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STUDIES ON BRAIN CYTOSOL NEURAMINIDASE

I. ISOLATION AND PARTIAL CHARACTERIZATION OF TWO FORMS OF THE ENZYME FROM PIG BRAIN

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Summary

1. Two forms of cytosol neuraminidase (EC 3.2.1.18) (neuraminidase A and neuraminidase B) were isolated and purified from pig brain homogenate, by proceeding through the following steps: centrifugation of brain homogenate at 105 000 × *g* (1 h); ammonium sulphate fractionation (35—55% saturated fraction); column chromatography on Biogel A 5 m; column chromatography on hydroxy apatite/cellulose gel; affinity chromatography on Affinose-tyrosyl-*p*-nitrophenyloxamic acid. The separation of the two forms of neuraminidase was provided by chromatography on hydroxylapatite/cellulose gel. Neuraminidase A was purified about 500-fold; neuraminidase B about 400-fold.

2. The pH optima and the maximum activities in various buffers were different for neuraminidase A and B (for instance the pH optimum was, in sodium acetate/acetic acid buffer, 4.7 for neuraminidase A and 4.9 for neuraminidase B). Ions affected in a different way the two enzymes: K^+ activated neuraminidase A but not neuraminidase B; Na^+ and Li^+ inhibited neuraminidase A at a higher degree than neuraminidase B. Neuraminidase B seemed to be moderately activated by some bivalent cations (Ca^{2+}; Mg^{2+}; Zn^{2+}); neuraminidase A did not.

The K_m values for sialyllactose were different: $2.2 \cdot 10^{-3}$ M for neuraminidase A; $0.46 \cdot 10^{-3}$ M for neuraminidase B.

Introduction

The presence in brain cytosol of a neuraminidase (EC 3.2.1.18), first reported by Carubelli et al. [1] has been definitely established [2,3]. This

finding further supports the general concept that mammalian tissues contain a cytosol neuraminidase [4,5], as well as a membrane-bound form.

The interaction of the cytosol neuraminidase with the sialoderivatives occurring in brain and the relationships between the cytosol and particulate (membrane-bound [3,6—8] and lysosomal [9]) neuraminidases are still to be defined. Moreover, it is not known whether cytosol neuraminidase is present, in mammalian tissues, in different forms, as observed for the soluble neuraminidases of bacterial [10], viral [11,12] and protozoan [13] origin.

The present investigation was undertaken with the aim of developing a rapid and reproducible procedure for the purification of brain cytosol neuraminidase and for checking the possible occurrence of different forms of the enzyme. On the basis of preliminary indications [14] pig brain, particularly rich in this enzyme, was employed.

Materials and Methods

Commercial chemicals (C.Erba, Merck GmbH, Baker Chemicals) were of analytical or of the highest available grade. *N*-acetylneuraminic acid and crystalline bovine serum albumin were obtained from Sigma Chem. Co. Sialyl-lactose (isomer C-3) was isolated from 1 day colostrum according to Ohman and Hygstedt [15] (the final separation of isomers C-3 and C-6 was performed on a silica gel H column). Silica gel H was purchased from Merck GmbH; Biogel A 5 m (100—200 mesh), Biogel HTP (hydroxy apatite), Cellex N-1 (highly purified cellulose powder not containing ion exchange groups), Affinose 201 A (succinylaminoalkyl agarose from BioRad Lab); *p*-nitrophenyl oxamic acid from K and K; Dowex 2-X8 (200—400 mesh) from Dow Chem. Co. Dialysis tubing was obtained from A. Thomas Co.

The Affinose-tyrosyl-*p*-nitrophenyloxamic acid was prepared according to the procedure of Cuatrecasas and Illiano [16]. Dowex 2-X8 (200—400 mesh) was prepared in acetate form according to Svennerholm [17]. The hydroxy apatite/cellulose (2/1, W/W) gel was prepared according to Tulsiani and Carubelli [5].

Brains of adult pigs were obtained at the slaughterhouse, immediately after death, and kept on ice until processed (generally 20—30 min). Meninges were removed and the gray matter, grossly dissected, after washing in ice-cold homogenizing solution, was homogenized as described below.

The homogenizations were carried out with a Waring Blendor at top speed (about 800 rev./min), and centrifugations performed in an International E.C. Mod. B 60, refrigerated centrifuge. The elution of the proteins from chromatographic columns was followed by automatic recording of the absorbance at 280 nm, and fractions from 5 to 15 ml were collected. The incubations were performed in an incubator shaker at 37° C.

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

(a) Separation and purification of cytosol neuraminidase (two enzyme forms)

The gray matter, obtained from 10—12 pig brains, was homogenized with 9 vol 0.156 M KCl for 3 min and centrifuged for 1 h at 105 000 × *g*. The pellet was washed with the same above volume of 0.156 KCl, centrifuged, and the

two supernatants pooled. The purification of the enzyme developed through the following steps:

Step 1: ammonium sulphate fractionation. Solid ammonium sulphate was added to the pooled supernatants to 35% saturation (pH adjusted to 6.8) and the mixture centrifuged at 40 000 × *g* (30 min). The supernatant, after addition of solid ammonium sulphate to 55% saturation, (final pH 6.8) was stirred for 1 h, than centrifuged for 1 h at 105 000 × *g*.

Step 2: chromatography on Biogel A 5m. The pellet obtained was dissolved with the minimum volume of 0.01 M K phosphate buffer (pH 6.8) and carefully poured on the top of a Biogel A 5m (100—200 mesh) column (8 × 110 cm), previously equilibrated (overnight) with 0.02 M sodium acetate buffer, pH 6.8. The elution was carried out with 0.02 M sodium acetate buffer (pH 6.8), flow rate 120 ml/h, and fractions of 13 ml were automatically collected. The proteins, eluted in the most retarded peak, containing the enzyme (see Fig. 1), were precipitated at 85% ammonium sulphate saturation (pH 6.8) and collected by centrifugation for 1 h, (105 000 × *g*). The pellet, dissolved with 0.01 M potassium phosphate buffer (pH 6.8) was dialyzed for 8—10 hours against 50 vol. of the same buffer, changed 3—4 times.

Step 3: chromatography on hydroxy apatite/cellulose gel. The dialyzed solution was poured on the top of a column (4 × 14 cm) of hydroxy apatite/cellulose gel equilibrated (overnight) with 0.01 M potassium phosphate buffer, pH 6.8. A stepwise elution was carried out using suitable volumes of potassium phosphate buffer at the following concentrations: 0.05 M, 0.1 M, 0.15 M, 0.175 M, 0.2 M, 0.3 M, 0.75 M. Each buffer was passed through the column until no more protein was detectable in the eluate (extinction at 280 nm). The flow rate was 20 ml/h and fractions of 5 ml were collected. The neuraminidase was present in the eluates obtained separately with 0.15 M ("enzyme A") and with 0.2 M ("enzyme B") buffer (see Fig. 2). Proteins from both eluates were separately precipitated using 85% ammonium sulphate and collected by centri-

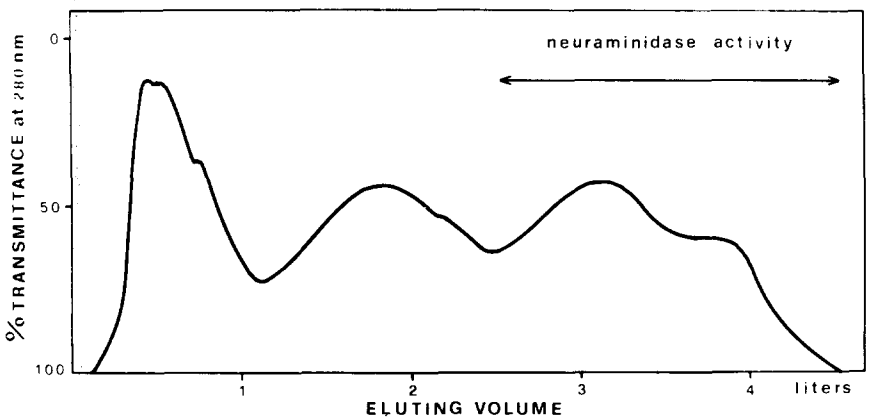

Fig. 1. Elution of brain cytosol neuraminidase from Biogel A 5m (100—200 mesh) column. The assay of neuraminidase activity was performed, as described in Materials and Methods, on samples of the eluate (sodium acetate/acetic acid buffer was employed). The eluate portion carrying neuraminidase activity is indicated. Protein, 3298 mg.

fugation (105 000 × *g*, 1 h). The pellets were dissolved with the minimum volume of 0.01 M K phosphate buffer, pH 6.8, and dialyzed against four changes of 50 vol. each of the same buffer for 12—14 h.

Step 4: affinity chromatography on Affinose 201 A-tyrosyl-p-nitrophenyloxamic acid. Portions of each dialyzed solution, containing 40—45 mg of protein, were dialyzed for 8—10 h against 4 changes of 30 vol. of 0.025 M sodium acetate, 1 mM $CaCl_2$, 0.1 mM EDTA pH 5.0, and poured into an Affinose 201 A-tyrosyl-*p*-nitrophenyloxamic acid column (0.4 × 5 cm), previously equilibrated with the same above buffer until the eluate showed pH 5.0. Then the column was washed with 1—2 bed vol. of the above buffer. The enzyme was eluted with 0.1 M $NaHCO_3$ buffer, pH 9.1, added at first slowly, in order to provide a sharp elution front. Through the whole affinity chromatography process, fractions of 1 ml were collected (the tubes were kept in an ice-water bath). The samples, eluted with 0.1 M $NaHCO_3$ buffer, were immediately adjusted to pH 6—7 by adding few drops of 0.2 M citric acid.

(b) Assay of neuraminidase activity

The incubation mixtures, containing, in a volume of 0.65 ml, 0.15 M sodium acetate/acetic acid or sodium citrate/citric acid buffer, adequate amounts of protein (from 0.1 to 3 mg) and a saturating amount of sialyllactose (from 2 to 5 mM) were incubated in an incubator shaker, at the optimum pH, for a convenient time. The optimum assay condition, determined for each stage of the purification process, are reported in detail under Results.

The amount of liberated *N*-acetylneuraminic acid (AcNeu) was determined by the Warren's [18] reaction following the indications of Preti et al. [19]. When substances, that were known to interfere in the above reaction, had to be used (e.g. ions), the mixtures, after incubation, were subjected to ion exchange chromatography on Dowex 2-X8 columns [17] and then the purified AcNeu was assayed. The control incubation mixtures (blanks) were prepared with the boiled (15 min) enzyme. One unit of neuraminidase is defined as the amount of enzyme releasing 1 mol of AcNeu per min under the given conditions.

(c) Other methods

Protein was determined by the method of Lowry et al. [20], bovine serum albumin being used as the standard.

Results

(1) Separation and purification of two forms of cytosol neuraminidase (neuraminidase A and neuraminidase B)

After consideration of the procedure set up for separating and purifying two forms of cytosol neuraminidase from pig brain homogenate, from a technical point of view the following points are worth noting:

(a) The aim to retard the protein carrying neuraminidase activity, by gel filtration (3rd step of our procedure), was more easily carried out using Biogel A 5m instead of Sephadex G (used in previous work [21]) or Biogel P materials. The main advantages were: the faster and more adjustable rate, the higher

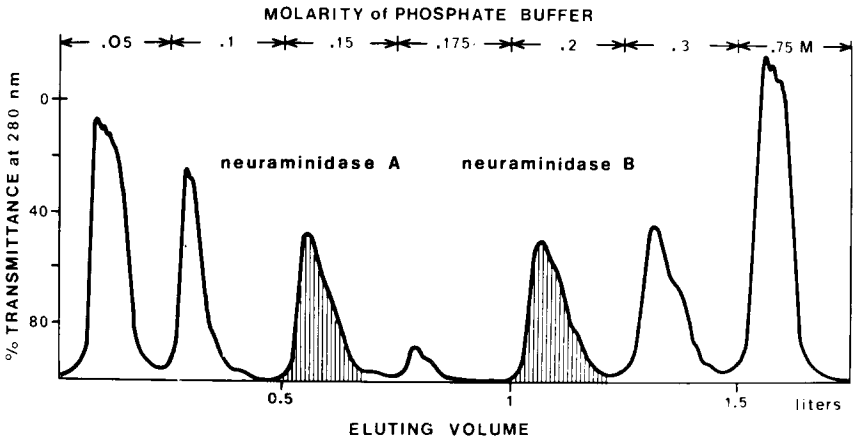

Fig. 2. Separation of two forms of brain cytosol neuraminidase (neuraminidase A and neuraminidase B) by hydroxy apatite/cellulose (2:1, w/w) gel column chromatography. The neuraminidase activity was assayed, as described in Materials and Methods (in 0.17 M acetate buffer), on samples obtained from each eluted protein peak after protein precipitation with 85% saturated ammonium sulphate, centrifugation and dialysis of the redissolved pellet against 0.01 M potassium phosphate buffer at pH 6.8 (for more details see Materials and Methods). Protein, 588 mg.

level of reproducibility, the possibility to re-use the columns many times (8—10) with no change in the flow rate and resolution.

(b) The separation of two different protein peaks carrying neuraminidase activity (neuraminidase A and neuraminidase B) was attained by hydroxy apatite/cellulose gel chromatography (Fig. 2). The peaks were eluted with 0.15 M (neuraminidase A) and with 0.2 M (neuraminidase B) potassium phosphate buffer. An intermediate peak, eluted with 0.175 M potassium phosphate buffer showed no neuraminidase activity. The re-chromatography of the "0.15 M protein peak" (after dialysis against 0.01 M potassium phosphate buffer, pH 6.8), under the same conditions, yielded over 90% protein and 95% enzyme activity in the newly eluted 0.15 M potassium phosphate buffer peak. Re-chromatography of the "0.2 M protein peak" also yielded a corresponding recovery of protein and enzyme activity in the newly eluted 0.2 M potassium phosphate buffer peak. The protein fractionation and flow rate on hydroxy apatite cellulose gel was reproducible and the same column could be satisfactorily re-used 4—5 times.

(c) The behaviour of neuraminidase A and B on affinity chromatography was somewhat different, the former enzyme remaining attached to the resin only up to pH 5.0; the second up to pH 6.0. The treatment by affinity chromatography gave, in our case, only a relatively modest increase of the specific activity of both neuraminidase A and B (2—3 times). This is in partial agreement with recent reports [22,23] showing that the oxamate moiety in the "oxamate affinity column", has no effect on the purification of neuraminidase.

As indicated by the data in Table I the final purification was 153-fold for neuraminidase A, 109-fold for neuraminidase B. The final recovery of the enzyme activity (neuraminidase A plus neuraminidase B) was 51.2%. The chromatography on Biogel A 5m provided the greatest increase in purification

TABLE I

PURIFICATION OF BRAIN CYTOSOL NEURAMINIDASE (S)

The figures reported are the average of 5 experiments. The incubations were carried out in 0.17 M sodium acetate/acetic acid buffer

Purification step	Protein		Neuraminidase activity*			
	mg	%	Units/mg protein	total units	recovery (%)	purification (fold)
105 000 × *g* (1 h) supernatant	10 400	100	0.021	218.4	100	0
35%—55% ammonium sulphate fraction	3 298	30.7	0.060	198	90.6	2.85
Biogel A 5m column (retarded fraction)	588	5.6	0.30	176	80.7	14.30
Hydroxy apatite/ cellulose gel column:						
0.15 M fraction	61	—	1.21	73.8	—	57.60
0.2 M fraction	48.4	—	0.92	44.2	—	43.85
Total	109.4	1.05	—	118.0	54.0	—
Affinity chromatography						
Enzyme A	19.9	—	3.22	64.2	—	153.30
Enzyme B	20.7	—	2.30	47.5	—	109.50
Total	40.6	0.39	—	111.8	51.2	—

* Expressed in units (amount of enzyme yielding 1 mmol released Ac Neu/min under the experimental conditions described in Materials and Methods).

(5-fold); while the chromatography on hydroxy apatite/cellulose gel caused the greatest loss of enzyme activity (about 25%).

(II) General properties of brain cytosol neuraminidase A and neuraminidase B

(a) pH optimum and effect of buffers. The pH optimum of brain cytosol neuraminidase activity, contained in the preparations before the separation of the two enzyme forms was 4.8 (0.15 M sodium acetate buffer). As shown in Table II and in Fig. 3 the optimum pH of neuraminidase A was 4.1, 4.2, 4.7, and 5.2 in sodium acetate/acetic acid, sodium citrate/citric acid; sodium phosphate/citric acid and Tris/sodium maleate buffers, respectively. The pH optima of neuraminidase B were, 4.5, 4.2, 4.9, 5.0, respectively, in these buffers. Thus the two enzyme forms had different pH optima except in citrate buffer.

Neuraminidase A showed the highest activity (Table II and Fig. 3) in citrate buffer, followed by phosphate/citric acid buffer (87.4% of the maximum), acetate buffer (75%) and Tris/maleate buffer (50.2%). For neuraminidase B, on the contrary, the values of maximum activity obtained with acetate buffer, phosphate/citric acid buffer and citrate buffer were very close (96%—100%), but also in this case, the lowest activity was provided in Tris/maleate buffer.

The range of optimum molarity was 0.1—0.2 M for acetate, phosphate/-citric acid and citrate buffers; 0.01—0.05 M for Tris/maleate buffer.

TABLE II

pH OPTIMUM AND MAXIMUM ACTIVITY OF BRAIN CYTOSOL NEURAMINIDASES A AND B IN DIFFERENT BUFFER SYSTEMS

The enzyme preparations at the final step of purification were employed. The data reported are the average of 5 experiments.

Buffer	Neuraminidase A			Neuraminidase B		
	Optimum pH	Spec. act.*		Optimum pH	Spec. act.*	%
		units/mg protein	%			
Sodium acetate/acetic acid, 0.17 M	4.7	3.22	75	4.9	2.30	95.8
0.17 M sodium phosphate/0.17 M citric acid	4.1	3.75	87.4	4.5	2.31	96.2
0.17 sodium citrate/citric acid	4.2	4.29	100	4.2	2.40	100
0.03 M Tris/ sodium maleate	5.2	1.87	43.6	5.0	1.20	50

* Referred to the highest activity observed (= 100%).

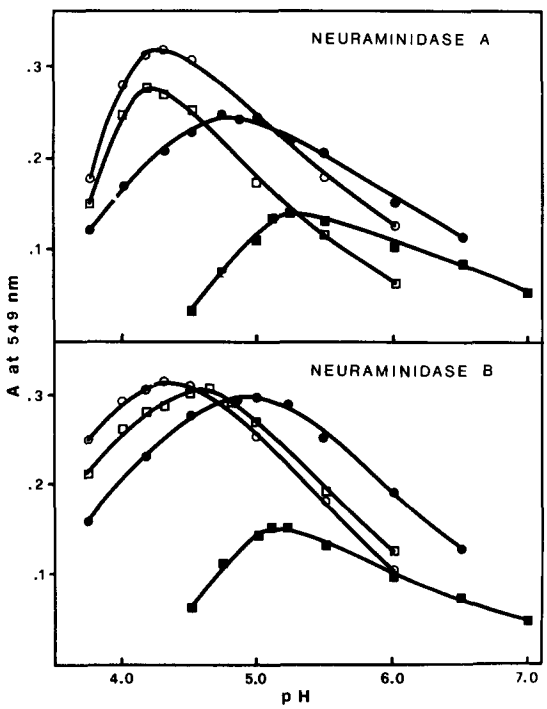

Fig. 3. Effect of pH and of different buffers on the activity of brain cytosol neuraminidase A and neuraminidase B. The enzymes were employed at the highest degree of purification. Protein, 265 μg for neuraminidase A, 310 μg for neuraminidase B. The data shown are the average of three experiments. ○———○, 0.17 M sodium citrate/citric acid buffer, ●———●, 0.17 M sodium acetate/acetic acid buffer, □———□, 0.17 M sodium phosphate/citric acid buffer, ■———■, 0.03 M sodium maleate/Tris buffer.

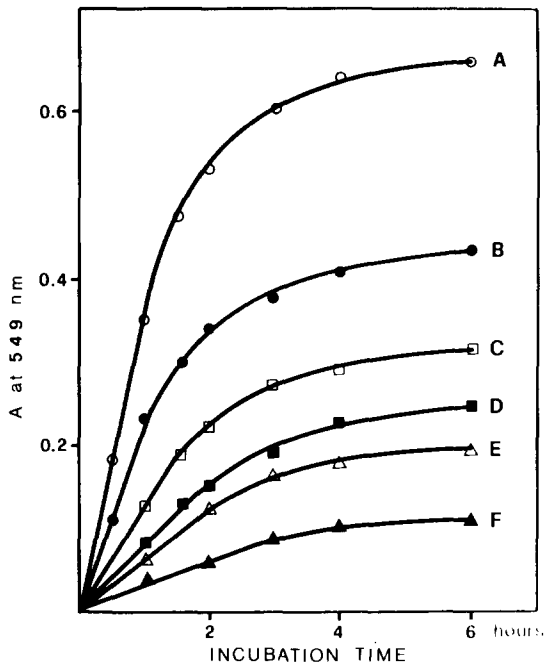

Fig. 4. Time course of brain cytosol neuraminidase activity at different steps of the purification procedure. The neuraminidase assay was performed in 0.17M acetate buffer at the pH optimum. These data are the average of three experiments. A, neuraminidase A, after affinity chromatography (protein: 415 μg, pH 4.7); B, neuraminidase B, after affinity chromatography (protein: 235 μg, pH 4.9); C, neuraminidase A, after hydroxy apatite cellulose gel column chromatography (protein: 453 μg, pH 4.7); D, neuraminidase B, after hydroxy apatite cellulose gel column chromatography (protein: 264 μg, pH 4.9); E, neuraminidase activity, after Biogel A 5m column chromatography (protein: 970 μg, pH 4.8); F, neuraminidase activity, after 35—55% saturated ammonium sulphate fractionation (protein: 2.37 mg, pH 4.8).

(b) Time course of the reaction. When the enzyme preparations from the initial steps of purification (ammonium sulphate fraction, Biogel A 5m preparation) were used, the hydrolysis of sialyl-lactose was linear with time during 2 h of incubation (see Fig. 4). After neuraminidases A and B were separated, sialyl-lactose was linearly affected for 1 h (the same time also after affinity chromatography).

(c) Proportionality between protein amount and reaction rate. The rate of hydrolysis of sialyl-lactose was proportional to the amount of protein from 0—5 mg when using the enzyme at the initial steps of purification (see Fig. 5). The proportionality was only up to 800 μg protein when employing the separated enzymes A and B.

(d) Stability. Standing at 4°C up to two days and at −20°C up to two months caused no significant change in the enzyme activity. The loss of enzyme activity after 4—5 freezing and thawing treatments was only 10%, when the enzyme preparation before the separation of the two enzyme forms was assayed, and about 20% when the individual purified enzymes A and B were assayed. Further freezing and thawing (up to 10 times) did not significantly change the enzyme activity.

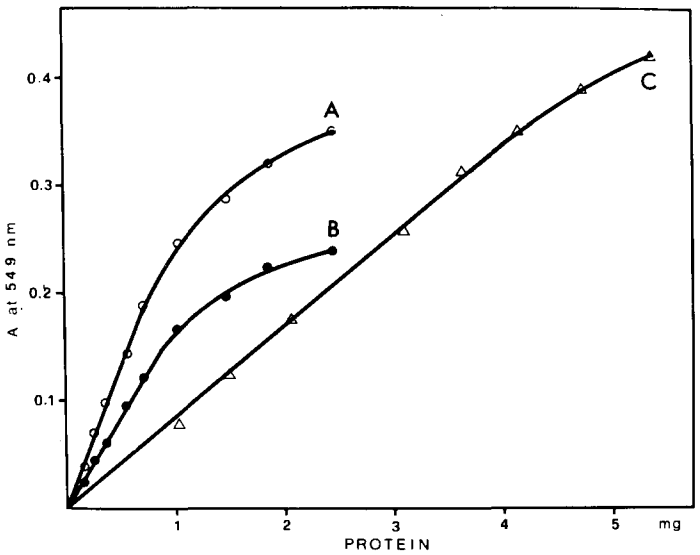

Fig. 5. Proportionality between enzyme activity and protein at the initial and the final steps of the purification procedure. The neuraminidase assay was performed in 0.17 M acetate buffer. The data reported are the average of three experiments. A, neuraminidase A (after affinity chromatography) (incubation time: 1 hour); B, neuraminidase B (after affinity chromatography) (incubation time: 1 hour); C, neuraminidase activity (after 35—55% saturated ammonium sulphate fractionation) (incubation time, 2 h).

The behaviour of the enzyme upon freezing and thawing was the same, regardless of the method of performing the thermal treatment (alternative immersion of the test tube into a dry ice/acetone bath and into tap water, or alternative standing overnight in the freezer at —20° C and in the cold room at 4° C until thawed).

(e) Solubility. Lowering the ionic strength under 0.01 or the pH below 5.0 caused a bulk precipitation of both neuraminidases A and B in an unsoluble form which was, however, still active. The unsolubilized enzyme could hardly be redissolved and only to a minor degree.

(f) Effect of ions. A comparative picture of the ion effects on neuraminidases A and B is shown in Fig. 6. NH_4^+ inhibited both enzyme forms, the same % of inhibition being obtained at a given ion concentration. Na^+ and Li^+ also had an inhibitory effect, but only above the concentration of 0.1 mM; the inhibition was significantly different for the two enzyme forms: at the ion concentration of 0.2 mM the inhibition was 40—45% for enzyme form A, while for enzyme form B it was 70—75%. K^+ exhibited a slight but reproducible activatory effect on both enzyme forms within a certain concentration (0.2 mM for enzyme form A, 0.1 mM for enzyme form B). Hence the inhibition was more marked with enzyme form B.

Bivalent cations affected differently the two enzyme forms (see Fig. 6). None of the tested bivalent cations was able to activate enzyme form A: Ca^{2+} and Mg^{2+} did not influence the enzyme activity up to 10^{-3} M while Zn^{2+}, Hg^{2+}, Cu^{2+} and Fe^{2+} has an inhibitory effect. The inhibition at 10^{-3} M ion concentration was 20% for Zn^{2+}, 45% for Hg^{2+}, 70% for Cu^{2+} and 90% for Fe^{2+}. On the

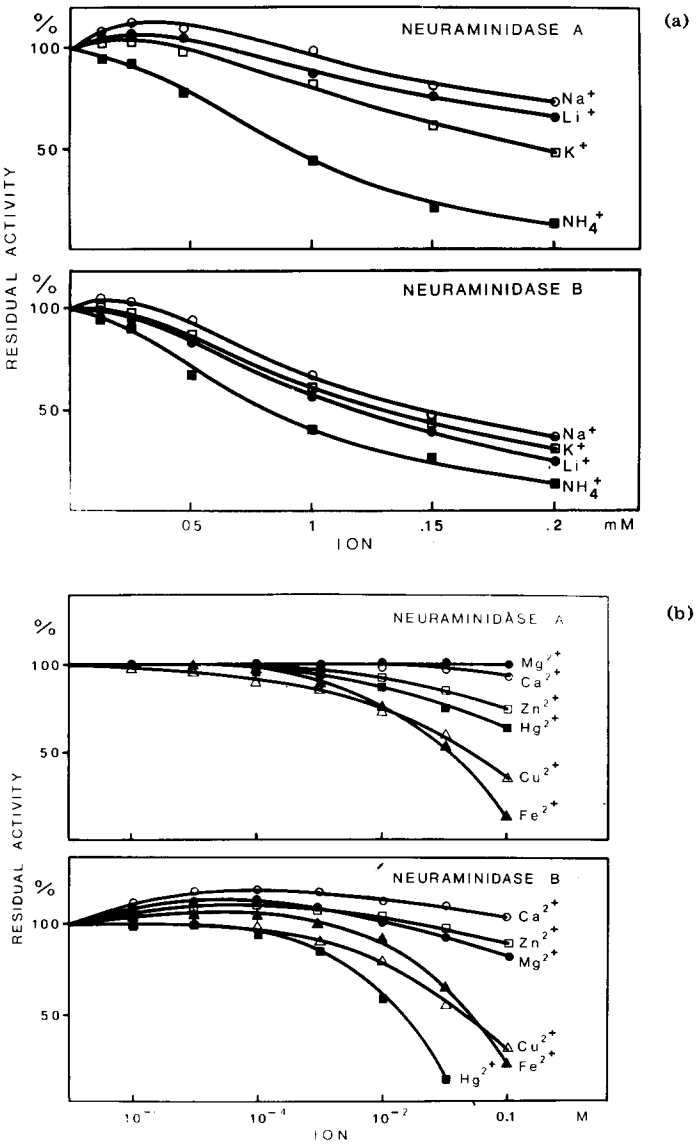

Fig. 6. Effect of different monovalent (a) and bivalent (b) cations on the activity of brain cytosol neuraminidase A and B. The enzymes at the final stage of purification were employed. The neuraminidase assay was performed in 0.17 M acetate buffer at the pH optimum. Protein, 234 μg for neuraminidase A, 285 μg for neuraminidase B. The data are the average of three experiments.

contrary, the above cations (except Cu^{2+} and Hg^{2+}) activated, at the lowest concentrations, the enzyme form B (from a minimum of 7% with Zn^{2+} to a maximum of 20% with Ca^{2+} and Fe^{2+}). With increasing ion concentration, an inhibitory effect appeared to be more marked in the case of Fe^{2+}. It cannot be excluded that part of the above-described effects are not due to direct action of ions on the enzyme molecule, but to ion interaction with the substrate, which is itself ionic. The different behaviour with the two enzymes, however, remains.

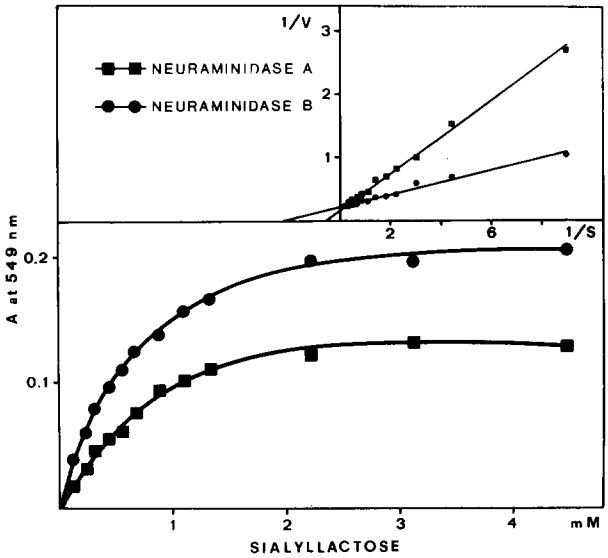

Fig. 7. Kinetics of brain cytosol neuraminidase A and B on sialyl lactose. The enzymes at the final stage of purification were employed. The neuraminidase assay was performed in 0.17 M sodium acetate/acetic acid buffer at the pH optimum. Protein, 130 μg for neuraminidase A, 140 μg for neuraminidase B. The data are the average of three experiments.

(III) Kinetics of the two forms of brain cytosol neuraminidase

The V over S relationship of cytosol neuraminidase forms A and B, acting on sialyl-lactose, followed the classical Michaelis Menten kinetics. (See Fig. 7). The K_m values of enzyme form A and form B for sialyl-lactose, established by the double reciprocal plot method of Lineweaver and Burk [24], were $2.22 \cdot 10^{-3}$ M and $0.46 \cdot 10^{-3}$ M, respectively.

Discussion

The presence in brain of a cytosol neuraminidase has been definitely established [1—3]. With the present investigation we succeeded in separating and purifying two forms of the enzyme, indicated as neuraminidase A and neuraminidase B.

The step providing the separation of the neuraminidase activity into two different entities was the column chromatography on mixed gel of hydroxy apatite and cellulose. More precisely, both the protein peaks eluted with 0.15 M and 0.2 M potassium phosphate buffer carried neuraminidase activity. The elution from the column of a protein peak at an intermediate buffer molarity (0.175 M) without enzyme activity, and the reproducibility of the elution after rechromatography indicated that the two enzyme forms were different. Further and direct proofs for the diversity of the two enzymes were provided by the study of the enzyme properties and kinetics. In fact the values of pH optimum and maximum activity in various buffers were different for neuraminidase A and B. Moreover, ions differently affected the two enzymes. Finally the K_m values for sialyl-lactose were different.

Referring to the 105 000 × g supernatant, neuraminidase A was purified

about 150 times; neuraminidase B, 110 times. Unfortunately the determination of cytosol neuraminidase activity in brain homogenate is practically impossible, due to the presence of a particulate neuraminidase having a close pH optimum (4.9) and similar profiles of ion inhibition [22]. Thus, in the absence of this information, the purification, referred to the homogenate, can be evaluated only on the basis of the protein ratio supernatant/homogenate and calculated as being 3—4 times higher.

Pilot experiments have shown that the interaction between neuraminidases A and B and gangliosides, the possible physiological substrates of the enzymes, were quite complex. This matter will be more thoroughly described and discussed in a separate and following paper.

Note added in proofs

Employing the new, recently introduced, stock of Biogel HTP (which is much lighter and softer than the former one) the protein peak carrying neuraminidase B is eluted from the hydroxy apatite cellulose gel column with 0.25 M (instead of 0.2 M) K phosphate buffer.

Acknowledgments

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