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ASSAY OF BRAIN PARTICULATE NEURAMINIDASE III. PREPARATION OF THE ENZYME DEVOID OF ENDOGENOUS SUBSTRATES

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SUMMARY

1. The crude preparation of brain particulate neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) (0–105 000 × *g* pellet, prepared from rabbit brain homogenate in isotonic sucrose) was depleted from endogenous (intrinsic) substrates by autolysis performed under the following conditions: 0.18 M sodium acetate buffer, 0.3% Triton X-100, final pH 4.2, 25°, 5 h incubation.

2. After this treatment only 54% protein, 51% sialoglycoproteins, 89% gangliosides, 75% total and lipid-bound phosphorus remained particulate. Neuraminidase remained firmly particulate and fully active (97% recovery). Thus, the 105 000 × *g* sediment obtained after the above treatment is the enzyme preparation devoid of endogenous substrates.

3. The basic properties of the enzyme present in the preparation devoid of endogenous substrates were the same observed in the starting crude preparation. The K_m for disialoganglioside GD1a was $4.8 \cdot 10^{-6}$ M; the v_{max} 1.6 nmoles released *N*-acetylneuraminic acid per min.

4. The examined properties of particulate neuraminidase depleted from endogenous substrates by our procedure were very similar to those of the enzyme prepared according to Z. LEIBOVITZ AND S. GATT (*Biochim. Biophys. Acta*, 152 (1968) 136). However, the final enzyme recovery was 5 times higher with our procedure.

INTRODUCTION

The presence in brain of a particle-bound and a soluble neuraminidase has been ascertained by several investigators[1–5]. Information concerning the optimum pH, substrate specificity, detergent requirements[2–4,6], the regional[4,7,8] and subcellular[9,10] distribution, as well as the developmental profiles[11–14] of the enzyme(s) have also been given.

Since the first report by MORGAN AND LAURELL[15] many authors have observed the constant presence, in both crude and purified preparations of particulate neur-

Abbreviation: NANA, *N*-acetyl-neuraminic acid.

aminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18), of sialo compounds easily hydrolyzed by the enzyme during the incubation (endogenous or intrinsic substrates). The recent intensive study of ÖHMAN *et al.*[6] on human brain neuraminidase, prepared by the method of LEIBOVITZ AND GATT[2], clearly showed that a meaningful determination of the kinetic features and substrate specificity of the particulate enzyme could be done only after the complete depletion of endogenous substrates.

Despite the above valuable information there is still much uncertainty as to the nature and specificity of particulate and soluble neuraminidase, as well as the differences in the distribution and properties of the two enzymes in various animal species.

The present investigation concerns the following experimental problems: (a) to prepare brain particulate neuraminidase devoid of the soluble enzyme and of endogenous substrates, using a simple procedure, mild enough to maintain the native arrangement of the enzyme; (b) to assess the suitability of the enzyme preparation for accurate kinetic studies.

The initial source of the particulate enzyme was the 0–105 000 × *g* sediment prepared from rabbit brain, homogenized in 0.32 M sucrose. The depletion of intrinsic substrates was accomplished by action of the bound enzyme itself, using the experimental conditions described by LOMBARDO *et al.*[16].

EXPERIMENTAL

Materials

Commercial Chemicals were of analytical or of the highest available grade. Solvents were distilled before use. Triton X-100, sodium cholate, *N*-acetylneuraminic acid (NANA) and crystalline bovine serum albumin were obtained from Sigma Chemical Co.

Ganglioside GD1a* was prepared, as the potassium salt, from beef brain according to TETTAMANTI AND ZAMBOTTI[3]. The purity of the final preparation was better than 96%, the contaminants being ganglioside GM1 and silica gel. It was stored in a vacuum desiccator at −10° and put into aqueous solution just before use.

Dowex 2-X8 resin, 200–400 mesh (Dow Chemical Co.) was prepared in acetate form according to SVENNERHOLM[18]. Dialysis tubing (0.25 inch width) was purchased from A. Thomas Co.; cellulose nitrate filter membranes from Sartorius-Membranfilter GmbH.

Adult male rabbits (Burgundy strain), 2 kg average weight, were used. The animals were killed by decapitation and the brain immediately removed, freed as much as possible of meninges and white matter, weighed, washed in ice-cold homogenizing solution and homogenized as described below.

The homogenizations were carried out with glass homogenizers, provided with a mechanically driven teflon pestle (0.25 mm clearance) (A. Terzano, Milan). Centrifugations were done in a Sorwall, RC-2 model, (runs below 30 000 × *g*) and an International E.C., model B-60, (runs over 100 000 × *g*) refrigerated centrifuge. The incubations were performed in an incubator shaker (Colora).

* The ganglioside nomenclature according to SVENNERHOLM[17] was followed.

Glass redistilled water was used for preparing the different solutions. All operations were conducted at 0–4° unless otherwise stated.

Enzyme preparations

Crude preparation of particulate neuraminidase. The crude preparation of particulate neuraminidase, that is the 0–105 000 × *g* (1 h) sediment, was prepared from the homogenate of brain in buffered 0.32 M sucrose according to PRETI *et al.*[19]. Prior to use, the final pellet was homogeneously suspended with bidistilled water (4 mg/g starting fresh tissue)[19].

Preparation of particulate neuraminidase devoid of endogenous substrates. The crude enzyme preparation was incubated in 0.18 M sodium acetate buffer, final pH 4.2., in the presence of Triton X-100 (0.3% final concentration) either at 25° for 5 h or at 19° for 20 h (ref. 16). The mixtures were then centrifuged at 105 000 × *g* for 1 h. The sediment was suspended with bidistilled water (4 ml/g starting fresh tissue) and homogenized at 1500 rev./min for 1 min (glass homogenizer clearance: 0.15 mm). This material is referred to as the enzyme preparation devoid of endogenous substrates. The supernatant was concentrated to a final protein concentration of about 10 mg/ml by either ultrafiltration on dialysis tubing under low vacuum, or filtration on Sartorius filter membranes (porosity 0.005μ; molecular weight exclusion limit: 10 000).

Other enzyme preparations. An enzyme preparation according to the method of LEIBOVITZ AND GATT[2] was made. The final supernatant at 105 000 × *g* (after treatment with 0.5% Triton X-100) was used.

Enzyme assay

The incubation mixtures contained 0.1–1.0 mg enzyme (as protein), 0.18 M sodium acetate buffer, 0.025–1.00 mM disialoganglioside GD1a, in a total volume of 0.65 ml. Triton X-100, when present, was added in aqueous solution. The pH of the reaction mixture and the length of the incubation varied according to the assay purpose. When the activity on endogenous substrates had to be measured the addition of GD1a was obviously omitted.

The incubation mixtures, set up at the temperature of melting ice, were immersed into the incubator shaker, adjusted at the desired temperature, and the incubation initiated. The determination of the enzyme reactions, storage of the test tubes, direct determination of liberated NANA and the calculation of the enzyme activity were done according to PRETI *et al.*[19]. In those experiments where the incubations were carried out in the presence of excess sucrose (for instance when using the concentrated supernatant), the incubation mixtures were subjected to ion exchange chromatography on Dowex 2-X8, according to the method described by PRETI *et al.*[19], in order to eliminate interferring sucrose prior to the determination of free NANA.

The control incubation mixtures (blanks) for the assay of neuraminidase activity on endogenous substrates were performed according to PRETI *et al.*[19] (boiled enzyme was used); the control incubation mixtures for the assay of the neuraminidase activity on added GD1a were performed according to TETTAMANTI *et al.*[20].

When the enzyme preparation according to LEIBOVITZ AND GATT[2] was used the enzyme assay was carried out following the indication of ÖHMAN *et al*[6].

One unit of neuraminidase is defined as the amount of enzyme liberating 1 n mole NANA per min in the given conditions.

Analytical methods

Protein was determined by the method of LOWRY *et al.*[21]. The extraction and separation of gangliosides and sialoglycoproteins from the enzyme preparation was accomplished by the method of TRAMS AND LAUTER[22] as modified by TETTAMANTI *et al.*[23].

Ganglioside-bound NANA was assayed by the resorcinol method of SVENNERHOLM[18]. Protein-bound NANA was determined by WARREN's[24] method after hydrolysis performed according to TETTAMANTI *et al.*[23].

Total and lipid-bound phosphorus was determined by the method of ZILVERSMIT AND DAVIS[25].

RESULTS

Neuraminidase activity of the crude enzyme preparation

The time course of neuraminidase activity contained in the crude enzyme preparation in the absence or presence of added substrate (GD1a), is reproduced in Fig. 1. The reaction rate, 0.73 unit/mg protein in the presence only of endogenous substrates, appeared not to be enhanced by the addition of GD1a (up to 0.075 mM). Higher concentrations of added GD1a (over 0.1 mM) caused a marked inhibition on neuraminidase activity.

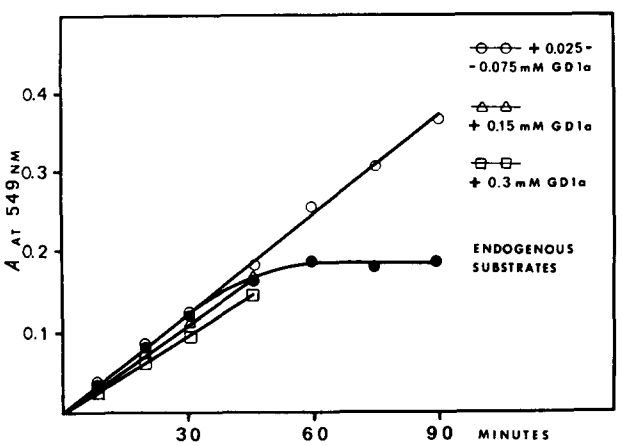


Fig. 1. Progress curve of brain particulate neuraminidase activity on endogenous (intrinsic) substrates in the absence or the presence of added substrate (disialoganglioside GD1a). Assay conditions as reported in EXPERIMENTAL. The assay mixtures, containing 0.48 mg protein, 0.3% Triton X-100, with final pH 4.2, were incubated at 37°.

Neuraminidase activity of the enzyme preparation devoid of endogenous substrates

The treatment used for depletion of endogenous substrates maintained the particulate nature and the full activity of the enzyme (Table I). In fact after the mixtures, incubated either at 25° for 5 h or at 19° for 20 h, were centrifuged at 105 000

TABLE I

NEURAMINIDASE ACTIVITY OF THE ENZYME PREPARATION DEVOID OF ENDOGENOUS SUBSTRATES

Protein and neuraminidase (expressed, respectively, as total mg and total units/g fresh tissue) present in the crude enzyme preparation before and after the treatment for the complete depletion of neuraminidase endogenous substrates. The treated mixtures were spun at 105 000 × *g* (1 h) and divided into a sediment and a supernatant. Neuraminidase specific activity (units/mg protein) was calculated from the maximum reaction rate measured under optimal assay conditions. The crude enzyme preparation was the 0–105 000 × *g* sediment obtained from rabbit brain homogenate in 0.32 M sucrose. The removal of endogenous substrates was accomplished by incubating in 0.18 M sodium acetate buffer, final pH 4.2, in the presence of 0.3% Triton X-100, for 5 h at 25° or 20 h at 19°.

Preparation	*Protein (mg)*	*Recovery (%)*	*Neuraminidase*			
			Specific activity (units/mg protein)		*Total activity (total units)*	*Recovery (%)*
			On endogenous substrates	*On added GD1a*		
Crude enzyme preparation	60.4	—	0.73	—	44.1*	—
Same after treatment at 25° for 5 h						
Sediment	32.6		0.00	1.31	42.7**	
Supernatant	27.2		0.00	0.00		
Total	59.8	99				96.8
Same after treatment at 19° for 20 h						
Sediment	32.1		0.00	1.29	41.4**	
Supernatant	27.4		0.00	0.00		
Total	59.5	98.5				94

* Neuraminidase assay: crude enzyme preparation, 1 mg (as protein), 0.18 M sodium-acetate buffer, final pH 4.2, 0.3% Triton X-100, 37°, 15 min (blank mixtures prepared with "boiled" enzyme). Substrate: endogenous oligosialogangliosides (available in concentration already saturating the enzyme).

** Neuraminidase assay: enzyme preparation devoid of endogenous substrates, 0.4 mg (as protein), 0.18 M sodium acetate buffer, final pH 4.1, 0.05 mM GD1a, 37°, 15 min.

× *g* for 1 h, the enzyme was recovered only in the sediment. No neuraminidase activity was detected in both supernatants within a wide range of experimental conditions (pH: from 3.5 to 7.0; protein: from 1 to 5 mg; GD1a concentration: from 0.025 to 2 mM). Neuraminidase specific activity shifted from 0.73 unit/mg protein (crude preparation) to 1.31 and 1.29 units/mg protein in the sediment obtained after incubation, respectively, at 25° for 5 h and 19° for 20 h (the values were based on measured maximum reaction rates at apparent GD1a optimum concentration). The enzyme recovery was, respectively, 96.8 and 94%. The procedure routinely used for the complete removal of endogenous substrates was the treatment at 25° for 5 h.

Behaviour of some constituents of the crude enzyme preparation during the treatment for the depletion of endogenous substrates

Efforts were made to evaluate the possible modifications encountered by the enzyme containing material during the treatment for the removal of endogenous

TABLE II

BEHAVIOUR OF PROTEIN, GLYCOPROTEIN-BOUND NANA, GANGLIOSIDE-BOUND NANA, FREE NANA, TOTAL PHOSPHORUS AND LIPID-BOUND PHOSPHORUS, CONTAINED IN THE CRUDE ENZYME PREPARATION, DURING THE TREATMENT FOR THE DEPLETION OF NEURAMINIDASE ENDOGENOUS SUBSTRATES

The treated mixtures and the correspondent controls blocked at o time were spun at 105 000 × *g* (1 h) and divided into a sediment and a supernatant. The reported values, which are referred to 1 g starting fresh tissue, are the average of 5 experiments. The crude enzyme preparation was the 0–105 000 × *g* sediment obtained from rabbit brain homogenate in 0.32 M sucrose. The treatment for removal of endogenous substrates was accomplished as referred in the legend of Table I using the condition: 25° for 5 h. The control reaction mixtures were blocked at zero time immediately after being set up. The % distribution was calculated on the total recovered material.

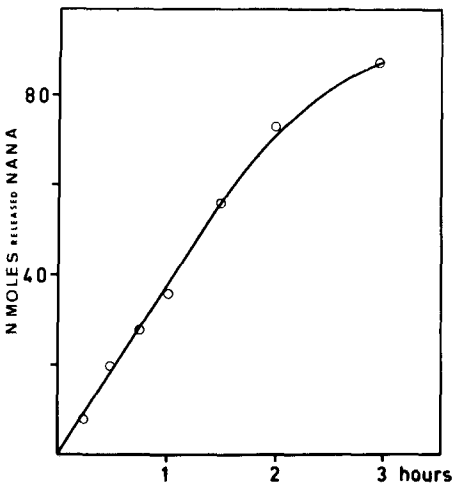
Preparation	*Protein*		*Glycoprotein-bound NANA*		*Ganglioside-bound NANA*		*Total phosphorus*		*Lipid-bound phosphorus*		*Free NANA*	
	(mg)	*(%)*	*(μg)*	*(%)*	*(μg)*	*(%)*	*(mg)*	*(%)*	*(mg)*	*(%)*	*(μg)*	*(%)*
Crude enzyme preparation (untreated)	60.4	—	332	—	1153	—	3.000	—	1.932	—	0.00	—
Same after treatment at 25° for 5 h												
Sediment	32.6	54.5	156	50.6	470	89	1.978	73.5	1.432	77.1	25	4.2
Supernatant	27.2	45.5	153	49.4	58	11	0.706	26.5	0.425	22.9	578	95.8
Recovery Total	59.8		309		528		2.684		1.857		603	
Recovery %		99		93.2		45.8		88.8		96.1		
Treated mixture control, blocked at o time												
Sediment	32.1	53.7	218	70	935	84	1.906	72.2	1.407	76.4	—	
Supernatant	27.4	46.3	94	30	183	16	0.734	27.8	0.435	23.6	—	
Recovery Total	59.5		312		1118		2.640		1.842			
Recovery %		98.5		94		97		88		95.4		

substrates. The following parameters were studied: gangliosides and sialoglycoproteins (both expressed as bound NANA), protein, total phosphorus and lipid-bound phosphorus (Table II). After incubation at 25° for 5 h under the conditions previously referred (see EXPERIMENTAL) and centrifugation at 105 000 × *g* for 1 h, approximately 11% gangliosides, 49% sialoglycoproteins, 46% protein and 23–26% of both total and lipid-bound phosphorus moved into the supernatant. The recovery was between 89 and 99% for all parameters except for gangliosides. Ganglioside recovery was only 46%, the remainder being free NANA, removed during the incubation and present in the supernatant.

The data obtained at zero time and after incubation (Table II) show that the "solubilization" of protein, gangliosides and phosphorus occurred at about the same extent at zero time and after incubation. Probably the phenomenon is not dependent on events taking place during incubation, but solely due to the solubilizing effect of Triton X-100. On the contrary, sialoglycoproteins underwent a 30% solubilization at zero time and an additional 20% during the incubation.

Properties of particulate neuraminidase present in the preparation devoid of endogenous substrates

The activity of bound neuraminidase present in the preparation devoid of



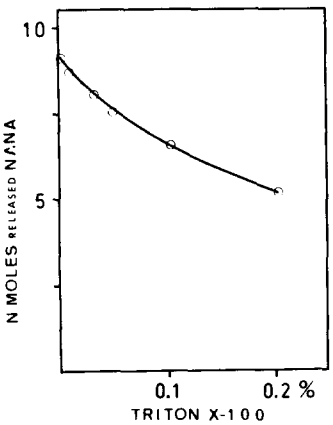
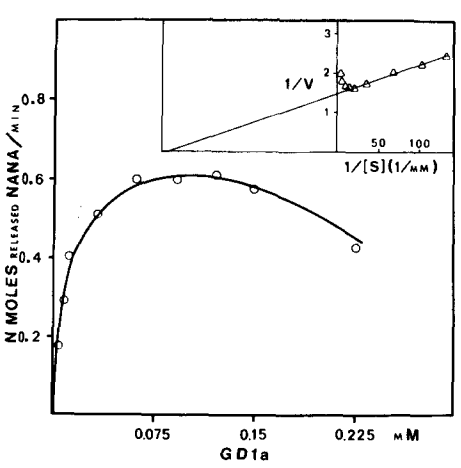


Fig. 2. Time course of brain particulate neuraminidase activity at 37°. The enzyme preparation devoid of endogenous substrates was used. Assay conditions as reported in EXPERIMENTAL; the assay mixtures contained 0.46 mg protein and 0.10 mM GD1a.

Fig. 3. Effect of Triton X-100 on the activity of brain particulate neuraminidase devoid of endogenous substrates. The assay mixtures, prepared as reported in EXPERIMENTAL, contained 0.5 mg protein, 0.1 mM GD1a and were incubated for 15 min.



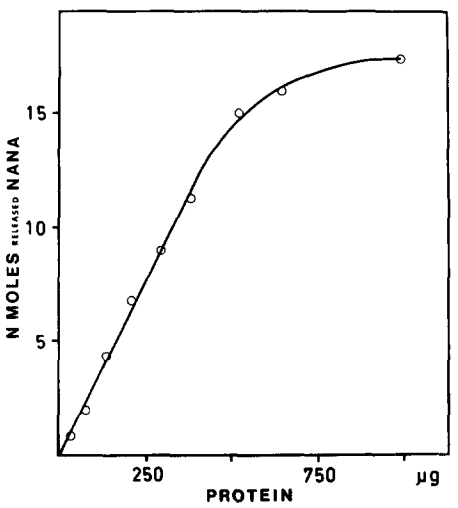


Fig. 4. Effect of ganglioside GD1a concentration on the activity of brain particulate neuraminidase devoid of endogenous substrates. The assay mixtures contained 0.4 mg protein.

Fig. 5. Protein–activity relationship of brain particulate neuraminidase devoid of endogenous substrates. The mixtures, containing 0.05 mM GD1a were incubated, at optimum pH, for 30 min.

endogenous substrates was linear with time till 90 min (Fig. 2). The enzyme had optimum pH 4.1 and was markedly inhibited by Triton X-100 (Fig. 3).

A typical substrate–velocity curve is demonstrated in Fig. 4. With the use of the LINEWEAVER AND BURK[26] plot, the calculated value for K_m was $4.8 \cdot 10^{-6}$ M and for v_{max} 1.6 nmoles released NANA per min. At high substrate concentration a pronounced fall in velocity was observed: a 50% decrease from the highest observed reaction velocity occurred at 0.5 mM GD1a concentration.

A linear relationship was observed between protein concentration and NANA released, under the experimental conditions referred in Fig. 5.

Storage of the enzyme preparation rid of endogenous substrates at 0–4° for 6 days, and frozen at −20° for 3 months (period of time actually checked) resulted in no appreciable changes of neuraminidase activity.

Comparative study of the neuraminidase prepared according to LEIBOVITZ AND GATT *and to the present procedure*

The preparation of neuraminidase obtained from rabbit brain by LEIBOVITZ AND GATT[2] method contained small amounts of endogenous substrates which were generally exhausted by 20–25-min preincubation carried out as indicated by ÖHMAN *et al.*[6]. The enzyme had optimum pH 4.3, apparent specific activity 1.26 units/mg protein, and required the presence of Triton X-100 for maximum activity (optimum final concentration of the detergent 0.5%).

The study of the effect of GD1a concentration on enzyme activity showed a marked inhibition at high concentrations (50% decrease at 3 mM GD1a concentration, in accordance with the evidences reported with human enzyme by ÖHMAN *et al.*[6]. The K_m and v_{max} values, calculated from the $1/v$ over $1/[S]$ plot[26] were, respectively, $2.8 \cdot 10^{-5}$ M and 1.4 nmoles released NANA per min.

A comment concerning the enzyme recovery (in terms of total units/g fresh tissue) with the procedure of LEIBOVITZ AND GATT[2] and the present one is worth making. The specific activity of the two enzymes, determined under optimal conditions were practically identical (1.26 and 1.31 units/mg protein) while the protein recovery was 7 mg/g fresh tissue for the LEIBOVITZ AND GATT[2] preparation, 32.6 mg for our preparation. Therefore the total activity (as units/g fresh tissue) obtained with our procedure was about 5 times higher (42.7 against 8.82 units).

DISCUSSION

The source of particulate neuraminidase used in this investigation was the 0–105 000 × *g* pellet prepared from rabbit brain homogenate in isotonic sucrose. The pellet, completely free of soluble neuraminidase, contained all the brain sialocompounds, gangliosides[27,28] and sialoglycoproteins[29]. Of them, as demonstrated by LOMBARDO *et al.*[16], only oligosialogangliosides (GD1a, GD1b, GT1b, GQ1) acted as endogenous substrates for particulate neuraminidase. The kinetic study of the hydrolysis of intrinsic substrates presented here, clearly showed that the initial reaction rate was not enhanced by added disialoganglioside GD1a. Therefore, in the case of crude particulate neuraminidase prepared from rabbit brain, the activity on endogenous substrates represented the maximal enzyme activity.

The depletion of endogenous substrates from the crude enzyme preparation,

necessary for meaningful kinetic studies, was accomplished by action of the bound enzyme, as suggested by ÖHMAN *et al.*[6], under the following conditions: 0.18 M sodium acetate buffer, pH 4.2; Triton X-100 (0.3% final); 25° for 5 h. After the treatment neuraminidase was fully active (97% recovery), firmly particulate (sedimented entirely at $105\,000 \times g$) and retained unmodified some properties, like optimum pH (4.1) and the strong inhibition by excess substrate. This is consistent, in our opinion, with the assumption that the enzyme molecule did not undergo relevant modifications during the autolysis treatment. Surprisingly enough Triton X-100, known to activate the enzyme in the crude preparation[16,20], behaved as a strong inhibitor. A possible explanation of the above phenomenon is that the enzyme molecule, after the depletion of endogenous substrates, remained fixed in the conformation most suitable for activity.

The enzyme preparation devoid of endogenous substrates was fairly suitable for kinetic studies. The initial reaction rate could be accurately evaluated, provided that pure substrate was used for the enzyme assay (in our case ganglioside GD1a). The effect of GD1a on the enzyme activity is worth discussing. The K_m value, $4.8 \cdot 10^{-6}$ M, indicates a high affinity of particulate neuraminidase for this ganglioside, which generally predominates in the mammalian brain. But in consideration of the pronounced inhibitory effect of substrate at high concentration, already reported by ÖHMAN *et al.*[6], it can be assumed that particulate neuraminidase, in the physiological milieu (ganglioside concentration range of the order 10^{-3}–10^{-4} M), is under constant inhibition. Consequently the action of the enzyme should be supposed to be "intermittent", and perhaps involved in highly specialized brain functions, which are intermittent. Of course, further and more detailed investigations are needed in order to assess the validity of this hypothesis.

The enzyme devoid of endogenous substrates resembled in many aspects (optimum pH; excess substrate inhibition) the enzyme prepared according to LEIBOVITZ AND GATT[2], while differing in others (action of Triton X-100; K_m value). From a practical point of view, however, the most relevant differences between LEIBOVITZ AND GATT[2] preparation and ours were the absence of soluble neuraminidase, the maintenance of the firmly particulate nature and the much higher recovery with our procedure.

A final comment concerns the applicability of the procedure presented here to the brain of other animal species. The experiments recently carried out in our laboratory gave excellent results with some animals (calf, human) and poor results with others (rat, pig), the success appearing to be solely due to the capacity of the bound enzyme, present in the crude preparation, to hydrolyze the endogenous substrates completely. This point, which is also essential for the choice of criteria for the precise determination of brain particulate neuraminidase, will be throughly considered and discussed in a future paper.

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