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STUDIES ON BRAIN CYTOSOL NEURAMINIDASE. II. EXTRACTABILITY, SOLUBILITY AND INTRANEURONAL DISTRIBUTION OF THE ENZYME IN PIG BRAIN

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Summary

The origin and properties of cytosolic neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) from pig brain were studied.

1. The brain extracts containing the cytosol derived from neuronal bodies and glial cells carry 0.69 munits neuraminidase/g fresh tissue. The behaviour of neuraminidase during extraction closely paralleled that of authentic cytosolic enzyme, lactate dehydrogenase; whereas, it differed from that of the lysosomal enzymes, β-hexosaminidase and β-galactosidase, also found in the extracts.

2. Nerve endings from either crude or purified preparations, when treated by hypoosmotic shock, released neuraminidase activity up to a maximum of 1.25 munits/g fresh tissue. The behaviour of releasable neuraminidase was always identical to that of lactate dehydrogenase and very similar to that of ATPase and acetylcholinesterase. Typical lysosomal enzymes, however, such as β-galactosidase and β-hexosaminidase, behaved differently under the same conditions. This neuraminidase activity is thought to be derived from the cytosol of nerve endings.

3. The specific activity of neuraminidase in nerve-ending cytosol is 15—20 times that in neuronal body and glial cell cytosol. Some properties (pH, K_m value, V/t relationship) of the cytosolic enzymes of different origin are similar; others (stability on standing at 4°C; resistance to freezing and thawing) are different. Hypoionic solutions caused both cytosolic neuraminidases to slowly precipitate and to assume a stable insoluble form which was still active.

Introduction

The cell sap from the brain of different animals displays neuraminidase activity (acylneuraminyl hydrolase, EC 3.2.1.18) [1,2].

The optimal conditions for the extraction of cytosolic neuraminidase have not yet been established; these would be necessary for the accurate quantitation of the enzyme and for assessing its origin from the cytosol of the different cells and of cell terminals present in brain. A further problem is the relationship between the cytosolic neuraminidase and the neuraminidases known to occur in brain lysosomes and in neuronal plasma membranes.

The present investigation was undertaken to find a solution to some of the above problems. The following studies were done: (a) the extractibility of the brain enzyme from the cytosol of neuronal bodies and glial cells and of nerve endings; (b) the solubility of the enzyme; (c) the possible links between enzymes occurring in cytosol and lysosomes. The experimental basis of our approach was: (a) to separate the cytosol of nerve endings from that of other origin (neuronal bodies, glial cells); (b) to monitor the presence of cytosolic material by assaying lactate dehydrogenase (EC 1.1.1.27) and that of lysosomal material by assaying β-hexosaminidase (EC 3.2.1.30) and β-galactosidase (EC 3.2.1.23); (c) to compare the behaviour of soluble neuraminidase with that of the marker enzymes. Following earlier studies [2,3], pig brain was employed.

Experimental

Materials

Commercial chemicals (C. Erba, Merck GmbH, Baker Co.) were of analytical or of the highest available grade. *N*-acetylneuraminic acid (NeuNAc), crystalline bovine serum albumin and all materials for marker enzyme assays were obtained from Sigma Chem. Co; 4-methylumbelliferyl-β-D-*N*-acetylglucosamine and 4-methylumbelliferyl-β-D-galactose from Koch-Light; Ficoll 400 from Pharmacia. Sialyllactose (isomer C-3) was isolated from 1-day cow colostrum according to the method of Ohman and Hygstedt [4]. Dowex 2-X8 resin (200—400 mesh, Bio-Rad Labs.) was prepared in acetate form according to the method of Svennerholm [5]. Dialysis tubing was purchased from A. Thomas Co.

Brains of adult pigs were obtained at the slaughterhouse immediately after death and kept on ice until processed (generally 30—40 min). Meninges were removed and the gray matter, grossly dissected, was washed in ice-cold homogenizing solution, carefully broken down in a mortar, weighed and homogenized as described below.

Glass-redistilled water was used to prepare the different solutions. All operations were conducted at 0—4°C unless otherwise stated.

Methods

(a) Preparation of the cytosol from neuronal bodies and glial cells. 10—12-g portions of gray matter were homogenized for 1 min with 9 vols. of one of the following, ice-cold solutions: (a) 0.156 M KCl; (b) 0.1 M potassium phosphate buffer; (c) 0.32 M sucrose/1 mM potassium phosphate buffer/0.1 mM EDTA. All solutions were adjusted to pH 7.2. The homogenizations were carried out with a glass homogenizer, provided with a mechanically driven Teflon pestle, rotating at 400 rev./min (stated radial clearance: 0.30 mm), following the recommendations for avoiding destruction of subcellular organelles and nerve

endings [6]. The mixtures were centrifuged at 105 000 X *g* for 1 h and the supernatant solutions decanted and stored in the cold. Each pellet was extracted 4 times and centrifuged each time at 105 000 X *g* (1 h). The five pooled supernatant solutions constituted the 'neuronal body and glial cell cytosol'.

(b) Preparation of the fraction P_2 and isolation of nerve endings. The brain homogenate fraction sedimenting between 1000 X *g* and 11 500 X *g* (fraction P_2) containing mitochondria, nerve endings, lysosomes and myelin fragments, was prepared according to the procedure of Gurd et al. [6]. Fraction P_2 was suspended in homogenizing buffer (0.32 M sucrose/1 mM potassium phosphate buffer/0.1 mM EDTA, pH 7.2) and diluted with 25% Ficoll solution in homogenizing buffer to the following final Ficoll concentrations: 14, 16, 18, 20 or 22%. The final volume was 10 ml. This suspension was overlaid with a solution of 7.5% Ficoll in homogenizing buffer (total vol.: 20 ml) and centrifuged at 60 000 X *g* for 2.5 h (International E.C., Model B-60 ultracentrifuge; swinging bucket rotor SB-110). Each tube contained fraction P_2 obtained from no more than 3-g fresh tissue. The materials sedimenting (a) as a well-defined band over the 7.5% Ficoll layer (myelin subfraction), (b) as a well-defined band at the 7.5—14% to 22% Ficoll interface (nerve-ending subfraction) and (c) the pellet (mitochondrial subfraction) were carefully removed, diluted with 3—4 vols. of 0.32 M sucrose and centrifuged at 105 000 X *g* for 30 min. Each pellet was washed with 0.32 M sucrose and centrifuged at 105 000 X *g* for 20 min.

Liberation of nerve endings cytosol by hypoosmotic shock: The material to be submitted to hypoosmotic shock (usually fraction P_2 and the nerve-ending subfraction) was homogenized for 1 min in 1—2 ml 1 mM potassium phosphate buffer/0.1 mM EDTA (pH 7.2) diluted with 4 vols. of the same buffer at room temperature, gently mixed and allowed to stand at 4°C for 45 min, with occasional stirring. This procedure followed, essentially, the method of Ledeen et al. [7]. The suspension was then centrifuged at 105 000 X *g* for 30 min.

(c) Enzyme assays. Cytosolic neuraminidase. The incubation mixture, containing (in a volume of 1 ml): 0.15 M sodium acetate/acetic acid buffer, adequate amounts of protein (0.25—4 mg) and 4 mM sialyllactose, was incubated at 37°C at the optimum established pH (4.9) for a proper time (in the routine experiments: 3 h). The reaction mixture, stopped by addition of 0.1 ml 0.5 M H_2SO_4, was quantitatively transferred on a 0.4 X 5 cm, Dowex 2-2-X8, acetate form (1 ml sedimented resin) column. Each column was washed with 5 ml water and *N*-acetylneuraminic acid eluted with 2.5 ml of 2 M sodium acetate/acetic acid buffer (pH 4.6). 1.25 ml eluate were submitted to the Warren's reaction [8], with the addition of 0.3 ml periodate reagent, 2 ml cyclohexanone being employed for the chromophore extraction. Under these conditions, 2—50 μg *N*-acetylneuraminic acid were retained on the column and recovery was 92—95%. The corrected extinction of 0.100 corresponded to 6.4 nmol of free *N*-acetylneuraminic acid.

Control experiments showed that enzyme preparations, incubated for up to 8 h (in the absence of sialyllactose) did not liberate measurable amounts of *N*-acetylneuraminic acid; this excluding the presence of sialoglycoconjugates acting as endogenous substrates. When necessary, prior to neuraminidase assay, proteins were concentrated by precipitation at 90% saturation $(NH_4)_2SO_4$; the

precipitate, dissolved in the minimum volume of 0.01 M potassium phosphate buffer (pH 6.8), was dialyzed overnight at 0—4°C against the same buffer. Protein and neuraminidase recovery was greater than 95%.

Marker enzymes: Lactate dehydrogenase was assayed by following the oxidation of NADH (in the presence of pyruvate) at 340 nm. Acetylcholine esterase (EC 3.1.1.7) was assayed by the method of Ellman et al. [9] in the presence of 10^{-4} M physostygmine and 0.25% Triton X-100. ($Na^+ + K^+$)-stimulated ATPase (EC 3.6.1.3) was assayed by measuring the ATPase activity which was inhibited by 1 mM ouabain in a medium containing 150 mM NaCl, 25 mM KCl and 3 mM $MgCl_2$ [10]. Inorganic phosphate released was estimated by the method of Zilversmit and Davis [11]. β-galactosidase and β-hexosaminidase were assayed by the fluorimetric procedure of Kint [12] using, respectively, 4-methylumbelliferyl-β-D-galactose and 4-methylumbelliferyl-β-D-*N*-acetylglucosamine as substrate.

The activity of neuraminidase and marker enzymes is expressed in International Units (μmol transformed substrate/min at 37°C).

(d) Other methods. Protein was determined by the method of Lowry et al. [13] with bovine serum albumin as standard. Prior to analysis, proteins were precipitated by 10% (final concentration) trichloroacetic acid, standing for 20 min at room temperature and centrifugation.

(e) Electron microscopy. Preparations were centrifuged at 105 000 × *g* for 1 h and fixed for 12—14 h in 5% glutaraldehyde/0.1 M sodium phosphate buffer (pH 7.4). After postfixation with 0.1% osmic acid, the fractions were dehydrated and embedded in Araldite. Sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiscope I.

Results

(1) Extractibility of neuraminidase from the cytosol of neuronal bodies and glial cells

As shown in Table I, the amount of protein and lactate dehydrogenase extracted after 5 treatments with each of the solutions: 0.156 M KCl, 0.1 M potassium phosphate buffer, 0.32 M sucrose was practically the same regardless of the solution employed. About 70% of the lactate dehydrogenase activity present in the homogenate was recovered in the pooled supernatant solutions. A substantial release of β-galactosidase and β-hexosaminidase occurred during the extraction procedure. It was higher, especially for β-hexosaminidase, with 0.156 M KCl (or 0.1 M potassium phosphate buffer) than with 0.32 M sucrose.

The total quantity of extracted neuraminidase obtained with 0.32 M sucrose was only 53% of that (about 0.7 munits/g fresh tissue) yielded with 0.156 M KCl or 0.1 M potassium phosphate buffer. However, the addition of KCl to 0.156 M in the sucrose homogenate prior to centrifugation, raised the relative yield of neuraminidase from 53 to 93%. Since sucrose proved to have very little effect on enzyme stability and exhibited only a slight inhibitory effect on the enzyme activity (about 25% inhibition at 0.32 M sucrose) (unpublished data), the loss of neuraminidase activity when homogenizing with 0.32 M sucrose was probably due to adhesion of neuraminidase to particulate material. This phenomenon was prevented by the addition of KCl. The data in Fig. 1 show

TABLE I

CONTENT OF PROTEIN, NEURAMINIDASE, LACTATE DEHYDROGENASE (LDH), β-HEXOSAMINIDASE (β-Hex) AND β-GALACTOSIDASE (β-Gal) IN THE 'NEURONAL BODY AND GLIAL CELL CYTOSOL' PREPARED FROM PIG BRAIN CORTEX (5—6 g) WITH DIFFERENT HOMOGENIZING SOLUTIONS. THE ENZYME ACTIVITIES ARE EXPRESSED IN INTERNATIONAL UNITS (μmol TRANSFORMED SUBSTRATE/min AT 37°C). THE DATA SHOWN, REFERRED TO 1 g STARTING FRESH TISSUE, ARE THE MEAN VALUES ± S.E. OF 6 EXPERIMENTS

Homogenizing solution	Protein		Neuraminidase **		LDH			β-Hex			β-Gal		
	Total (mg)	% *	Total (munits)	μunits/ mg protein	Total (munits)	% *	munits/ mg protein	Total (munits)	% *	munits/ mg protein	Total (munits)	% *	munits/ mg protein
(a) 0.156 M KCl	27.7 ± 0.9	22.7	0.69 ± 0.04	24.9	90.4 ± 6.2	70.6	3.26	470.3 ± 29.1	37.9	17.0	7.2 ± 0.47	16.6	0.26
(b) 0.1 M K phosphate buffer	27.8 ± 0.8	22.8	0.68 ± 0.05	24.5	90.2 ± 5.8	70.5	3.24	452.1 ± 25.3	36.4	16.3	7.0 ± 0.5	16.1	0.25
(c) 0.32 M sucrose	28.7 ± 0.7	23.5	0.41 ± 0.03	14.3	90.7 ± 0.75	70.8	3.16	161.1 ± 9.5	13.0	5.6	5.8 ± 0.42	13.2	0.20
(d) 0.32 M sucrose with the addition of KCl till 0.156 M after homogenization and before centrifugation	28.2 ± 1.0	23.1	0.64 ± 0.03	22.7	90.6 ± 0.8	70.7	3.21	300.3 ± 14.5	24.2	10.6	6.3 ± 0.51	14.5	0.32

* Calculated on the total amount in the homogenate.

** Due to the presence also of a membrane bound neuraminidase, the determination of cytostolic neuraminidase in the homogenate was not possible.

TABLE II

RELEASE OF PROTEIN, NEURAMINIDASE, LACTATE DEHYDROGENASE (LDH) AND β-HEXOSAMINIDASE (β-Hex) FROM BRAIN CRUDE MITOCHONDRIAL FRACTION P_2, UPON HYPOOSMOTIC TREATMENT. FRACTION P_2 WAS PREPARED FROM 10—12 g PIG BRAIN CORTEX. PRIOR TO CENTRIFUGATION AT 105 000 × *g* (1 h), TO THE HYPOOSMOTIC-SHOCKED MIXTURE, KCl (UP TO 0.156 M) WAS OR WAS NOT ADDED. THE DATA, REFERRED TO 1 g FRESH TISSUE, ARE THE MEAN VALUES ± S.E. OF 10 EXPERIMENTS.

Preparation	Protein		Neuraminidase		LDH			β-Hex		
	Total (mg)	Recovery (%)	Total (munits)	μunits/ mg protein	Total (munits)	Recovery (%)	munits/ mg protein	Total (munits)	Recovery (%)	munits/ mg protein
Homogenate	122 ± 10		—	—	128 ± 9.4		1.05	1239 ± 91		10.1
P_2 fraction	40.7 ± 6	33.4 *	—	—	9.35 ± 0.6	7.3 *	0.23	526 ± 45	42.4 *	11.3
Shocked P_2 supernate:										
— KCl	5.8 ± 0.7		0.82 ± 0.05	141.4	8.16 ± 0.5		1.41	410 ± 30.6		70.7
+ KCl	7.7 ± 0.6	18.9 **	1.25 ± 0.08	162.3	8.76 ± 0.5	93.7 **	1.14	416 ± 28	79.0 **	54

* Referred to homogenate.

** Referred to P_2 fraction.

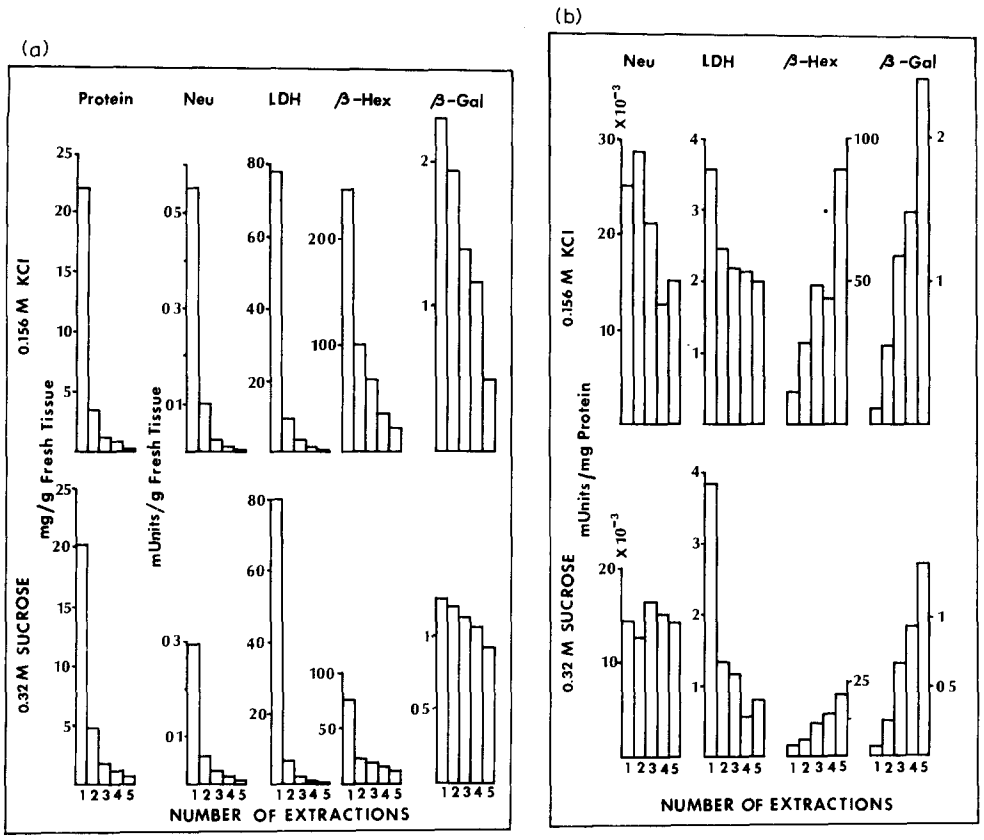


Fig. 1. Extraction of protein, neuraminidase, lactate dehydrogenase (LDH), β-hexosaminidase (β-Hex) and β-galactosidase (β-Gal) from the 'neuronal body and glial cell cytosol' of pig brain cortex. Extracting solutions: 0.156 M KCl and 0.32 M sucrose. The data presented are the mean values of 10 experiments. (a) Absolute content of the different parameters in the various extracts. (b) Specific activity of neuraminidase (Neu), lactate dehydrogenase (LDH), β-hexosaminidase (β-Hex) and β-galactosidase (β-Gal) in the various extracts.

TABLE III

DISTRIBUTION OF PROTEIN, RELEASABLE (SOLUBLE) NEURAMINIDASE, LACTATE DEHYDROGENASE (LDH), ACETYLCHOLINESTERASE (AchE), ATPase, β-HEXOSAMINIDASE (β-HEX) AND β-GALACTOSIDASE (β-Gal) IN THE SUBFRACTIONS I, II, III OBTAINED FROM PIG BRAIN FRACTION P_2 AFTER CENTRIFUGATION ON THE 7.5%/20% FICOLL GRADIENT. STARTING TISSUE:

Fraction, subfraction	Protein		Neuraminidase		LDH	
	mg	Distribution (%) *	Total (munits)	Distribution (%) *	Total (munits)	Distribution (%) *
"P_2"	177 ± 11		5.62 ± 0.41		41.8 ± 3.5	
"P_2" I	43.0 ± 6	32.4	undetectable		0.67 ± 0.04	2.1
"P_2" II	65.0 ± 7	49.0	3.8 ± 0.45	90.1	27.1 ± 2.2	84.4
"P_2" III	24.7 ± 3	18.6	0.42 ± 0.04	9.9	4.32 ± 0.8	13.5
Total	132.7	—	4.22	—	32.1	—
Recovery (%)	75		75.1		76.8	

* Of the total recovered.

the behaviour during the extraction procedure of neuraminidase and lactate dehydrogenase on one side and β-hexosaminidase and β-galactosidase on the other.

The bulk of neuraminidase and lactate dehydrogenase was extracted with the first treatment, the extraction being completed after 5 treatments (Fig. 1a). Conversely, the release of β-hexosaminidase and, especially, of β-galactosidase, continued appreciably with all treatments. Moreover (Fig. 1b) the specific activity of neuraminidase and lactate dehydrogenase in the extracts diminished throughout the extraction while that of β-hexosaminidase and β-galactosidase gradually increased up to 10-fold.

(2) Extractibility of neuraminidase from the cytosol of nerve endings

(a) Release of neuraminidase activity from P_2 fraction by hypoosmotic treatment. As shown in Table II, hypoosmotic treatment caused the relase of 19% of the protein, 93.7% of lactate dehydrogenase and 79% of β-hexosaminidase present in fraction P_2. This indicates, in particular, that (besides a substantial rupture of lysosomes) an almost complete lysis of the cytoplasm trapping-particles present in the P_2 (nerve endings) occurred. The released, soluble protein carried 1.25 munits neuraminidase/g fresh tissue, the specific activity of which was 8 times that in the cytosol derived from neuronal body and glial cells.

(b) Release of neuraminidase activity from isolated nerve endings by hypoosmotic treatment. The results of these experiments are shown in Figs. 2 and 3 and Table III. Morphological examination (Fig. 2) showed that the myelin subfractions contained mainly myelin fragments with various membranous contaminants. The nerve ending subfractions contained pinched-off nerve endings, few junctional processes, mitochondria, dense bodies (possibly lysosomes) and some contaminating myelin fragments. In general, nerve endings constituted more than 60% of the particle population. By increasing the Ficoll concentration in the second gradient layer from 14 to 22%, the nerve ending subfraction became more abundant, with some increase in the percentage of nerve endings. The mitochondrial subfractions contained mitochondria, dense bodies and

4.5 g. NEURAMINIDASE ACTIVITY WAS DETERMINED IN THE SUPERNATE OBTAINED AFTER HYPOOSMOTIC TREATMENT OF THE FRACTION P_2 OR ITS SUBFRACTIONS, ADDITION OF KCl UP TO 0.156 M AND CENTRIFUGATION AT 105 000 × *g* (1 h). THE DATA REPORTED ARE THE MEAN VALUES ± S.E. OF 6 EXPERIMENTS.

AchE		ATPase		β-Hex		β-Gal	
Total (munits)	Distribution (%) *	Total (munits)	Distribution (%) *	Total (munits)	Distribution (%) *	Total (munits)	Distribution (%) *
2647 ± 178		18.8 ± 1.5		1736 ± 142		82.1 ± 6.8	
239.7 ± 35	11.7	2.5 ± 0.3	16.7	59.1 ± 7	4.7	3.0 ± 0.4	5.3
1616.7 ± 186	78.9	9.9 ± 1.5	65.8	507.1 ± 60.7	40.3	20.1 ± 2.4	35.1
192.6 ± 28	9.4	2.6 ± 0.4	17.5	692.1 ± 95.8	55.0	34.1 ± 5.3	59.6
2049		15.0	—	1258.3	—	57.2	—
77.4		79.6		72.5		69.7	

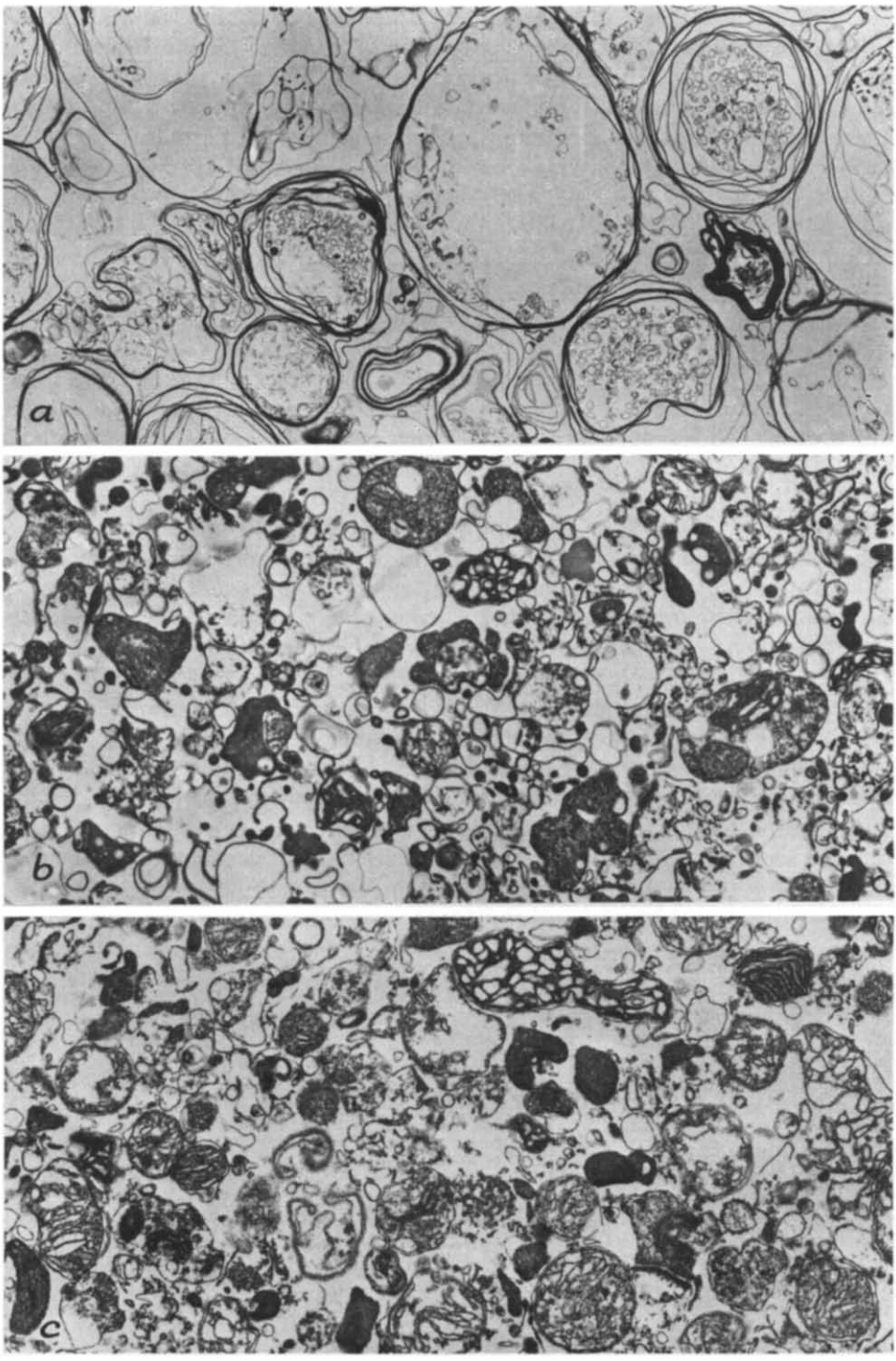


Fig. 2. Electron micrographs of the three subfractions obtained after centrifugation of pig brain fraction P_2 on the Ficoll density gradient 7.5%—20%. (a) Myelin subfraction (magnification X4000); (b) nerve-ending subfraction (magnification X9000); (c) mitochondria subfraction (magnification X9000).

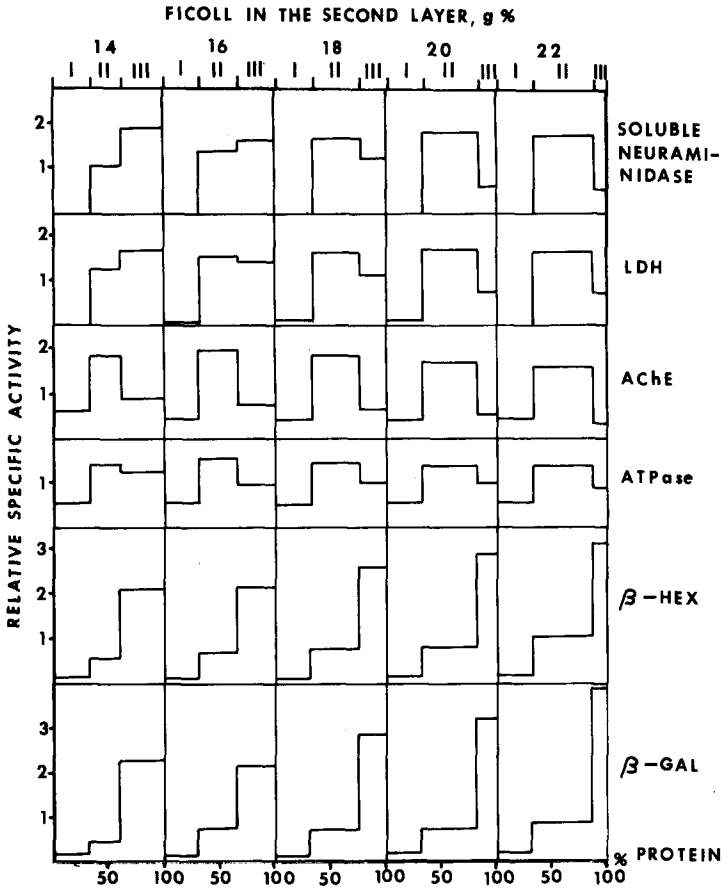


Fig. 3. Relative specific activity of soluble neuraminidase, lactate dehydrogenase (LDH), acetylcholine esterase (AchE), ATPase, β-hexosaminidase (β-Hex) and β-galactosidase (β-Gal) in the 3 subfractions (I, II, III) obtained after centrifugation of the P_2 on a gradient made by two layers of Ficoll: the upper one at 7.5% Ficoll concentration; the lower one at a Ficoll concentration varying, in the different experiments, from 14% to 22%. The data exposed are the average of 3 experiments.

nerve terminals. Nerve endings predominated in the mitochondrial subfractions obtained at the lowest Ficoll concentration used (14%), but diminished markedly passing from 14 to 22% Ficoll. In this latter case, the nerve endings in the mitochondrial subfraction were only minor contaminants. The biochemical findings (Fig. 3) confirmed the information obtained by morphological examination. The distribution of β-hexosaminidase and β-galactosidase in the three subfractions was quite parallel. The Ficoll experiments showed that the relative specific activity of the two enzymes progressively increased both in the nerve ending and the mitochondrial subfractions, with a marked enhancement of the recovery in the nerve ending subfractions. The amount of these lysosomal enzymes in the myelin subfractions was extremely low. ATPase and acetylcholinesterase provided the highest relative specific activity in the nerve ending subfractions and the recovery of enzyme activity in this subfraction markedly increased between the 14 and the 22% Ficoll gradient. The amount of these

enzymes in the lightest subfractions remained approximately equal, in terms of either total and relative specific activity. The behaviour of releasable neuraminidase closely paralleled that of lactate dehydrogenase; both enzymes were present, as expected, only in the nerve ending and mitochondrial subfractions. By increasing the Ficoll concentration from 14—22%, their relative specific activities showed a slight increase in the nerve ending subfraction and a marked decrease in mitochondrial subfraction. Concurrently, the content of both enzymes in the nerve ending subfraction increased until about 90% of the total recovered.

The data concerning the distribution of protein neuraminidase, β-hexosaminidase, β-galactosidase, ATPase, acetylcholinesterase and lactate dehydrogenase in the three subfractions obtained from fraction P_2 in the Ficoll gradient 7.5%/20% are shown in Table III. They indicate the good reproducibility and recovery (75% for protein and 70%—80% for the different enzymes) of the procedure.

The nerve ending subfractions, when submitted to hypoosmotic shock, released in the supernatant solution about 15% of protein and 93% of lactate dehydrogenase, this indicating an almost complete lysis of nerve endings. The amount of liberated neuraminidase was 0.84 munits/g fresh tissue, that is approx. 67% of that released by crude fraction P_2. Considering that the recovery during the subfractionation procedure was from 70 to 75%, it can be concluded that over 90% of the nerve endings contained in fraction P_2 were recovered in the nerve ending subfraction.The neuraminidase specific activity was 1.5 times that of the enzyme released from crude fraction P_2.

(c) Solubility of cytosolic neuraminidase. Preparations of cytosolic neuraminidase, derived from neuronal bodies and glial cells, or from nerve endings, on standing at 2—4°C up to 2 h in 0.156 M KCl, showed neither formation of

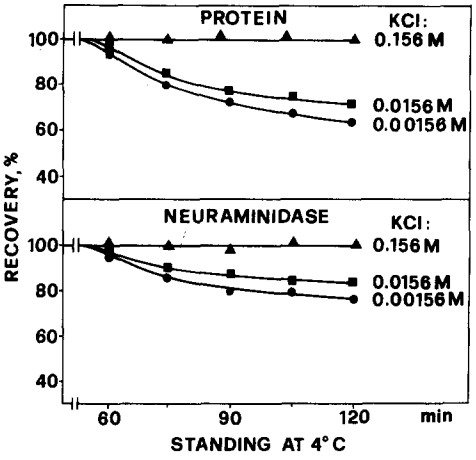
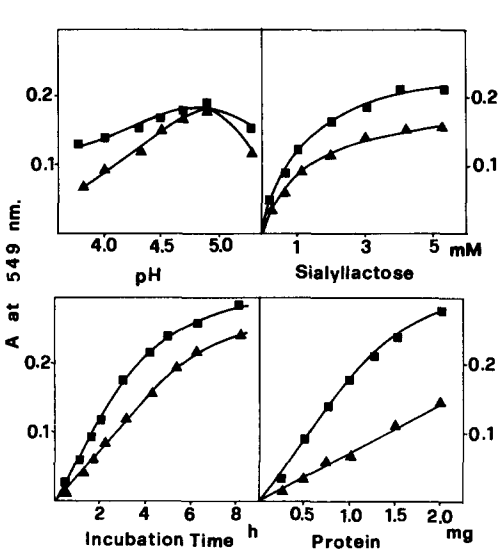


Fig. 4. Effect of ionic strength on solubility of protein and activity of neuraminidase extracted from brain cytosol (neuronal body and glial cell cytosol and nerve ending cytosol). The starting mixtures, in 0.156 M KCl, were properly diluted with distilled water, allowed to stand at 4° C for a fixed time, then centrifuged at 105 000 × *g* (20 min). The time indicated in the figure starts from the dilution and includes that taken up by centrifugation. The data exposed are the mean values of 6 experiments.

insoluble material nor appreciable loss of neuraminidase activity. On the contrary, when the same preparations were diluted 1 : 10 or 1 : 100 with distilled water, a time-dependent, partial protein insolubilization occurred (Fig. 4). For at least 45 min after dilution, proteins remained completely soluble. They then started precipitating and a parallel loss of neuraminidase was recorded. However, the neuraminidase activity was almost all recovered in the precipitate; the precipitated enzyme could not be resolubilized by restoring the initial ion concentration.

(d) Basic properties of the neuraminidase released after lysis from nerve endings. The optimum pH of the neuraminidase released by hypoosmotic shock from nerve endings was 4.9 (Fig. 5); the K_m value for sialyllactose was 0.7 mM; the V/t relationship was linear up to 3 h and the V/e relationship up to 1 mg protein.

The behaviour of the neuraminidase present in cytosol from neuronal body and glial cells (studied in parallel) was quite similar. Substantial differences in the enzymes of different cytosolic origin were observed with regard to their stability. The nerve ending neuraminidase lost 70% of its activity after 3-days standing at 4°C and 55% activity when submitted to 5 freezing-and-thawing treatments (Fig. 6). The other neuraminidase lost only 15% activity on standing at 4°C for the same time and was resistant to freezing-thawing. When the two enzyme preparations were mixed together, at equal neuraminidase activities and submitted to the same treatments, an exactly intermediate behaviour was observed.



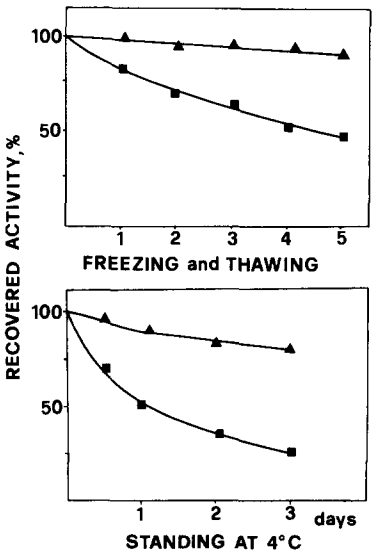


Fig. 5. Main properties of neuraminidase deriving from the cytosol of neuronal bodies and glial cells (▲———▲) and from the cytosol of nerve endings (■———■). The data presented are the average of three experiments.

Fig. 6. Behaviour of neuraminidase deriving from the cytosol of neuronal bodies and glial cells (▲———▲) and from the cytosol of nerve endings (■———■) upon freezing and thawing and upon storage at 4°C.

Discussion

It is generally accepted that mammalian tissues, including brain, contain a cytosol [1,2,14—16], a plasma-membrane-bound [17—20] and a lysosomal [16,21,22] neuraminidase. If any relationships exist among the three neuraminidases it is not known. The membrane enzyme is so firmly bound that all attempts to solubilize it by physical and mild chemical methods were unsuccessful. A possible leakage of neuraminidase from lysosomes into the cytoplasm cannot be excluded. With regards to the cytosol neuraminidase from brain tissue, an open problem is whether it originates from the neuronal body and glial cells and/or from nerve endings. With the present work, the situation of brain cytosol neuraminidase was focused.

Procedurally, the cell sap moves to the supernatant solution after tissue homogenization in buffered isotonic media and centrifugation at 105 000 × g for 1 h. In the case of brain, if proper precautions are taken not to destroy nerve endings, the above supernatant fraction contains cytosol derived from neuronal bodies and glial cells. Of course, any loosely-bound protein and material from ruptured subcellular particles is also found in the supernatant fraction. In fact, after homogenization with either isoionic and hypoionic isotonic solutions, we observed the liberation of 19% of the brain protein and 70% of that of lactate dehydrogenase; this is expected for the cytosol derived from neuronal bodies and glial cells. However a substantial release of β-hexosaminidase and β-galactosidase was also found to occur. Neuraminidase was extracted at about 0.7 munits/g fresh tissue. The most likely origin of this from the cytosol and not from lysosomes is shown by the fact that the behaviour of the enzyme during extraction paralleled that of lactate dehydrogenase. Lysosomal enzymes followed a quite different trend: the amount of neuraminidase recovered in the supernatant fraction using 0.32 M sucrose as homogenizing solution was markedly lower than that obtained with isoionic solutions. A similar finding was reported by Tulsiani and Carubelli [16] for the cytosol neuraminidase from chick liver. Sucrose facilitates adhesion or non-specific adsorption of soluble neuraminidase to particular material, the phenomenon being prevented by the addition of KCl. This effect, which is absent with lactate dehydrogenase, should be checked for other enzymes.

Pinched-off nerve endings, formed during brain homogenization, are contained in the so-called 'fraction P_2', together with mitochondria, lysosomes, myelin fragments, etc. By sumitting this fraction to hypoosmotic treatment, the cytosol entrapped into nerve endings could be released. In fact, we observed that about 20% of the total content of protein and 93% of lactate dehydrogenase, present in fraction P_2 moved into the supernatant fraction proving that lysis of nerve endings was almost complete. Under the same conditions, 1.25 munits neuraminidase/g fresh tissue were also released. However, about 80% of the total fraction P_2 content of β-hexosaminidase and β-galactosidase was also liberated into the medium. Thus, the appearance of a 'soluble' neuraminidase after lysis may mean that the enzyme was derived from the cytosol of nerve endings, but does not exclude its possible origin from the lysosomes contained in fraction P_2.

Direct evidence for the nerve ending origin of this neuraminidase activity was

provided by subfractionation studies. These studies were devised in order to follow the distribution of particles in the different bands formed after centrifugation of the P_2 on Ficoll gradients. The amount of nerve endings equilibrating in the interface band became more and more abundant by increasing the Ficoll concentration in the lower gradient; it was almost complete when this was 20%. Since, upon hypoosmotic treatment of the nerve-ending subfraction, the release of protein was about 15% and that of lactate dehydrogenase 93%, lysis of nerve endings was complete. The behaviour of soluble neuraminidase during subfractionation was substantially identical to that of lactate dehydrogenase, while different from that of β-hexosaminidase and β-galactosidase. These two lysosomal enzymes had increased relative specific activities, upon changing the Ficoll concentration in the second layer from 14% to 22%, both in the pellet and in the interface band. This indicates that one population of lysosomes behaved like nerve terminals (intra- and extra-synaptosomal lysosomes, [24,26]) and a second population, denser, precipitated in the pellet, confirming previous work [23,24]. The pellet obtained with 20 and 22% Ficoll had the highest relative specific activities of β-hexosaminidase and β-galactosidase and, conversely, the lowest relative specific activity of neuraminidase. Thus lysosomes precipitating in these pellets contained very little neuraminidase. However, a population of lysosomes floated also at the gradient interface; therefore at least a portion of the neuraminidase released from the interface band might be of lysosomal origin. This hypothesis relies on the assumption that only the lysosomes of the lightest population contain releasable neuraminidase activity. Thus far, no evidence can support this assumption, even if differences in the behaviour of lysosomal enzymes from diverse subcellular populations have been reported [24].

It seems reasonable to assume that the neuraminidase liberated from the nerve-ending subfractions was derived from nerve-ending cytosol. In consequence, the neuraminidase liberated by hypoosmotic treatment of crude fraction P_2 was also derived from nerve endings contained in this fraction.

The total content of neuraminidase from the cytosol trapped in neuronal bodies and glial cells was 0.7 munits/g fresh tissue; that from nerve ending cytosol 1.25 munits. The figures for lactate dehydrogenase from the same two pools of cytosol were, respectively, 90.4 and 9.35 munits/g fresh tissue. The figures of lactate dehydrogenase reasonably fit the proportion between the cytosol from neuronal bodies and glial cells and that from nerve endings. This means that the cytosol of nerve endings (at least in pig brain) is highly enriched in neuraminidase (more than 15-fold, with respect to that surrounding nuclei and of glial cells).

It is notable that neuraminidase from nerve-ending cytosol is labile on prolonged standing at 4°C and to freezing and thawing, while that derived from neuronal body and glial cell cytosol was stable. Since this latter neuraminidase maintained its stability even after more than 100-fold purification [3], the above findings are sufficient to state that the molecular properties of the two enzymes are different. It is known [3] that the neuraminidase derived from neuronal bodies and glial cells occurs in two forms, A and B. It is possible that the cytosol of nerve endings contains multiple forms of neuraminidase different from those occurring in the neuronal body and in glial cells, perhaps with different degree of stability. This situation is currently under investigation.

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