Biochimica et Biophysica Acta, 350 (1974) 406–414

BBA 67245

STUDIES ON BRAIN MEMBRANE-BOUND NEURAMINIDASE

I. GENERAL PROPERTIES OF THE ENZYME PREPARED FROM CALF BRAIN

AUGUSTO PRETI, ADRIANA LOMBARDO, BENVENUTO CESTARO, SANDRO ZAMBOTTI and GUIDO TETTAMANTI

Department of Biological Chemistry, Medical School, University of Milan, Milan, 20133 (Italy)

(Received December 27th, 1973)

SUMMARY

1. A crude preparation of membrane-bound neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18), obtained from calf brain, was used. This preparation was free from the soluble and lysosomal neuraminidases and depleted of endogenous substrates.

2. The enzyme showed: (a) optimum pH 4.0 for gangliosides GD1a, GD1b, GT1b and GQ1; 3.8 for ovine submaxillary mucin, ovine submaxillary mucin-sialoglycopeptides and brain sialoglycopeptides; 3.1 for sialyllactose (C-3 isomer); (b) apparent higher affinity for gangliosides (K_m of the order 10^{-5} M) than for sialyllactose (K_m $0.68 \cdot 10^{-3}$ M), ovine submaxillary mucin and sialoglycopeptides; (c) higher V for gangliosides (1.26–2.12 units/mg protein), lower for sialyllactose (1.18 units/mg protein) and sialoglycoprotein substrates (0.27–0.41 units/mg protein); (d) inhibition by excess substrate (over 0.15–0.2 mM) only in the case of gangliosides; (e) maximum rate of hydrolysis of gangliosides at 70 °C (24 units/mg protein in the case of ganglioside GD1a); (f) considerable stability.

3. Na^+ and Li^+ did not influence the enzyme activity; K^+ activated below 0.1 M; NH_4^+ started inhibiting at 0.01 M. All bivalent cations tested inhibited the enzyme: Hg^{2+} from 10^{-6} M, Cu^{2+} from 10^{-5} M, Ca^{2+} from 10^{-3} M. Anions had no appreciable influence on the enzyme activity, at concentrations up to $5 \cdot 10^{-2}$ M.

INTRODUCTION

Two particulate neuraminidases (mucopolysaccharide *N*-acetylneuraminyl-

Abbreviations: The formulas of the used gangliosides, named according to Svennerholm [9], are the following: ganglioside GM3, AcNeuα2-3Galβ1-4Glcβ1-1Cer; ganglioside GM2, GalNAcβ1-4(AcNeuα2-3)Galβ1-4Glcβ1-1Cer; ganglioside GM1, Galβ1-3GalNAcβ1-4(AcNeuα2-3)Galβ1-4Glcβ1-1Cer; ganglioside GD1a, AcNeuα2-3Galβ1-3GalNAcβ1-4(AcNeuα2-3)Galβ1-4Glcβ1-1Cer; ganglioside GD1b, Galβ1-3GalNAcβ1-4(AcNeuα2-8AcNeuα2-3)Galβ1-4Glcβ1-1Cer; ganglioside GT1b, AcNeuα2-3Galβ1-3GalNAcβ1-4(AcNeuα2-8AcNeuα2-3)Galβ1-4Glcβ1-1Cer; ganglioside GQ1, AcNeuα2-8AcNeuα2-3Galβ1-3GalNAcβ1-4(AcNeuα2-8AcNeuα2-3)Galβ1-4Glcβ1-1Cer; AcNeu, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; Cer, ceramide.

hydrolase, EC 3.2.1.18) are currently reported to be present in the brain [1–5]. The first one affects chiefly (if not specifically) multisialogangliosides, but is not active on hexosamine-containing monosialogangliosides. This enzyme is linked to synaptosomal plasma membranes [6] and also to neuronal plasma membranes with different cytological localization ("membrane-bound neuraminidase") [7]. The second enzyme, localized in the lysosomes, is able to hydrolyze the above mentioned monosialogangliosides and seems to be very labile ("lysosomal neuraminidase") [5, 8].

It seems reasonable to assume that the membrane-bound and the lysosomal neuraminidases play different physiological roles. However, it is difficult to formulate a meaningful hypothesis on which role each has. This is mainly due to the lack of knowledge on the properties, substrate specificity, substrate interaction and kinetics of the two enzymes and on the metabolic association of the two enzymes with the several sialo-derivatives present in the brain.

A systematic study on the properties and kinetics of brain neuraminidases, starting from the membrane-bound enzyme, has therefore been undertaken in our laboratory.

In this paper the general properties of brain membrane-bound neuraminidase, prepared from calf brain, are described.

MATERIALS

Commercial chemicals were of analytical or of the highest available grade. *N*-acetylneuraminic acid (AcNeu), sodium cholate, Triton X-100 and crystalline bovine serum albumin were obtained from Sigma Chem. Co., and pronase from Merck GmbH.

Gangliosides GM3, GM2, GM1, GD1a, GD1b, GT1b and GQ1 were prepared from beef brain according to Tettamanti and Zambotti [10] (purity higher than 95%). Ovine submaxillary mucin ("major") was prepared according to Tettamanti and Pigman [11]. Ovine submaxillary mucin-glycopeptides were obtained by pronase treatment following the method of Carubelli et al. [12]. Brain sialoglycopeptides were prepared, after pronase digestion, according to Roukema and Heijlman [3]. Sialyllactose (C-3 isomer) was isolated from 1 day cow colostrum according to Ohman and Hygstedt [13].

Precoated silica gel thin layer plates and silica gel (0.05–0.2 mm 60–325 mesh ASTM), were purchased from Merck GmbH; Dowex 2-X8 resin (200–400 mesh; Dow Chem. Co.) was prepared in acetate form according to Svennerholm [14]. Dialysis tubing (0.25 inch width) was purchased from A. Thomas Co.

The brains of calves (average age 4 months) were removed at the slaughter house within 20 min after sacrifice and processed within the next 30 min. They were freed as much as possible of meninges and white matter, weighed, washed in ice-cold homogenizing solution and homogenized as described below. The homogenizations and centrifugations were carried out as described in a previous work (Tettamanti et al. [4]). The incubations were performed in a shaking incubator (Colora). The temperature inside the incubation mixtures was measured by a telethermometer YSI (model 43 TC) provided with a proper thermistor probe. Double glass-distilled water was used for preparing the different solutions. All operations were conducted at 0–4 °C unless otherwise stated.

METHODS

Preparation of the enzyme devoid of endogenous substrates

The crude preparation of membrane-bound neuraminidase, that is the 105 000 $\times$ *g* (1 h) sediment, was prepared from the homogenate of brain in buffered 0.32 M sucrose according to Preti et al. [15]. The removal of endogenous (intrinsic) substrates was attained by incubating the crude enzyme preparation, homogeneously suspended in distilled water (4 ml/g fresh starting tissue), in 0.15 M sodium acetate buffer, final pH 4.2, in the presence of Triton X-100 (0.3% final concentration) at 25 °C for 5 h (Tettamanti et al. [4]). The mixture was then centrifuged at 105 000 $\times$ *g* for 1 h. The pellet, suspended in distilled water (4 ml/g fresh starting tissue) was the enzyme preparation used for subsequent assays.

Enzyme assay

The incubation mixtures contained 0.5–1.0 mg enzyme (as protein), 0.15 M sodium acetate buffer, in a final volume of 0.65 ml. All the substrates were added in an aqueous solution. The pH of the reaction mixtures and the incubation time varied according to the assay purpose. The incubation mixtures, set up at the temperature of melting-ice, were immersed in the shaking incubator (adjusted to the desired temperature) and the incubation started.

The termination of the enzyme reaction, the direct determination of liberated AcNeu (Warren's method [16]) purified, if necessary, on Dowex 2-X8 columns, and the calculation of the enzyme activity were done according to Preti et al. [15]. Control incubation mixtures (blanks) were performed according to Preti et al. [15], using enzyme boiled for 15 min. The following substrates were employed: gangliosides GM2, GM1, GD1a, GD1b, GT1b and GQ1; ovine submaxillary mucin; ovine submaxillary mucin-sialoglycopeptides; brain sialoglycopeptides; sialyllactose (C-3 isomer). The V and K_m values were calculated from the $1/v$ vs $1/[S]$ plot according to Lineweaver and Burk [17].

One unit of neuraminidase is the amount of enzyme which liberates, under the assay conditions, 1 nmole AcNeu/min.

Other analytical methods

Protein was determined by the method of Lowry et al. [18] with bovine serum albumin as standard. The extraction and fractionation (on thin-layer plates) of gangliosides was accomplished according to Tettamanti et al. [19].

RESULTS

Assessement of the suitability of the preparation of membrane-bound neuraminidase for kinetic studies

After depletion of endogenous substrates the following observations were made.

(a) up to 95% of the enzyme activity present in the initial crude preparation (assayed as reported elsewhere [4]) was recovered in the sediment (105 000 $\times$ *g*, 1 h) and no activity could be detected in the corresponding supernatant.

(b) the sediment of the final enzyme preparation contained, as sialoderivatives,

the monosialoganglioside GM1 (which is resistant to the enzyme action) and sialoglycoproteins in lower amounts (about 75%); from these sialoderivatives no detectable release of AcNeu occurred upon incubation (under the described conditions) for 1 h.

(c) the sediment showed no detectable activity on added ganglioside GM2 within a wide range of experimental conditions [pH 3.0–7.0, presence or absence of various detergents (sodium cholate, Triton X-100), 1–5 mg of protein, incubation time up to 6 h, ganglioside GM2 (0.05–0.2 mM)].

General properties of membrane-bound neuraminidase

Optimum pH. The optimum pH was 4.0 for gangliosides GD1a, GD1b, GT1b and GQ1; 3.8 for ovine submaxillary mucin, ovine submaxillary mucin-sialoglycopeptides and brain sialoglycopeptides; 3.1 for sialyllactose; using sodium acetate–acetic acid buffer, sodium citrate–citric acid buffer or sodium citrate–mono sodium phosphate buffer. The optimum concentration for acetate buffer was 0.05–0.2 M and 0.015–0.06 M for the other buffers. In the routine experiments, 0.15 M sodium acetate buffer was used; when employing sialyllactose as substrate 0.025 M sodium citrate–monosodium phosphate buffer was used.

Time course of reaction. The rate of hydrolysis was linear for 10 min for

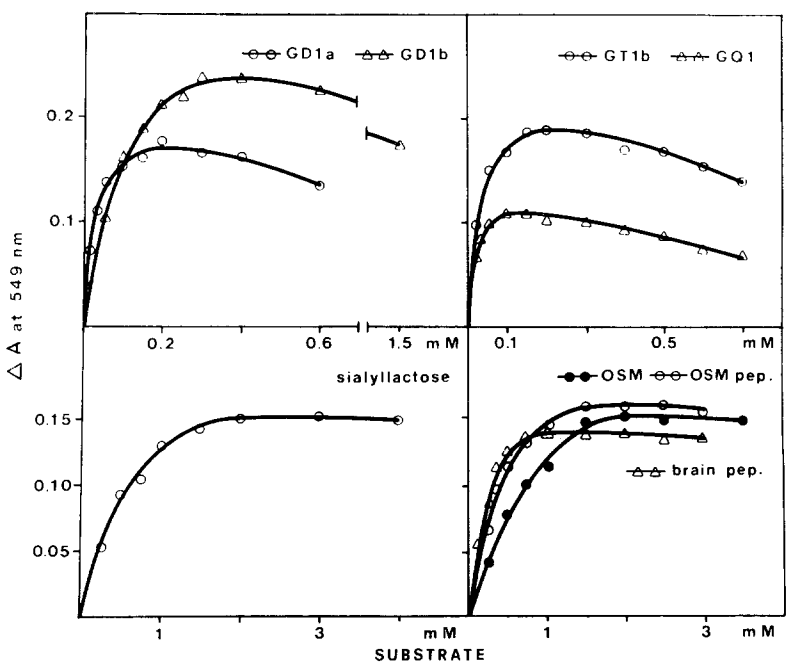

Fig. 1. Effect of the concentration of different substrates (sialoglycolipids; sialoglycoproteins; sialooligosaccharides) on the activity of calf brain membrane-bound neuraminidase. The assay mixtures, containing 1 mg protein, were incubated, under the conditions specified in Methods, for the following times: 10 min (tetrasialoganglioside GQ1); 15 min (disialoganglioside GD1a and trisialoganglioside GT1b); 20 min (sialyllactose); 35 min (disialoganglioside GD1b); 40 min (ovine submaxillary mucin-sialoglycopeptides); 45 min (ovine submaxillary mucin and brain sialoglycopeptides). In all cases the incubation time was within the linear time course of the enzyme catalyzed reaction. The reported data are the average of 5 experiments. OSM = ovine submaxillary mucin.

ganglioside GQ1 (with reference to the hydrolysis of a single residue, ganglioside GT1b being chromatographically ascertained as the only reaction product); 15 min for gangliosides GD1a and GT1b; 40 min for sialyllactose; 45 min for ganglioside GD1b, ovine submaxillary mucin and the different sialoglycopeptides.

Kinetics and substrate specificity. With all tested substrates, under the assay conditions used, the $v/[S]$ relationship followed an hyperbola (see Fig. 1) and the $1/v$ versus $1/[S]$ plot was a straight line. The highest V values (at 37 °C) were observed with the gangliosides and were as follows: 2.12, 1.5, 1.3 and 1.26 units/mg protein, respectively, for gangliosides GT1b, GD1a ,GQ1 and GD1b; the lowest values were obtained with sialoglycoprotein substrates: 0.3, 0.41 and 0.27 units/mg protein, respectively for ovine submaxillary mucin, ovine submaxillary mucin-glycopeptides and brain glycopeptides. For sialyllactose the V value was 1.18 units/mg protein.

The highest affinity was shown with gangliosides GQ1, GD1a and GT1b (K_m values: 1.2, 2.1 and $3.0 \cdot 10^{-5}$ M, respectively). With GD1b, the K_m was significantly higher ($11.6 \cdot 10^{-5}$ M) and with sialyllactose still greater ($6.8 \cdot 10^{-4}$ M). The enzyme showed an apparently poor affinity for sialoglycoprotein substrates. However, (see Fig. 1) a significant increase of apparent affinity was observed from ovine submaxillary mucin to the correspondent ovine submaxillary mucin-sialoglycopeptides, that is, with decrease of the molecular weight of substrate. A marked and early inhibition of enzyme activity by excess of substrate (Fig. 1) was present only in the case of gangliosides.

Stability. Freezing and thawing, up to 10 times, performed either very rapidly (freezing in dry ice–acetone bath followed by thawing under tap water) or very slowly (standing overnight in a freezer at –20 °C followed by thawing in the cold room at 2–4 °C) caused no appreciable change of the enzyme activity on the following sub-

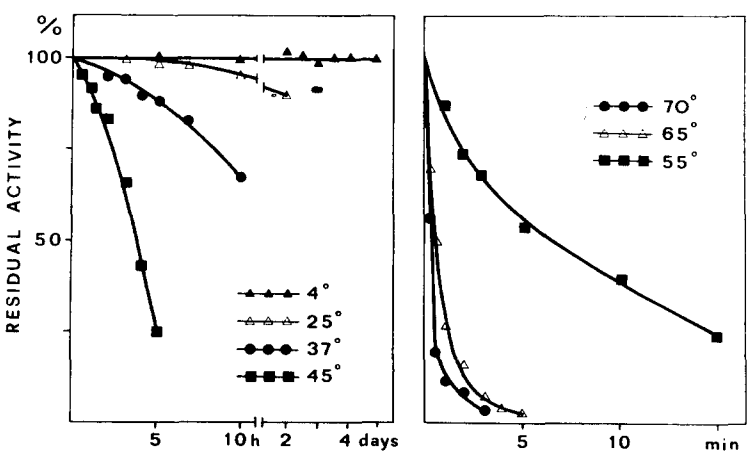

Fig. 2. Effect of the exposure to different temperatures on the activity of calf brain membrane-bound neuraminidase. The enzyme preparation (at pH 6.5) was allowed to stand at the different temperatures for the specified period of time, then incubated under the conditions reported in Methods, disialoganglioside GD1a being used as the substrate. The assay mixtures contained 810 μg of protein. The reported data are the average of 5 experiments.

strates: ganglioside GD1a, sialyllactose and ovine submaxillary mucin. At 4 °C, no change in enzyme activity, measured on ganglioside GD1a, was observed within 4 days (see Fig. 2). However, an increase of temperature caused a loss of enzyme activity. The loss was 15% after 24 h at 25 °C, 35% after 10 h at 37 °C, 75% after 5 h at 44 °C, 80% after 15 min at 55 °C, 50% and 75% after 1 min at 65 °C and 70 °C. Similar results were obtained with sialyllactose and ovine submaxillary mucin as the substrates.

Optimum temperature. As shown in Fig. 3 the maximum reaction rate (24

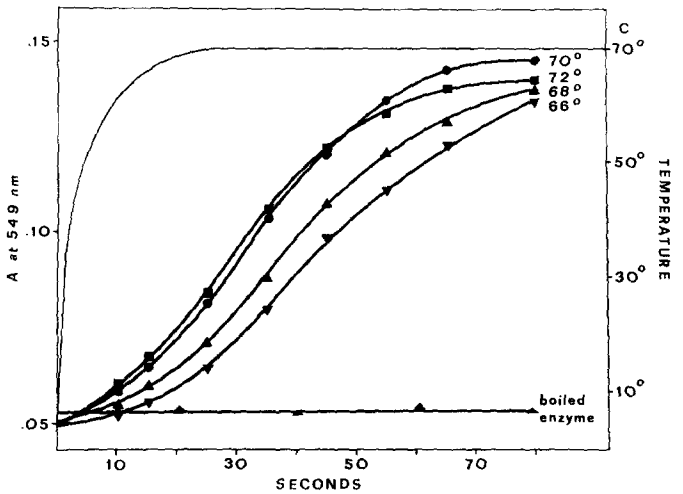

Fig. 3. Time course of the activity of calf brain membrane-bound neuraminidase at high temperatures (66, 68, 70 and 72 °C). The assay mixtures contained 870 μg of protein and 0.2 mM disialoganglioside GD1a. They were set up in an ice bath, immersed in the shaking incubator adjusted to the given temperature, and allowed to incubate. The thin line indicates the temperature measured inside the test tubes a by YSI telethermometer. The reported data are the average of 4 experiments.

units/mg protein), with ganglioside GD1a as substrate, was obtained at 70 °C. This rate was maintained only for 10 s (from 25–35 s after the immersion of the test tube in the incubator), then rapidly diminished with the onset of thermal degradation. The same value of optimum temperature (70 °C) was observed also with gangliosides GD1b and GT1b.

Effect of ions. A pilot study on the effect of cations (Na^+, K^+, Li^+, NH_4^+, Hg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}) and anions ($H_2PO_4^-$, HPO_4^{2-}, Cl^-, HCO_3^-, SO_4^{2-} acetate, citrate) on the activity of membrane-bound neuraminidase was undertaken, ganglioside GD1a at a saturating concentration being used as the substrate.

The only cation (see Fig. 4) found to activate the enzyme was K^+. The activating effect (+15%), present at an ion concentration below 0.1 M, was followed, at a higher concentration, by inhibition. Na^+ and Li^+, up to 0.2 M did not show any significant effect on enzyme activity; NH_4^+ caused inhibition even at a low concentration (0.01 M). All the examined bivalent cations inhibited the enzyme. Hg^{2+} started inhibiting at very low concentrations (about 10^{-6} M); Cu^{2+} from 10^{-5} M; Zn^{2+}, Mg^{2+},

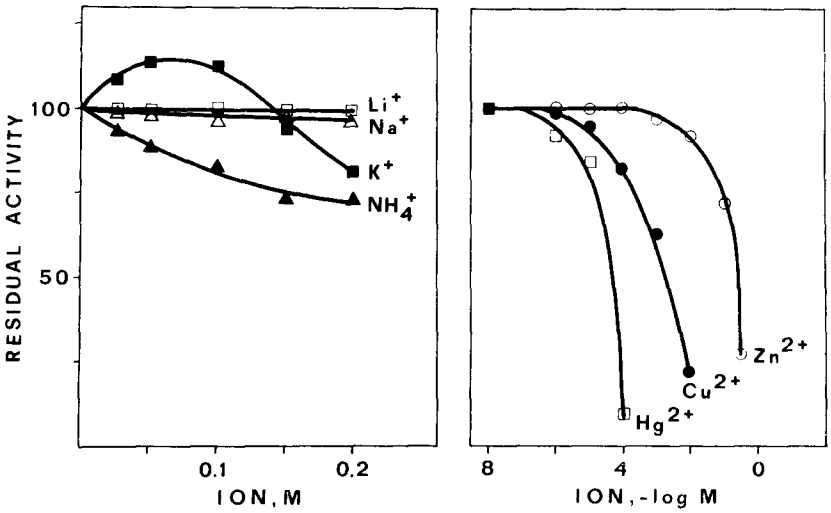

Fig. 4. Effect of monovalent and divalent cations on the activity of calf brain membrane-bound neuraminidase. The assay mixtures contained 820 μg protein and 0.15 mM disialoganglioside GD1a.

Ca^{2+} and Co^{2+} from 10^{-3} M. With all examined anions, both inorganic and organic, no appreciable influence on enzyme activity was observed up to concentrations of $5 \cdot 10^{-2}$ M.

DISCUSSION

The current information [2–4, 20, 21] on the properties and kinetics of brain membrane-bound neuraminidase is largely incomplete. In addition, the possible contamination of the enzyme preparations, as used by various authors, with the lysosomal [5] and soluble [22] neuraminidase, shown recently, may invalidate a number of data. The subject needed complete reinvestigation starting with an enzyme preparation proven to contain only the membrane-bound neuraminidase, and suitable for kinetic studies.

Our enzyme preparation was, simply, the 105 000 $\times$ g (1 h) pellet obtained from calf-brain homogenate in isotonic sucrose, washed, and treated by a previously described procedure [4] so that the endogenous subtrates were exhausted. Moreover, the absence of activity on added (or intrinsic) ganglioside GM1 and GM2 excluded the presence in the same preparation of lysosomal neuraminidase (see refs 5 and 8).

Using this enzyme preparation, the following information on the properties of the membrane-bound neuraminidase was obtained: acidic optimum pH (4.0 for gangliosides; 3.8 for sialoglycoproteins and sialoglycopeptides; 3.2 for sialyllactose); affinity apparently higher for multisialogangliosides (K_m values: around 10^{-5} M) and lower for sialyllactose ($K_m = 0.68 \cdot 10^{-3}$ M), sialoglycoproteins and sialoglycopeptides; different affinity for the two isomer disialogangliosides, GD1a ($K_m = 1.5 \cdot 10^{-5}$ M) and GD1b ($K_m = 11.6 \cdot 10^{-5}$ M); early inhibition by excess substrate (above 0.15 mM) only in the case of gangliosides; activation by K^+; early inhibition by NH_4^+; inhibition by divalent cations, but starting at different concentrations

(10^{-6} M for Hg^{2+}, 10^{-2} M for Ca^{2+}); no action by Na^{+} and Li^{+} up to 0.2 M; maintenance of full activity after freezing and thawing (10 times); appreciable degree of thermal stability.

Some of these data, for instance those concerning the optimum pH, the action on gangliosides and the inhibition by excess of gangliosidic substrate, confirm and give full validity to previous reports by Leibovitz and Gatt [1], Ohman et al. [2], Heijlman and Roukema [21], Schengrund and Rosenberg [20] and Tettamanti et al. [4] and do not need further discussion. It is worth adding that they provide a substantial indication for the high degree of stability of the enzyme, this being a further proof of the difference between the membrane-bound and the lysosomal neuraminidase.

A particular point, already stressed by Heijlman and Roukema [21], which our experiments largely support, concerns the capacity of the enzyme to act on sialoglycoproteins, besides gangliosides.

At present, it is hard to speculate on the true affinity of membrane-bound neuraminidase for native brain sialoglycoproteins and to evaluate, in quantitative terms, the effect of the enzyme action on these macromolecules. Anyway, the study reported here on ovine submaxillary mucin, a large molecule, and on ovine submaxillary mucin-sialoglycopeptides, much smaller, at least indicates that the enzyme acts more readily on the lower molecular weight molecules. Similar findings were reported by Heijlman and Roukema [21]. Thus, also in the case of brain neuraminidase, the action of the enzyme on sialoglycoproteins appears to be greatly facilitated by a previous proteolysis. The assessement of the possible relevance of this situation in the catabolic pathway of brain sialoglycoproteins would be quite interesting.

A peculiar property of membrane-bound neuraminidase is the ability to act at high temperatures. The maximum velocity of the enzyme catalyzing hydrolysis of gangliosides, reached at 70 °C, exceeded 16 times that observed at 37 °C. Since such high temperatures are hardly within physiological range, any physiological significance of this observation seems questionable.

Concluding, the present investigation confirms and substantiates previous reports and contributes further evidence on the properties and kinetics of brain membrane-bound neuraminidase. In addition it provides a simple and precise methodological approach for a deeper study of the enzyme–substrate(s) interactions.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Consiglio Nazionale delle Ricerche (C.N.R.), Italy.

REFERENCES

1 Leibovitz, Z. and Gatt, S. (1968) Biochim. Biophys. Acta 152, 136–143
2 Ohman, R., Rosenberg, A. and Svennerholm, L. (1970) Biochemistry 9, 3774–3782
3 Roukema, D. A. and Heijlman, J. (1970) J. Neurochem. 17, 773–780
4 Tettamanti, G., Preti, A., Lombardo, A., Gasparini, M. and Zambotti, V. (1972) Biochim. Biophys. Acta 258, 228–237
5 Tallman, J. F. and Brady, R. O. (1972) J. Biol. Chem. 247, 7570–7575
6 Tettamanti, G., Morgan, I., Gombos, G., Vincendon, G. and Mandel, P. (1972) Brain Res. 47, 515–518

7 Tettamanti, G., Preti, A., Lombardo, A., Bonali, F. and Zambotti, V. (1973) Biochim. Biophys. Acta 306, 466–477
8 Kolodny, E. H., Kanfer, J., Quirk, J. M. and Brady, R. O. (1971) J. Biol. Chem. 246, 1426–1431
9 Svennerholm, L. (1970) in Comprehensive Biochemistry (Florkin, M. and Stotz, E. H., eds), Vol. 18, pp. 201–227, Elsevier, Amsterdam
10 Tettamanti, G. and Zambotti, V. (1968) Enzymologia 35, 61–74
11 Tettamanti, G. and Pigman, W. W. (1968) Arch. Biochem. Biophys. 124, 41–53
12 Carubelli, R., Bhavanandan, V. P. and Gottshalk, A. (1965) Biochim. Biophys. Acta 101, 67–75
13 Ohman, R. and Hygstedt, O. (1968) Anal. Biochem. 23, 391–402
14 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611
15 Preti, A., Lombardo, A. and Tettamanti, G. (1970) Ital. J. Biochem. 19, 371–385
16 Warren, L. (1959) J. Biol. Chem. 234, 1971–1974
17 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 659–665
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Tettamanti, G., Bonali, F., Marchesini, S. and Zambotti, V. (1973) Biochim. Biophys. Acta 296, 160–170
20 Schengrund, C. L. and Rosenberg, A. (1970) J. Biol. Chem. 245, 6196–6200
21 Heijlman, J. and Roukema, P. A. (1972) J. Neurochem. 19, 2567–2575
22 Carubelli, R. and Tulsiani, D. R. P. (1971) Biochim. Biophys. Acta 237, 78–87
23 Tettamanti, G., Lombardo, A., Preti, A. and Zambotti, V. (1970) Enzymologia 39, 65–71