermocirculator, and was enclosed in a Faraday cage. Before each experiment the trough was wiped clean with 70% ethanol and rinsed with bidistilled water. Monolayers formed by pure individual gangliosides were prepared at 34 ± 0.2°C by spreading 5–10 nmol of gangliosides in less than 10–25 μl of chloroform/methanol/water (2:1:0.15); 5 min were allowed for solvent evaporation and monolayer stabilization before the film was compressed to the desired initial surface pressure.

2.3. Enzymatic activity against ganglioside monolayers

100% ganglioside films were prepared as described previously [16]. The gangliosides used form stable monolayers [13,16], in the present work their surface pressure- and surface potential-molecular area compression isotherms are reproducible within ±0.03 nm^2, ±1 mN/m, and ±10 mV after three successive compression-decompression cycles (one complete cycle lasted 20 min) and at different rates of compression

(2–10 cm^2/min). The ganglioside monolayers were equilibrated at a pre-determined initial surface pressure over both the reaction compartment and an adjacent one separated from the first by a shallow and narrow slit (see above). The adjacent compartment served as a monolayer reservoir that was continuously supplied to the surface of the reaction compartment as required in order to maintain a constant surface pressure when studying the enzymatic reaction. When ganglioside films were kept at constant surface pressures, between 5–35 mN/m, onto enzyme-free subphases there was a maximum of 5% decrease of the surface area after 90 min. This decrease (indicated by the dotted base-line slope (labelled b) starting at 100% area in Fig. 2 is due to slight leakage of the monolayer film past the enclosing compression barriers and was always subtracted from the changes due to Ndase activity.

An aliquot of a concentrated enzyme solution (27 μg protein/ml, spec. act. 240 U/mg protein) was injected into the subphase (18 ml, 20 cm^2 of surface area) of the reaction compartment (continuously stirred with a miniature Teflon-coated rod spinning at 150–250 rpm) to reach the desired final concentration. The subphase consisted of an aqueous solution of 100 mM NaCl, 2 mM $CaCl_2$ and either 50 mM cacodylate (pH 4.8) or 50 mM Tris-HCl (pH 6.8 or 8). The course of the enzymatic reaction at 34 ± 0.2°C was followed by recording the decrease of surface area as a function of time that was automatically compensated by the surface barostat to keep the surface pressure constant. The activity of Ndase is calculated from the linear portion of this curve after the latency period (see Fig. 2). The reaction rate is expressed as the number of ganglioside molecules degraded per min and is normalized per unit of enzyme concentration in the subphase. The number of ganglioside molecules at each surface area is calculated on the basis of the known area per molecule at the particular surface pressure used, obtained from the surface pressure-area compression isotherm (see Fig. 1). Some experiments were done at constant area in order to measure the changes of surface pressure and surface (dipole) potential during the reaction. The extent of ganglioside degradation was corroborated by HPTLC. After the rate of the reaction decreased to zero (see Fig. 2) the monolayer was collected by adsorption to a hydrophobic paper (Whatman 1PS) previously washed with chloroform:methanol (2:1) or by surface aspiration of the film (in a total vol. of about 0.3 ml); success of collection was controlled by the decrease of surface pressure to less than 0.5 mN/m. The lipid was immediately extracted with chloroform/methanol/0.1 M NaOH (2:1:0.15) to prevent ganglioside lactonization [13] and the extent of degradation was analyzed by HPTLC and scanning densitometry [14].

3. Results

The products of degradation of ganglioside GM_3 and GD_{1a} by Ndase are LacCer and ganglioside GM_1, respectively. Fig. 1 shows the surface pressure-area isotherms of gangliosides GM_3, GM_1 and GD_{1a}, and of LacCer. The basis for the continuous measurement of the Ndase activity in monolayers is the marked difference in the molecular area of the ganglioside substrates and the products of the reaction, especially below the surface pressure of 33 mN/m. This permits following the enzymatic reaction by the continuous decrease of monolayer area, at constant surface pressure. Conversely, if the degradation of gangliosides is measured at constant surface area, the formation of product as a function of time is reflected by a continuous decrease of the surface pressure (see also Fig. 7).

Fig. 2 shows the tracing of an actual experiment, using GM_3 and GD_{1a} as substrates, under conditions of constant surface pressure. The enzymatic reaction exhibits a lag-time before the onset of more rapid and constant rate of ganglioside degradation. The linear decrease of surface area as a function of time, at constant surface pressure, reflects a surface kinetics that may be defined as pseudo-zero order. After this, the velocity of the enzymatic reaction decreases to zero. This behavior is different from the true zero-order kinetics observed when monolayers of short-chain phospholipids are degraded by phospholipase A_2, in which the enzymatic rate does not decrease as long as substrate is continuously supplied from the reservoir monolayer [11,17]. This is because the products of degradation of short-chain phospholipids by phospholipase A_2 (short-chain lysolecithin and fatty acid) are continuously released from the monolayer interface into the subphase solution. In the case of the reaction catalyzed by Ndase the products of ganglioside degra-

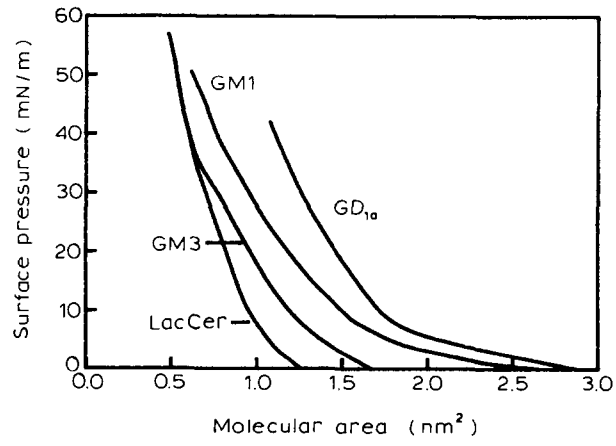


Fig. 1. Surface pressure-molecular area isotherms determined at 34°C for LacCer, GM_3, GM_1 and GD_{1a}. The subphase was 50 mM cacodylate buffer, 100 mM NaCl and 2 mM $CaCl_2$ (pH 4.8).

dation (LacCer for GM_3, or GM_1 for GD_{1a}) remain at the interface; when the mole fraction of the product increases in the monolayer the kinetics deviates from the zero-order region and the reaction eventually stops. The velocity of the reaction in the linear portion is proportional to the concentration of enzyme in the subphase for both ganglioside substrates (Fig. 2, inset).

The latency period of the reaction is generally longer for GM_3 than for GD_{1a} and it varies with the surface pressure (Fig. 3a). The duration of the latency period is inversely related to the enzyme concentration in the subphase (inset in Fig. 3a). The maximum degradation of GM_3 depends on the surface pressure and it can reach 100% (Fig. 3b, see also Fig. 2). The smaller extent of GM_3 degradation at 5 mN/m is not due to irreversible inactivation for the following reasons. After the surface reaction was allowed to proceed at 5 mN/m to its maximum extent (58% hydrolysis) the monolayer was compressed (within 30 s) to 10 mN/m and the reaction was recorded further. The rate of reaction observed was almost double and within ±10% of that obtained with the film initially set at 10 mN/m, and the extent of degradation reached 100% after 15 min. In addition, a film initially set at 5 mN/m and degraded by 58% was transferred, at constant surface pressure, onto an enzyme-free subphase and the surface pressure was subsequently changed to 10 mN/m. In these conditions the reaction continued to an extent of about 100% hydrolysis with a rate similar to that observed in films initially set at 10 mN/m.

At comparable surface pressures and enzyme concentration in the subphase the extent of degradation of GD_{1a} is less than that for GM_3 (see Fig. 2), and the maximum amount hydrolysed corresponds to about 60% of the total GD_{1a} on the surface of the reaction compartment. Densitometric quantitation after HPTLC of the lipid recovered from the monolayer at the maximum extent of degradation corroborated that 62% of GD_{1a} and over 90% of GM_3 were hydrolysed (Fig. 4). Increasing the enzyme concentration in the subphase causes an increase of the rate of the reaction for both

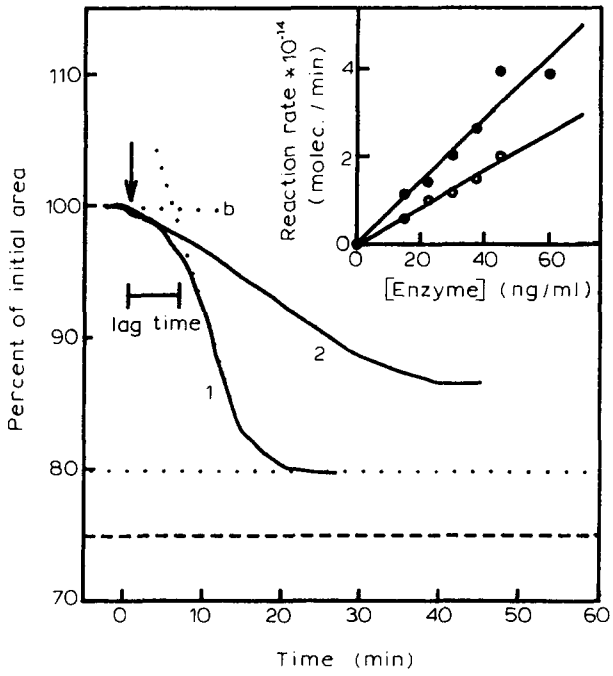


Fig. 2. Change in monolayer area vs. time at constant surface pressure. Tracing of an actual experiment using GM_3 (1) or GD_{1a} (2) as substrates. At time zero (arrow) Ndase at a final concentration of 15 ng protein/ml (GM_3) or 30 ng protein/ml (GD_{1a}) was injected into the subphase (pH 4.8) of the reaction compartment. The surface pressure was kept constant at 10 mN/m (GM_3) or 15 mN/m (GD_{1a}). The dotted line marked *b* starting at 100% area represents the spontaneous decrease in area due to film leakage. The lag-time for reaching a constant velocity with GM_3 is indicated. The horizontal lines at the bottom shows the minimum percent of initial area that is possible to achieve by degradation of all initial GM_3 (·····) and GD_{1a} (— — —). (Inset) Reaction rate from the slope of the linear portion of the curves, expressed as the number of molecules of GM_3 (●) or GD_{1a} (○) degraded per min, as a function of the enzyme concentration in the subphase.

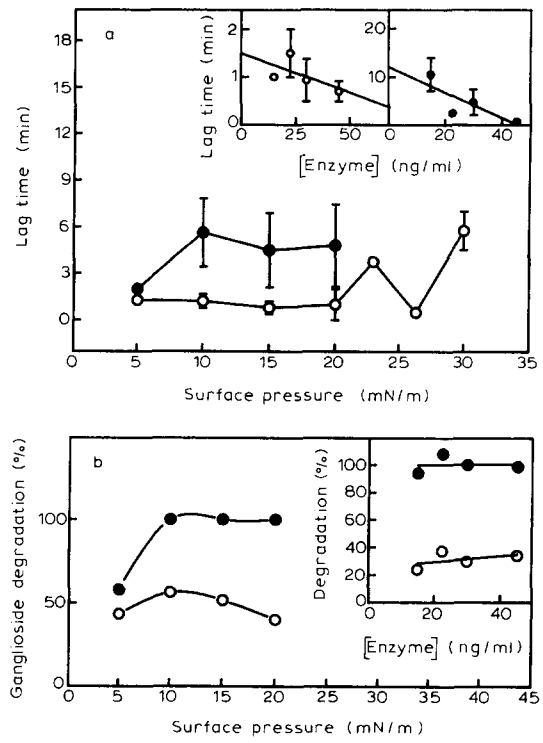
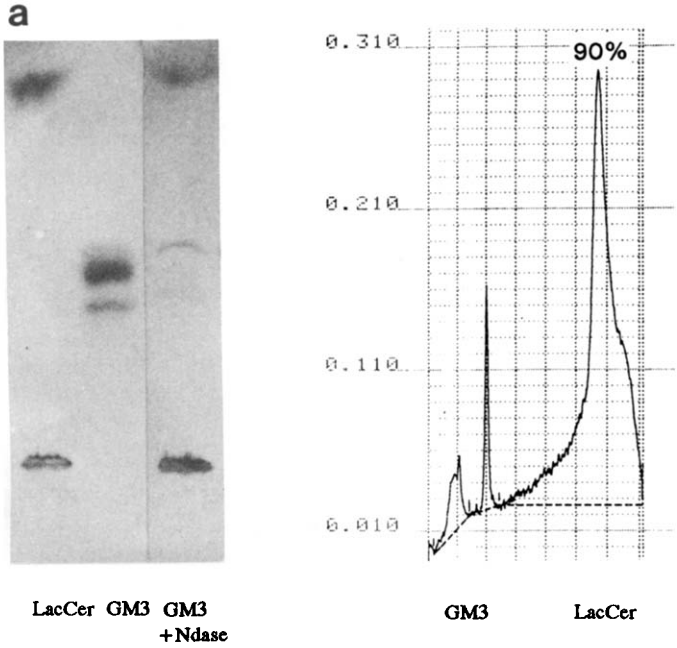


Fig. 3. (a) Dependence of lag time on the surface pressure: GD_{1a} (●); GM_3 (○). The enzyme concentration in the subphase (pH 4.8) was 30 ng protein/ml. (Inset) Variation of lag times with enzyme concentration in the subphase. The surface pressure of the monolayer was 20 mN/m. (b) Extent of ganglioside degradation by Ndase at different surface pressures. The substrate was GD_{1a} (○) or GM_3 (●). The final enzyme concentration was 30 ng protein/ml. (Inset) Variation of the extent of degradation with enzyme concentration in the subphase at constant surface pressure of 20 mN/m. S.E. values are within the points' size.

GM_3 and GD_{1a} (inset, Fig. 2) and of the total amount of GD_{1a} degraded (inset, Fig. 3b).

The dependence of the rate of the enzymatic reaction with the surface pressure is shown in Fig. 5. For GD_{1a} the rate of the reaction decreases rapidly between 10 and 20 mN/m while for GM_3 a broad optimum is observed in this range and the reaction rate decreases outside both these limits. No degradation could be found with GM_1 at any surface pressure.



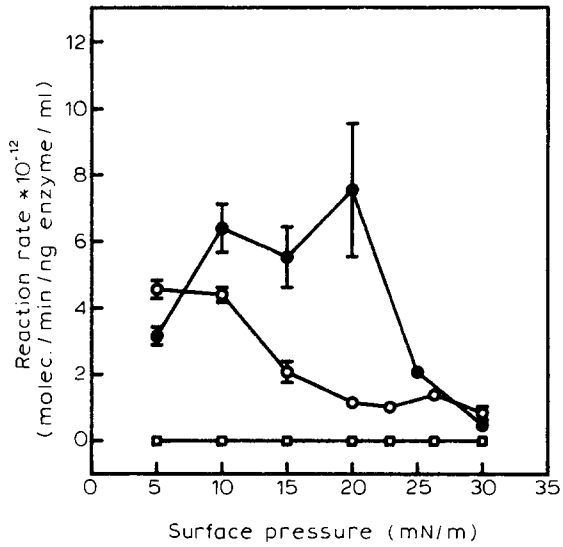


Fig. 5. Reaction rate as a function of the surface pressure. Rate of Ndase-catalyzed hydrolysis of ganglioside GM_3 (●), GD_{1a} (○) and GM_1 (□) at different surface pressures. The rates were calculated from the slope of the straight portion of curves recording the decrease of monolayer area vs. time (see Fig. 2). The changes in area were converted to molecules degraded by taking into account the difference in molecular areas of substrates and their corresponding products, obtained from the pressure-area isotherms (see Fig. 1). Data shown are the means ± S.E. The enzyme concentration was 30 ng/ml and the subphase pH was 4.8.

Fig. 4. HPTLC plates with their corresponding densitometric chromatograms. (a) Lac-Cer, GM_3 and the products of the hydrolysis of GM_3 catalyzed by Ndase. Monolayers of GM_3 (5 nmol) were formed at a lateral surface pressure of 12.5 mN/m. Ndase was injected in the subphase at a final concentration of 30 ng/ml. The reaction was recorded at constant surface pressure and when the rate decreased to zero the monolayer was collected with hydrophobic paper and the lipids were extracted with chloroform/methanol/0.1% (w/v) NaOH (60:30:4.5, v/v) as indicated in Materials and methods. In parallel experiments monolayers of Lac-Cer or GM_3 were prepared as described but in the absence of enzyme. After 20 min the lipids were collected and extracted in identical manner. The extract was concentrated under N_2, 5 μl were applied to HPTLC plates, run with chloroform/methanol/water (60:35:5, v/v), and developed by dipping the plates into a 3% (w/v) cupric acetate-8% (v/v) phosphoric acid solution and heating them at 180°C for 15 min. (left). The line corresponding to the reaction products was scanned at 600 nm in the reflectance mode from the origin to the solvent front (right). The splitting of the band corresponding to GM_3 is due to the heterogeneity in fatty acid composition and has already been described (cf. [40]). (b) GM_1, GD_{1a} and the products of the hydrolysis of GD_{1a} catalyzed by Ndase. Collection (after 40 min) and extraction of monolayers was done as indicated in part (a). The chromatograms were run with chloroform/methanol/0.2% (w/v) $CaCl_2$ (50:45:10, v/v) and developed by spraying the plate with resorcinol reagent and heating it at 100°C for 15 min (left).

Preliminary experiments in which the activity of Ndase was studied against GM_3 in mixed monolayers with 1-stearoyl-2-oleoylphosphatidylcholine at ganglioside mole fractions of 0.9, 0.8 and 0.5 indicated that the rate of enzymatic activity (normalized to take into account the surface dilution of the ganglioside substrate by the phospholipid) and the extent of ganglioside degradation were decreased in correspondence to the proportion of phospholipid in the film. Studies regarding the molecular aspects of the influence of phospholipid-ganglioside organization in mixed monolayers on the Ndase activity are being continued.

Fig. 6 shows the change of surface pressure observed at constant surface area, and using buffers of different pH to abolish or reduce the enzymatic activity. Ndase can adsorb to a lipid-free interface. At pH 8, this produces a maximum increase of surface pressure of 4–6 mN/m (Fig. 6a) and an increase of surface

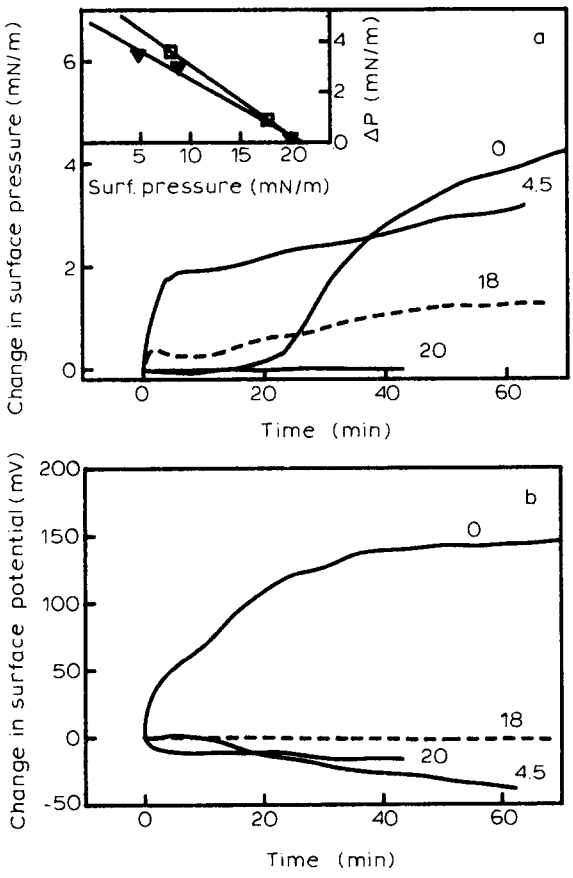


Fig. 6. Change of surface pressure and surface potential induced by Ndase as a function of time. Change in surface pressure (a) and surface potential (b) due to enzyme penetration into a lipid free interface (0 mN/m) or into GM_1 monolayers at the initial surface pressure of 4.5 mN/m, 18 mN/m or 20 mN/m as indicated on the curves. The pH of the subphase was 8 (———) or 4.8 (·····). (Inset) Change of surface pressure upon enzyme penetration in the GM_1 monolayer over a subphase at pH 8 (▼) or pH 4.8 (□) as a function of initial surface pressure. The enzyme concentration was 150 ng/ml.

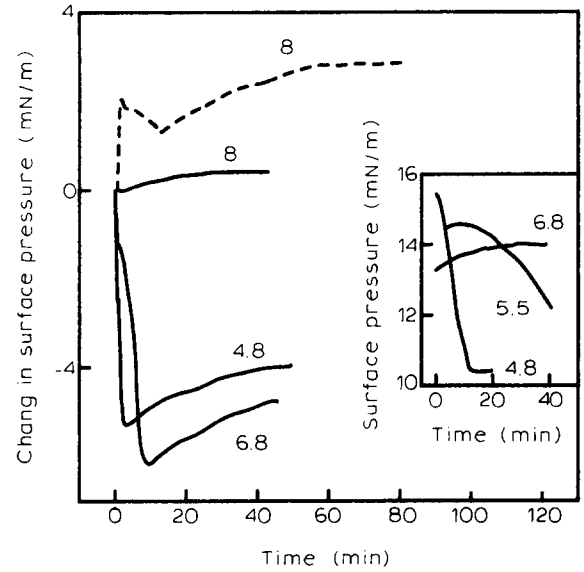


Fig. 7. Penetration of Ndase into monolayers of GD_{1a} at constant surface area. The change in surface pressure as a function of time was determined at an initial surface pressure of 5.8 mN/m (·····) or 21 mN/m (———). The subphase was at pH 8, pH 4.8 or pH 6.8 as indicated on the curves. The enzyme concentration in the subphase was 150 ng/ml. (Inset) Penetration of Ndase, at a low concentration in the subphase (60 ng/ml), into GD_{1a} monolayers at the subphase pH of 6.8, 5.5 and 4.8 as indicated on the curves.

potential of 150 mV (Fig. 6b). Monolayers of GM_1 are not degraded but the enzyme can interact well with this ganglioside (Fig. 6). This is clearly shown by the increase of surface pressure caused by the adsorption and penetration of the enzyme into the ganglioside monolayer at initial surface pressures that are above the maximum pressure caused by adsorption of Ndase to lipid-free interfaces (Fig. 6a). This also indicates that the enzyme acquires a higher surface stability in the presence of ganglioside and, similar to most other proteins [18], the penetration of Ndase decreases when the surface pressure is increased, with an approximate cut-off pressure of 21 mN/m (see inset in Fig. 6a). The penetration of the enzyme at low pressures is slightly higher at pH 4.8 but the cut-off point is similar at pH 8 (inset in Fig. 6a). Contrary to the increase of surface potential caused by the adsorption of *C. perfringens* Ndase to lipid-free interfaces, the interaction with monolayers of ganglioside GM_1 causes the surface potential to decrease by a maximum of about 30–50 mV, depending on the surface pressure and the subphase pH (Fig. 6b).

With GD_{1a} the increase of surface pressure caused by the penetration of Ndase is accompanied by a decrease of surface pressure due to the enzymatic reaction producing a more condensed product (GM_1) at the interface (see Fig. 1). Both phenomena can be dissected by abolishing or reducing the activity of the enzyme using a different subphase pH. Fig. 7 shows

that, at a constant surface pressure of 21 mN/m, the rate of pressure decrease is less at pH 6.8 (where the enzyme is less active) compared to pH 4.8. If the enzyme concentration is kept low enough, it is also possible to detect differences in the duration of the latency period before the onset of constant enzymatic rate. During the latency period, an increase of surface pressure can be observed when the reaction rate is measured at pH 5.5 or above (inset Fig. 7). At pH 8 degradation of GD_{1a} does not occur and only the increase of surface pressure due to penetration is observed. As expected, this is more marked at low than at high surface pressures, with a cut-off point of about 22 mN/m. At pH 4.8 and 6.8, the increase of surface pressure observed after the initial decrease is probably due to the penetration of Ndase into an interface that has become enriched in GM_1 after the maximum amount of GD_{1a} has been degraded (the increase of surface pressure observed in these conditions is similar to that found with GM_1). The variations of surface potential, in conditions of pH where no catalytic reaction occurs, show that the dipolar changes caused by interaction of the enzyme with a monolayer of GD_{1a} are also similar to those observed with GM_1.

With GM_3, the activity of the enzyme is higher than against GD_{1a} and degradation of GM_3 can be observed even at pH 8 (Fig. 8a). Similar to GD_{1a}, when the changes of surface pressure caused by the reaction with GM_3 are measured at low pH values and at constant area the surface pressure decreases after a long latency period. The decrease stops rather abruptly and is followed by a slight but reproducible increase of surface pressure (less than 1.5 mN/m). Fig. 8 also shows the accompanying changes of the surface potential. The surface pressure and surface potential changes are due to enzyme adsorption and the subsequent degradation of GM_3 to LacCer that has a more positive surface potential [19,20]; after the reaction has stopped the changes are similar to those observed with interfaces of pure LacCer (Fig. 8b).

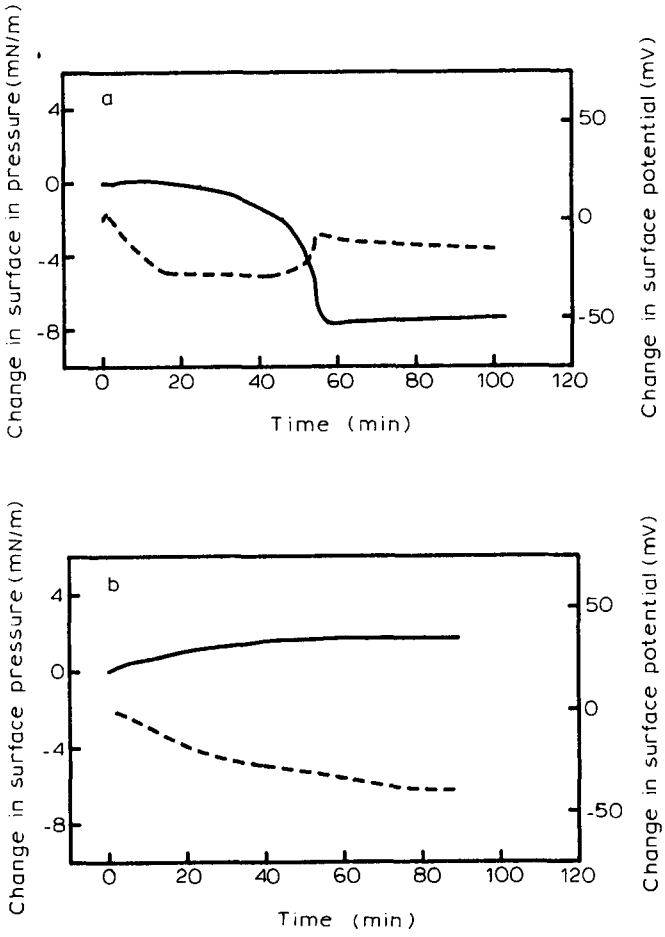


Fig. 8. Penetration of Ndase into monolayers of GM_3 and LacCer. The change in surface pressure (———) and surface potential (·····) as a function of time due to Ndase penetration into monolayers of GM_3 (a) and of LacCer (b) was measured. The subphase was at pH 8 and the enzyme concentration was 150 ng protein/ml. The initial surface pressure was 22 mN/m.

4. Discussion

In natural membranes gangliosides seldom represent more than 10 mol% of the total lipids [21], although they may be enriched in localized regions with respect to the average proportions [22–25]. Since the monolayers employed in our work were 100% ganglioside they should not be taken to represent the behavior of complex interfaces such as natural membranes. However, the dynamic regulation by the ganglioside organization of their own recognition and degradation by neuraminidase under continuously controlled molecular parameters during the catalytic reaction described in this work has revealed the existence of important control mechanisms acting at the supramolecular level on the membrane surface, besides those derived from classical substrate specificity. The activity of Ndase from *C. perfringens* against pure ganglioside monolayers is markedly regulated by the intermolecular organization of each of the ganglioside substrates as determined by the lateral surface pressure. For GD_{1a} the enzymatic activity is generally higher at low than at high surface pressures. For GM_3, the enzymatic activity at the lowest surface pressure studied is also higher than that found at the highest pressures but a broad surface pressure optimum is observed. The minimum pressure at which the activity of Ndase is inhibited is higher by about 5–10 mN/m for GM_3 than for GD_{1a}.

The buffer used in our experiments contained 2 mM Ca^{2+}. It has been shown that Ndases from different sources are, per se, either insensitive or activated by Ca^{2+} [1] and their activity is not influenced by a possible binding of the cation to pure gangliosides [7].

We and others have previously shown that the phase transition temperature of ganglioside GM_3, GM_1 and GD_{1a}, either in individual micelles or incorporated into dipalmitoylphosphatidylcholine bilayers, was shifted slightly upwards by Ca^{2+} above 5 mM [7,22,23,26]. The gangliosides have a relatively low affinity for Ca^{2+} (when their double layer electrostatic potential is properly screened in physiological NaCl solutions [27]) and the presence of up to 20 mM Ca^{2+} does not alter the intermolecular packing areas of gangliosides at all the surface pressures studied [28]. Alterations by Ca^{2+} of the ganglioside molecular areas were described by others [29] but these signify less than 30% of the variation of molecular area that is caused by a change of surface pressure of 5 mN/m. According to our results, a decrease of about 50% of the activity of Ndase against GD_{1a} is caused by an increase of surface pressure from 10 to 15 mN/m. The isotherms in Fig. 1 show that this corresponds to a decrease of about 0.2 nm^2 in the molecular area of GD_{1a}. For GM_3, the reduction of Ndase activity to similar values occurs at 25 mN/m where the decrease in molecular packing form 20 mN/m is about 0.1 nm^2. Thus, the different dependence of Ndase activity on the surface pressure observed for GM_3 and GD_{1a} can not be due to a simple effect of Ca^{2+} on the ganglioside organizational packing. As far as we know the local alterations that Ca^{2+} may induce in the ganglioside polar head group have not been described.

The amount of product formed at the interface also depends on the surface pressure. Thus, not all of the substrate ganglioside available to the enzyme can be degraded. In addition, our results suggest that the kinetics and the extent of the catalytic reaction are regulated independently by the intermolecular packing of gangliosides.

A relatively high rate of enzymatic activity at a particular surface pressure is not necessarily accompanied by a greater extent of the reaction (compare Figs. 3 and 5). On the other hand, the extent of hydrolysis of GM_3 at 5 mN/m reaches only about 58% of the total ganglioside available but it can proceed to 100% at higher surface pressures. This also indicates that even if the enzyme can interact with the product LacCer, this lipid does not affect the continuation of the catalytic process. We can not rule out a small degree of denaturation of Ndase at the surface, especially at low surface pressures. However, we demonstrated that the enzyme associated to the interface is not irreversibly denatured, that it remains in the film after transfer, and that it probably exchanges very slowly with the bulk subphase.

The total amount of GD_{1a} that can be degraded does not exceed about 60% of the total, irrespective of the surface pressure. This effect may be due to changes of the organization of the surface as a whole when it becomes enriched in GM_1 but inhibition caused by irreversible binding of GM_1 to the enzyme active site is not likely: at an enzyme concentration of 30 ng/ml in the subphase, the total amount of GD_{1a} degraded represents more than 100 molecules of GM_1 formed per molecule of enzyme indicating that each enzyme molecule performs many catalytic cycles. It was previously reported that only GM_1 micelles but not the ganglioside oligosaccharide moiety can inhibit *C. perfringens* Ndase [30,31]. This suggests that the surface interaction of Ndase with GM_1 does not directly involve the protein domain responsible for the cleavage of sialic acid, or that this can be rapidly and reversibly dissociated to act on substrate gangliosides.

GM_1 is a poor substrate for the various neuraminidases of viral, bacterial and mammalian origin (cf. [32,33]) but this ganglioside can be degraded in the presence of bile salts. The degradation of GM_1 by *C. perfringens* Ndase was reported to have an absolute requirement for bile salts [32,34] although below the critical micellar concentration (CMC) ganglioside GM_1 is susceptible to degradation in the absence of detergents [35]. Neuraminidase from *A. ureafaciens*, in the absence of detergents, has low but measurable activity against GM_1 and this can increase by more than 800-fold in the presence of cholate [32]. *V. cholerae* Ndase, which in the test tube showed low activity against GM_1 even in the presence of taurocholate, could hydrolyse GM_1 on thin layer chromatographic plates in the absence of detergents [36]. We found that pure monolayers of ganglioside GM_1 are not degraded by *C. perfringens* Ndase in the range of surface pressures studied. However, the enzyme can penetrate interfaces of ganglioside GM_1 at surface pressures that are well above the maximum adsorption pressure of Ndase on lipid-free interfaces. This indicates favorable interactions of the protein with GM_1 that increase the enzyme stability at the interface. This is in keeping with the reported formation of a complex of GM_1 with Ndase (from *V. cholerae*), and the incorporation of ^{125}I-labeled *C. perfringens* Ndase into GM_1 micelles in the presence and absence of cholate [1,6].

The molecular packing, surface potential and charge of ganglioside monolayers are unchanged in the pH range from 3 to 11 [19,20]. On the other hand, the isoelectric point of *C. perfringens* Ndase is 4.7 [37]. Thus, since both the enzyme and the interface are negatively charged at pH values above 4.7, an increase of Ndase penetration into ganglioside monolayers due to simple electrostatic effects is unlikely. The adsorption and penetration of *C. perfringens* Ndase into GM_1 monolayers causes a decrease of the surface potential and this is of opposite sign to the variation of surface potential caused by adsorption of Ndase onto a lipid-free interface. This suggests that the interactions occurring with GM_1 reverse the enzyme dipole moment

contributions, or that the protein remains mostly located below the air-water interfacial plane. With the substrate gangliosides GM_3 and GD_{1a} at optimum pH, penetration occurs prior to or simultaneous with ganglioside degradation and both phenomena can be separated depending on the activity of the enzyme (see Figs. 7 and 8). When the enzymatic activity is progressively decreased, only monolayer penetration is observed. The fact that Ndase can penetrate ganglioside interfaces in the absence of catalysis suggests that domains other than the catalytic site in the protein molecule participate in the initial interfacial recognition event. The non-catalytic interaction of the enzyme with monolayers of GD_{1a} and GM_3 induces decreases of the surface potential similar to those found for the interaction of Ndase with monolayers of LacCer and ganglioside GM_1. This suggests that the overall dipolar changes induced by interaction with and/or location of the protein molecule in the ganglioside interfacial region is similar for the substrates and products of Ndase activity.

A definite lag-time occurs before the onset of a constant rate of enzymatic reaction. In the case of lipolytic enzymes (such as phospholipase A_2 and other lipases) acting on organized substrates, the latency period can often be related to pre-catalytic interfacial steps of adsorption and activation [10–12]. As far as we know a latency period for the degradation of gangliosides by Ndase has not been previously reported and it suggests that activatory steps prior to catalysis are likely to occur at the interface before effective ganglioside degradation takes place.

For GD_{1a} and GM_3, the variation of the reaction lag-time with surface pressure is not correlated in a simple manner with the rate of enzymatic activity nor with the total extent of ganglioside degradation. With GD_{1a} the lag-time remains constant between 5 and 20 mN/m while the reaction rate decreases by 70% and the extent of degradation increases to a maximum of about 60%. With GM_3, when the surface pressure increases from 5 to 20 mN/m the lag-time, the rate of enzymatic activity and the extent of the reaction are all increased. In addition, the increase of the lag-time in the reaction with GM_3 does not impair the degradation of all the ganglioside available. Conversely, a short lag-time for GD_{1a} is not reflected in total degradation. All these observations indicate that the initial pre-catalytic steps of the reaction (i.e., substrate recognition, adsorption, or requirement for reaching an active state of the enzyme at the surface), the rate of ganglioside degradation, and the maximum amount of product formed are regulated by the intermolecular organization of the ganglioside substrate in an independent manner.

Enzyme penetration causes an increase in area and a delay for detection of the onset of pseudo-zero order reaction; conversely, an increase of the enzymatic rate of activity leads to a decrease in area in a short period of time due to rapid accumulation in the interface of product molecules with smaller areas than the substrates. Conceivably, changes of the reaction lag-time could only be an apparent consequence of the resultant effect of two phenomena causing opposite changes in the surface area measured at constant pressure. However, the rates of degradation of GD_{1a} and GM_3 observed at different surface pressures and the cut-off pressure of about 21 mN/m for enzyme penetration into the ganglioside interface (similar for GD_{1a}, GM_3 and GM_1) also have to be taken into account. On these basis it can be calculated that, at pH 4.8, the sole interplay of differences in penetration and rate of catalysis should cause the latency period to exhibit a decrease near 20 mN/m for GD_{1a} (no penetration, low activity) and a marked minimum between 15 and 20 mN/m for GM_3 (no penetration, high enzymatic activity). The experimental results are clearly contrary to this and it is not possible to ascribe the observed increase of the lag-times with the surface pressure to simple competition between catalytic rate and penetration. In addition, the decrease of the latency period that occurs when the enzyme concentration increases in the subphase (inset in Fig. 3a) suggests that neuraminidase activity is facilitated when an increased number of enzyme molecules become associated to the interface. This may be due to facilitation of activity against a lipid interface containing defined proportions of protein, to increased catalysis aided by initial formation of a small amount of products that may enhance reversible enzyme binding, or by increased activity due to a surface activation of Ndase.

Several evidences have suggested that the rate of ganglioside hydrolysis by neuraminidases may be modulated by changes of the membrane physical state as well as by changes in the aggregation of gangliosides in the membrane [7,8]. Our results indicate directly that, even against interfaces constituted by pure gangliosides, neuraminidase activity depends markedly on the substrate organization and is higher at lower surface pressures which corresponds to more loosely packed ganglioside molecules. This is in agreement with reports of other authors indicating a higher activity of *V. cholerae* neuraminidase against gangliosides that are dispersed rather than aggregated along the membrane plane [7,8], and with an enhancement of degradation caused by agents that increase membrane fluidity [38]. In brain microsomal membranes *V. cholerae* Ndase did not randomly degrade GD_{1a} but preferentially hydrolysed the species containing the 20:1 long-chain base, presumably more dispersed in the membrane, compared to that having the 18:1 base [24]. On the other hand, the Ndase used in this work can interact strongly with GM_1 without leading to ganglioside degradation.

This is in keeping with the idea that a neuraminidase endogenous to myelin [2,39] may have an important role in establishing the ganglioside pattern in myelin and strengthen membrane-membrane interactions through recognition and binding to GM_1 [3,21,40].

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References

[1] Corfield, A.P., Michalski, J.C. and Schauer, R. (1981) in Perspectives in Inherited Metabolic Diseases, Vol. 4, Sialidases and sialidoses (Tettamanti, G., Durand, P. and Di Donato, S., eds.), pp. 3–70, Ermes, Milano.
[2] Yohe, H.C., Saito, M., Ledeen, R.W., Kunishita, T., Sclafani, J.R. and Yu, R.K. (1986) J. Neurochem. 46, 623–629.
[3] Saito, M. and Yu, R.K. (1992) J. Neurochem. 58, 83–87.
[4] Barton, N.W. and Rosenberg, A. (1973) J. Biol. Chem. 248, 7353–7358.
[5] Noble, E.P., Syapin, P.J., Vigran, R. and Rosenberg, A. (1976) J. Neurochem. 27, 217–221.
[6] Cestaro, B., Cervato, G. and Tettamanti, G. (1978) Bull. Mol. Biol. Med. 3, 159–169.
[7] Myers, M., Wortman, C. and Freire, E. (1984) Biochemistry 23, 1442–1448.
[8] Masserini, M., Palestini, P., Venerando, B., Fiorilli, A., Acquotti, D. and Tettamanti, G. (1988) Biochemistry 27, 7973–7978.
[9] Brockman, H.L. (1984) in Lipases (Bergstrom, B. and Brockman, H.L., eds.), pp. 4–46, Elsevier, Amsterdam.
[10] Jain, M.K. and Berg, O. (1989) Biochim. Biophys. Acta 1002, 127–156.
[11] Ransac, S., Moreau, H., Riviere, C. and Verger, R. (1991) Methods Enzymol. 197, 49–65.
[12] Bell, J.D. and Biltonen, R.L. (1992) J. Biol. Chem. 267, 11046–11056.
[13] Fidelio, G.D., Ariga, T. and Maggio, B. (1991) J. Biochem. 110, 12–16.
[14] Macala, L.J., Yu, R.K. and Ando, S. (1983) J. Lipid Res. 24, 1243–1250.
[15] Bianco, I.D. and Maggio, B. (1989) Colloids Surfaces 35, 97–99.
[16] Perillo, M.A., Polo, A., Guidotti, A., Costa, E. and Maggio, B. (1993) Chem. Phys. Lipids 65, 225–238.
[17] Bianco, I.D., Fidelio, G.D. and Maggio, B. (1989) Biochem. J. 258, 95–99.
[18] Fidelio, G.D., Maggio, B. and Cumar, F.A. (1986) Anal. Asoc. Quim. Arg. 74, 801–813.
[19] Maggio, B., Cumar, F.A. and Caputto, R. (1978) Biochem. J. 171, 559–565.
[20] Maggio, B. (1992) in Charge and Field Effects in Biosystems-3 (Allen, M.J., Cleary, S.E., Sowers, A.E. and Shillady, D.D., eds.), pp. 69–80, Birkhäuser, Boston.
[21] Yu, R.K., Sato, S., and Saito, M. (1990) in NATO ASI Series, Vol. H43, Cellular and Molecular Biology of Myelination (Jesserich, G., Althaus, H.H. and Waehneldt, T.V., eds.), pp. 391–404, Springer-Verlag, New York.
[22] Maggio, B., Ariga, T., Sturtevant J.M. and Yu, R.K. (1985) Biochim. Biophys. Acta 818, 1–12.
[23] Maggio, B., Sturtevant, J.M. and Yu, R.K. (1987) Biochim. Biophys. Acta 901, 173–182.
[24] Palestini, P., Masserini, M., Fiorilli, A., Calappi, E. and Tettamanti, G. (1991) J. Neurochem. 57, 748–753.
[25] Maggio, B., Montich, G.G. and Cumar, F.A. (1988) Chem. Phys. Lipids 46, 137–146.
[26] Masserini, M., Palestini, P. and Freire, E. (1989) Biochemistry 28, 5039–5034.
[27] McDaniel,R and McLaughlin, S. (1984) Biochim. Biophys. Acta 819, 153–160.
[28] Maggio, B., Cumar, F.A. and Caputto, R. (1980) Biochem. J. 189, 435–440.
[29] Rahmann, H., Schifferer, F. and Beitinger, H. (1992) Neurochem. Int. 20, 323–338.
[30] Corfield, A.P., Schaver, R., Schwarzmann, G. and Wiegandt, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. 301, 231–231.
[31] Veh, R.W., and Schaver, R. (1978) Adv. Exp. Med. Biol. 101, 447–462.
[32] Saito, M., Sugano, K. and Nagai, Y. (1979) J. Biol. Chem. 254, 7845–7854.
[33] Miyagi, T. and Tsuiki, S. (1986) FEBS Lett. 206, 223–228.
[34] Wenger, D.A. and Wardell, S. (1973) J. Neurochem. 20, 607–612.
[35] Rauvala, H. (1976) FEBS Lett. 65, 229–233.
[36] Portoukalian, J. and Jouchon, B. (1986) J. Chromatogr. 380, 386–392.
[37] Bouwstra, J.B., Deyl, C.M. and Vliegenthart, F.G. (1987) Biol. Chem. Hoppe-Seyler 368, 269–375.
[38] Sandhoff, K., Scheel, G and Nehrkorn, H. (1981) in Perspectives in inherited metabolic diseases, Vol. 4, Sialidases and sialidoses (Tettamanti, G., Durand, P. and Di Donato, S., eds.), pp. 125–173, Ermes, Milano.
[39] Saito, M. and Yu, R.K. (1986) J. Neurochem. 47, 632–641.
[40] Saito, M. and Yu, R.K. (1992) J. Neurochem. 58, 83–87.
[41] Sekine, M., Ariga, T., Miyatake, T., Kuroda, Y., Suzuki, A. and Yamakawa, T. (1984) J. Biochem. 95, 155–160.