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STUDIES ON BRAIN MEMBRANE-BOUND NEURAMINIDASE

II. EFFECT OF DETERGENTS ON THE KINETICS OF THE ENZYME PREPARED FROM CALF BRAIN

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SUMMARY

1. The influence of various detergents on the kinetics of membrane-bound neuraminidase acting on gangliosides (amphipatic substances) was studied. The enzyme was prepared from calf brain and disialoganglioside GD1a was used as substrate.

2. In the absence of detergents the enzymatic hydrolysis of GD1a follows hyperbolic kinetics with a maximum rate reached at a substrate concentration close to the critical micellar concentration, 0.1 mM.

3. In the presence of sodium dodecylsulphate, cholate, deoxycholate and Triton X-100 the hyperbolic kinetics maintained with the maximum rate still reached at 0.1 mM GD1a. These detergents behave as non-competitive inhibitors although Triton X-100, at low concentrations, exhibits an activating effect.

4. Triton QS-31 and Lubrol WX cause a specific change of the enzyme kinetics. With Triton QS-31 the $v/[S]$ relationship shifts to a double-shouldered curve with a transition around 0.07–0.08 mM GD1a. With Lubrol WX the $v/[S]$ curve is sigmoidal with transition around 0.3 mM and a maximum rate at 1.0 mM GD1a. In the case of Ttriton QS-31 the addition of albumin restores the hyperbolic kinetics.

5. Under our experimental conditions, brain membrane-bound neuraminidase appears to act on the monomeric form of the gangliosidic substrate. In the presence of Triton QS-31 the enzyme apparently gains the capacity to work on the micellar form of substrate, while with Lubrol WX it recognizes and affects only the micellar form of substrate.

INTRODUCTION

It has already been reported that the neuronal plasma membranes are rich in sialoglycolipids (gangliosides) [1–3] and in sialidase (mucopolysaccharide *N*-acetyl-

Abbreviations: The ganglioside nomenclature follows Svennerholm's [14] suggestions: GD1a, AcNeu-α2-3Galβ1-3GalNAc(AcNeu-α2-3)Galβ1 4Glcβ1-1Cer; AcNeu, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; Cer, ceramide; CMC, critical micellar concentration.

neuraminylhydrolase, EC 3.2.1.18) [3, 4], which suggests the hypothesis that the two parameters are connected with, and possibly related to, a specialized brain function.

The above hypothesis could be strongly substantiated by a knowledge of the mechanism of the hydrolysis of gangliosides by membrane-bound neuraminidase. The experimental approach to this subject is particularly difficult because of the particulate nature of the enzyme [5–7] and the ability of gangliosides to undergo phase transition from monomer to micelle [8, 9].

Latest findings on bacterial neuraminidases [10, 11] suggest that a solution to this problem should be looked for with the proper use of detergents.

Various authors [5, 6, 12, 13] have already demonstrated an activating effect of Triton X-100 and less definite influences by other detergents, on the activity of brain particulate neuraminidase. However, all these studies, conducted at a fixed substrate concentration, are not suitable (nor was this their purpose) for inspecting the kinetics of the enzyme action.

The present investigation on the effect of non-ionic and anionic detergents on membrane-bound neuraminidase activity was carried on at different concentrations of the gangliosidic substrate (disialoganglioside GD1a). When useful for comparative purposes, sialyllactose was also employed as a substrate. An enzyme preparation obtained from calf brain by the method previously reported [15] was employed.

MATERIALS

Commercial chemicals were of analytical or of the highest available grade. Triton X-100, Triton X-305, Triton QS-5, Triton QS-55, Triton QS-31, Triton CF-34 and Triton DF-18 were obtained from Haas and Rohme through the Italian dealer (Nymco, Milan); sodium dodecylsulphate, sodium cholate, sodium deoxycholate, *N*-acetylneuraminic acid (AcNeu) and cristalline bovine serum albumin were from Sigma Chem. Co. Tween 20, Tween 40, Tween 60, Tween 80 were from Merck GmbH and Lubrol WX was from General Biochemicals.

Ganglioside GD1a was prepared (purity: 96%) from beef brain according to Tettamanti and Zambotti [16].

Sialyllactose, the C-3 isomer, was isolated from 1-day cow colostrum according to Ohman and Hygstedt [17].

All the detergents, ganglioside GD1a and sialyllactose were put into an aqueous solution just before use.

Dowex 2-X8 resin (200–400 mesh; Dow Chem. Co) was prepared in the acetate form according to Svennerholm [18].

Calves, average age of 4 months, were employed. The brains were processed as described by Preti et al. [15]. The homogenizations and centrifugations were carried out as described by Tettamanti et al. [7].

The incubations were performed in a shaking incubator (Colora). Double glass-distilled water was used for preparing the different solutions.

All operations were conducted at 0–4 °C unless otherwise stated.

METHODS

Analytical methods

The preparation of crude membrane-bound neuraminidase was carried out

according to Preti et al [15]. Protein was determined by the method of Lowry et al. [19], bovine serum albumin being used as the standard. The AcNeu content of ganglioside GD1a and sialyllactose was established by Svennerholm's method [18]. Free AcNeu was determined by Warren's method [20].

Enzyme assay

The incubation mixtures contained, in a total volume of 0.65 ml, 0.5–1.0 mg enzyme (as protein), 0.15 M sodium acetate buffer, definite amounts of ganglioside GD1a and of detergent, and had a final pH of 4.0. When using sialyllactose as substrate, 0.025 M sodium citrate–phosphate buffer was employed, the final pH being 3.3. The incubation mixtures, set up at the temperature of melting ice, were immersed in the incubating shaker and incubated at 37 °C for 10 min (ganglioside GD1a) or 20 min (sialyllactose). The determination of liberated AcNeu, the calculation of enzyme activity and the setting up of the appropriate blanks (with 15 min boiled enzyme) were carried out as described in previous papers [21, 15]. When used at concentrations above 1%, most of the detergents employed interfered in the reaction for the assay of AcNeu. In these cases, AcNeu, prior to the assay, was purified on Dowex 2-X8 column according to Preti et al. [21]. While studying the influence of the various detergents on the enzyme activity, the effect of added albumin was also examined, following the suggestions of Gatt et al. [22].

The V and K_m values were calculated from the $1/v$ over $1/[S]$ plot according to Lineweaver and Burk [23]. One unit of neuraminidase is the amount of enzyme liberating 1 nmole AcNeu/min under the given conditions.

RESULTS

The detergents used were: Triton X-100, X-305, CF-34, QS-5, QS-55, QS-31, DF-18, sodium cholate, sodium deoxycholate, sodium dodecylsulphate, Tween 20, 40, 60 and 80 and Lubrol WX.

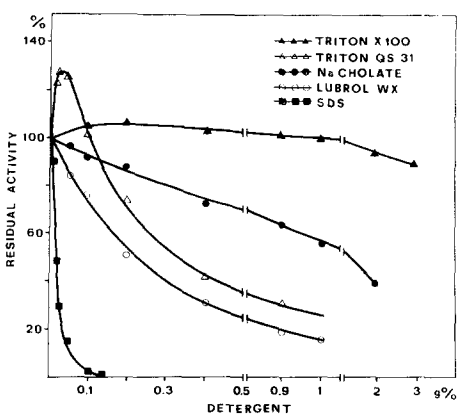

Fig. 1. Effect of increasing concentrations of various detergents on the activity of calf brain membrane-bound neuraminidase. Substrate: disialoganglioside GD1a, used at the saturating concentration established in the absence of detergent (0.15 mM). Enzymatic protein: 680 μg. The assay conditions, described in Methods, were followed. The data shown are the average of 4 experiments. SDS: sodium dodecylsulphate.

Pilot experiments, conducted at a fixed substrate concentration (0.15 mM ganglioside GD1a) and at increasing detergent concentration, showed that all the above detergents, except Triton QS-31, provided only an inhibitory effect on the activity of brain membrane-bound neuraminidase (see the most significant results in Fig. 1 and Fig. 2). Conversely, Triton QS-31 was also able to exhibit a substantial activation (about 25%).

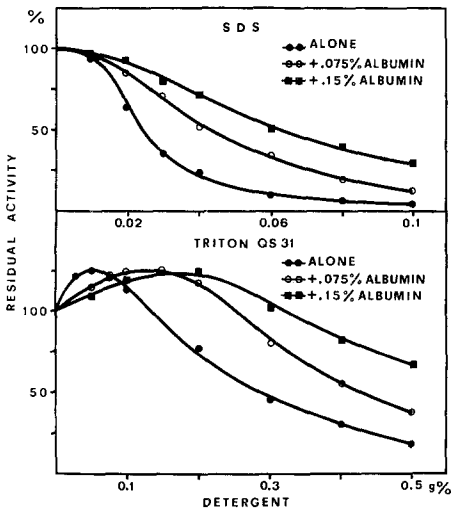

Fig. 2. Effect of increasing concentrations of sodium dodecylsulphate (SDS) and of Triton QS-31 on the activity of calf brain membrane-bound neuraminidase in the presence of added albumin. Substrate: disialoganglioside GD1a used at the saturating concentration established in the absence of detergent (0.15 mM). Enzymatic protein: 700 μg. The assay conditions described in Methods were followed. The data shown are the average of 4 experiments.

The graphs reproduced in Fig. 2 (concerning the effect of sodium dodecylsulphate and Triton QS-31), show that the addition of albumin caused a shifting of the inhibition (or activating effect, in the case of Triton QS-31) toward higher detergent concentrations. This clearly indicates that it is the ratio of detergent to total protein which is critical in determining the effect on the enzyme activity, rather than the absolute concentration of detergent. Similar results were obtained with the other detergents.

On the basis of the above findings we studied the influence of Triton X-100, Triton QS-31, sodium cholate, sodium dodecylsulphate and Lubrol WX on the reaction rate of membrane-bound neuraminidase at different substrate concentrations.

With sodium dodecylsulphate (Fig. 3) and sodium cholate (Fig. 4) the $v/[S]$ relationship at different detergent concentrations followed a hyperbolic curve and gave a straight line when plotted according to Lineweaver and Burk [23]. In this latter representation, the intercept on the abscissa was the same as in the absence of detergent (identical K_m values). The addition of albumin, which partially removed the inhibiting effect of both detergents, maintained, in both cases, the hyperbolic shape of the $v/[S]$ curves and the K_m values.

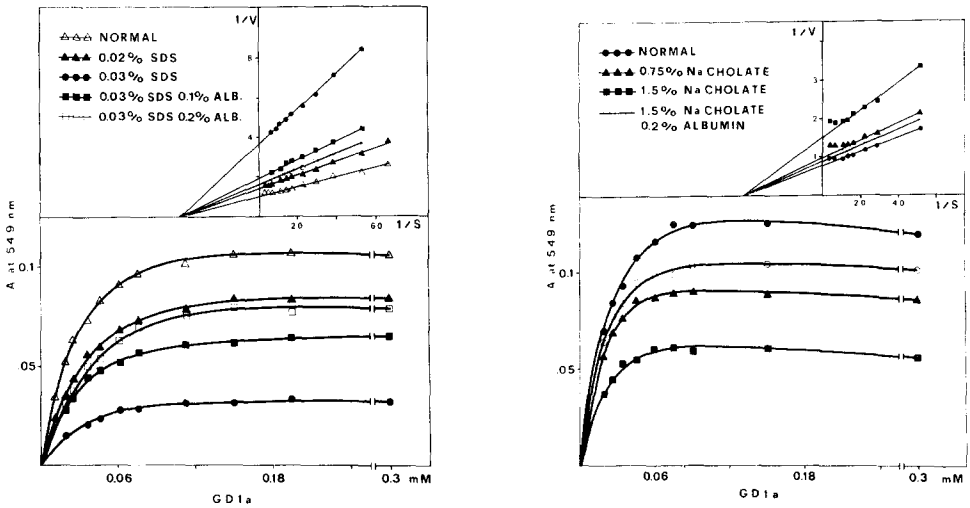

Fig. 3. Effect of sodium dodecylsulphate (SDS) on the activity of calf brain membrane-bound neuraminidase at different concentrations of the ganglioside substrate (disialoganglioside GD1a). The effect of detergent was studied in the absence and presence of different amounts of albumin. The assay conditions, described in Methods, were followed. Enzymatic protein: 646 μg. The data shown are the average of 4 experiments.

Fig. 4. Effect of sodium cholate on the activity of calf brain membrane-bound neuraminidase at different concentrations of the ganglioside substrate (disialoganglioside GD1a). The effect of detergent was studied in the absence and presence of albumin (at optimum concentration). The assay conditions, described in Methods, were followed. Enzymatic protein: 770 μg. The data shown are the average of 4 experiments.

Triton X-100 (Fig. 5) at low detergent/protein ratios exhibited a slight activation of the hydrolysis rate of ganglioside GD1a, with a significant diminution of the K_m value for the same ganglioside (from $2.1 \cdot 10^{-5}$ M in the absence of detergent, to 0.77 and $0.83 \cdot 10^{-5}$ M, respectively, at 0.1% and 0.3% detergent concentration).

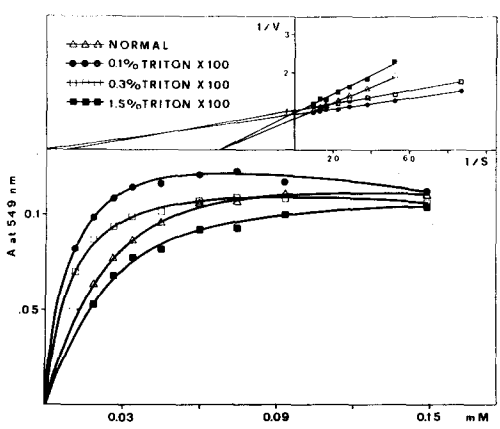

Fig. 5. Effect of Triton X-100 on the activity of calf brain membrane-bound neuraminidase at different concentrations of the ganglioside substrate (disialoganglioside GD1a). The assay conditions, described in Methods, were followed. Enzymatic protein: 690 μg. The data shown are the average of 5 experiments.

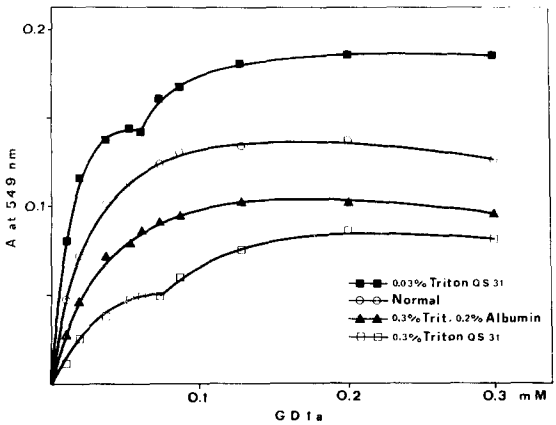

Fig. 6. Effect of Triton QS-31 on the activity of calf brain membrane-bound neuraminidase at different concentrations of the ganglioside substrate (disialoganglioside GD1a). The effect of detergent was studied in the absence and presence of albumin (at optimum concentration). The assay conditions are those described in Methods. Enzymatic protein: 846 μg. The data shown are the average of 6 experiments.

Conversely, at high detergent/protein ratios, Triton X-100 caused diminution of the V with maintenance of the K_m value (non-competitive inhibition).

Triton QS-31 (Fig. 6) exibited, as reported above, either an activating or an inhibiting effect depending on the concentration. However, in both cases the $v/[S]$ curve showed a reproducible double shoulder with a transition at about 0.07–0.08 mM ganglioside GD1a. Albumin (optimum concentration of 0.2%), added to the mixture containing 0.3% Triton QS-31, had a double effect: (a) the conversion of the $v/[S]$ relationship from the double-shoulder shaped curve to a hyperbola (with linear Lineweaver and Burk plot and unchanged K_m value); (b) the partial removal, as expected, of the inhibiting effect caused by detergent.

The graphs reported in Fig. 7 show shat, using sialyllactose as substrate, the kind of kinetics (hyperbolic) was not influenced by the presence of Triton QS-31.

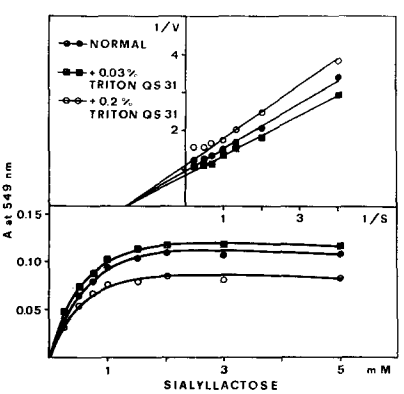

Fig. 7. Effect of Triton QS-31 on the activity of calf brain membrane-bound neuraminidase at different concentrations of sialyllactose (C-3 isomer). The assay conditions are those reported in Methods. Enzymatic protein: 620 μg. The data shown are the average of 6 experiments.

In this case, Triton QS-31 did not change the enzyme affinity, (the K_m value, $0.68 \cdot 10^{-3}$ M in the absence of detergent, remained unmodified) while slightly affecting the V (1.18 units/mg protein in the absence of detergent; 1.30 and 0.84 units/mg protein in the presence of 0.03% and 0.2% detergent) thus reproducing the activating and inhibiting effect observed on ganglioside.

In the presence of Lubrol WX (0.5%) the $v/[S]$ curve for ganglioside GD1a was sigmoidal (see Fig. 8) with a transition around 0.3 mM substrate. The maximum

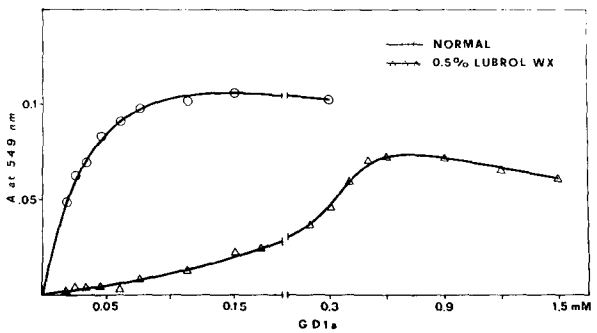

Fig. 8. Effect of Lubrol WX on the activity of calf brain membrane-bound neuraminidase at different concentrations of the ganglioside substrate (disialoganglioside GD1a). The assay conditions, described in Methods, were followed. Enzymatic protein: 640 μg. The data shown are the average of 5 experiments.

activity was only 70% of that measured in the absence of detergent and was reached at a very high substrate concentration: 1.0 mM. The addition of albumin, up to 0.2%, did not modify either the kind of kinetics or the partial inhibition by Lubrol WX.

When sialyllactose was used as substrate (Fig. 9) the presence of Lubrol WX maintained the hyperbolic behaviour of the $v/[S]$ relationship and caused only a slight diminution of the V (from 1.18 to 1.03 units/mg protein); the K_m value remained unchanged (non-competitive inhibition).

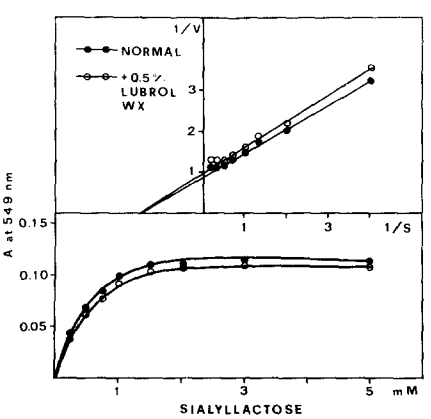

Fig. 9. Effect of Lubrol WX on the activity of calf brain membrane-bound neuraminidase at different concentrations of sialyllactose (C-3 isomer). The assay conditions, described in Methods, were followed. Enzymatic protein: 746 μg. The data shown are the average of 5 experiments.

DISCUSSION

Brain membrane-bound neuraminidase is a firmly bound particulate enzyme [3, 5, 6, 7, 12]. Gangliosides, the putative physiological substrates of neuraminidase, are amphipatic substances, existing as monomers below the critical micellar concentration, and as monomers and micelles above the critical micellar concentration [8, 9]. The nature of the interaction between gangliosides and neuraminidase, as well as the enzyme capacity to recognize the monomeric and/or micellar forms of gangliosides are not known.

We attempted, on the basis of reported evidence [10, 11], to obtain meaningful information on this matter by following the modifications of the enzyme kinetics caused by detergents.

In a system like ours, detergents can be expected to act: (a) at the level of the enzyme molecule changing its conformation or arrangement within the membrane; (b) at the level of substrate modifying the phase transition from monomer to micelle and causing the formation of mixed micelles (ganglioside plus detergent). Either ways the catalytic attitude of the enzyme or the availability of the substrate to the enzyme may change.

The situation in our system, in the absence of detergents, is as follows. The $v/[S]$ curve of the enzyme with ganglioside GD1a is hyperbolic and the maximum velocity is reached around 0.1 mM ganglioside GD1a concentration. Then, above 0.15 mM, the enzyme activity drops. Considering that the critical micellar concentration of ganglioside GD1a is 0.11 mM, as recently established by Gatt and coworkers (personal communication), it can be concluded that membrane-bound neuraminidase acts on the monomeric form of the ganglioside substrate with kinetics following the Michaelis–Menten theory of enzyme-substrate interaction. Moreover, the inhibition above a 0.15 mM substrate concentration may be due to the presence of the micellar form of substrate, as already suggested by Ohman et al. [6].

Our experiments showed that the addition of detergents to the system causes either the modification of the reaction rate without any change of the kind of kinetics, or a change of the kind of kinetics. The first situation is provided by sodium dodecylsulphate, sodium cholate (and deoxycholate) and Triton X-100, and the second one by Triton QS-31 and Lubrol WX.

Sodium dodecylsulphate and sodium cholate, causing a decrease of the V with no change of the K_m values, behave clearly as non-competitive inhibitors. The addition of albumin restores the enzyme activity, even if incompletely. This means that albumin competes with the enzyme molecule for the detergent, the enzyme being partly removed from the complex with detergent and made available for activity.

Triton X-100 provides a double effect depending on the detergent/protein ratio. At high ratios it inhibits non-competitively, together with sodium dodecylsulphate and sodium cholate; at low ratios it causes a significant diminution of the K_m value. Evidently, in this latter condition the enzyme gains in affinity, and, to a lesser extent, in activity towards the gangliosidic substrate.

With all the above-mentioned detergents, the enzyme kinetics are hyperbolic and the maximum activity is still reached around 0.1 mM substrate concentration. In other words, the enzyme continues to work on the monomeric form of the ganglioside substrate following the classical kinetics. Therefore, in these cases, the detergent

should be assumed to act at the level of the enzyme molecule or of its interactions with the other membrane components, rather than at the level of the physical state of substrate.

When membrane-bound neuraminidase acts on ganglioside GD1a in the presence of Triton QS-31 the $v/[S]$ curve shifts from the original hyperbola to a double shoulder curve showing a transition around 0.07–0.08 mM ganglioside concentration, that is in the range of the critical micellar concentration. Of note is the fact that if the substrate is sialyllactose the kinetics remain hyperbolic. This means that Triton QS-31 changes specifically the nature of the interaction between membrane-bound neuraminidase and the gangliosidic substrate. A possible interpretation of the phenomenon is that the enzyme, in the presence of Triton QS-31, is able to recognize either the monomeric and the micellar form of the amphipatic substrate and acts on both of them. It may also be that Triton QS-31 forms, with gangliosides, mixed micelles which behave as a good substrate for the enzyme. The addition of albumin restores the hyperbolic behaviour of the $v/[S]$, that is, cancels the capacity of the enzyme to act on the micellar form of the substrate. A further effect of Triton QS-31, which makes the matter still more complex, is its ability to activate or inhibit the enzyme. These effects are obtained either with ganglioside GD1a (with the "double shoulder" kinetics) or with sialyllactose (with hyperbolic kinetics).

With Lubrol WX the situation changes further. At relatively high detergent/protein ratios, the ability of the enzyme to act at low concentrations of ganglioside dramatically falls and only above 0.1 mM ganglioside concentration does the enzyme start acting and reaches, at 1.0 mM substrate concentration, the maximum activity, which is only 70% of that recorded in the absence of detergent. The kinetics become clearly sigmoidal. When using sialyllactose the effect of detergent is very modest and of the type of a non-competitive inhibition, with hyperbolic kinetics. Thus, also in the case of Lubrol WX, the detergent specifically affects the nature of the interaction between membrane-bound neuraminidase and ganglioside. The enzyme, apparently, does not recognize the monomeric form of the gangliosidic substrate, while acting on the micelle, which may be of the mixed, ganglioside-detergent, type.

However, it cannot be excluded that, in the presence of Lubrol WX, ganglioside is largely involved in the formation of mixed micelles with detergent. Therefore the enzyme still acts on the monomeric form of the ganglioside but the concentration of monomer becomes appreciable only at very high levels of "total" ganglioside.

We are fully aware that our line of interpretation is hypothetical and requires further evidence. However we are convinced that this approach, properly perfected, would be useful for studying the interactions between neuraminidase, gangliosides and the various other lipids, putative physiological detergents, present in the neuronal membranes.

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