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## OPTIMAL CONDITIONS FOR THE ASSAY OF FIBROBLAST NEURAMINIDASE WITH DIFFERENT NATURAL SUBSTRATES

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### Summary

A method for the assay of neuraminidase in human cultured fibroblasts has been worked out. The substrates, all naturally occurring, were: sialyloligosaccharides ( $\alpha(2 \rightarrow 3)$ sialyllactose,  $\alpha(2 \rightarrow 6)$ sialyllactose, disialyllactose), sialylglycolipids (disialogangliosides GD1a and GD1b), sialylglycoproteins and sialylglycopeptides (ovine submaxillary glycoprotein and its pronase-glycopeptides). The method was based on the determination of the enzymically liberated *N*-acetylneuraminic acid (NeuAc) by a chromatographic-colorimetric micro-procedure.

The enzyme acted on sialyloligosaccharides and, in the presence of Triton X-100, on gangliosides, while it did not appreciably affect sialylglycoproteins and sialylglycopeptides. The optimum pH was 4.0 for all tested substrates; the  $K_m$  values were higher for sialyloligosaccharides (about  $10^{-3}$  M), lower for gangliosides (about  $10^{-4}$  M); the apparent maximum velocity was higher with  $\alpha(2 \rightarrow 3)$ sialyllactose (400 mU/mg protein); the reaction rate was linear with time for up to 2 h, and with up to 0.6 mg of enzymic protein.

The assay method proved to be sufficiently sensitive (3–4 nmol liberated NeuAc), simple, and reproducible (mean activity on pooled fibroblasts with  $\alpha(2 \rightarrow 3)$ sialyllactose: 400 mU  $\pm$  6 S.E.).

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### Introduction

The metabolism of sialylglycoconjugates appears to be impaired in a variety of syndromes linked with the general term of mucopolidoses [1–9]. Assuming

an inborn error of metabolism as the cause of these diseases, several investigators attempted to characterize the missing enzyme and demonstrated the occurrence of a sialidase (neuraminidase) deficiency in several forms of mucopolipidosis.

The extent of neuraminidase deficiency appeared to be depending of the type of disorder (higher in mucopolipidosis I, lower in mucopolipidosis II and III) as well as of the assay conditions, namely the substrate used for the enzyme determination [24]. In fact in cases of mucopolipidosis I the neuraminidase deficiency was evident only using a substrate carrying a  $\alpha(2 \rightarrow 6)$ sialyl linkage [9]; in mucopolipidosis II and III the deficiency was evident with various substrates. However, the data given by different laboratories were at variance, and the question whether the neuraminidase deficiency is the primary or secondary lesion in these diseases remains open.

In our opinion most of the controversy is due to the different methodology used for the neuraminidase assay. Furthermore in the only study so far published [10] on the standardization of neuraminidase assay in fibroblasts, an artificial substrate was used. Therefore, relying on our experience in the study of brain neuraminidases, we decided to set up a sensitive and reproducible method for assaying neuraminidase in cultured fibroblasts.

The strategy of our approach was as follows: (a) to use as substrates naturally occurring sialylglycoconjugates of different nature, carrying all types of known sialyl linkages; (b) to measure the enzyme activity in all cases in terms of released sialic acid (*N*-acetylneuraminic acid); (c) to determine released sialic acid by a chromatographic microprocedure accomplishing the removal of occurring contaminants.

## Materials and Methods

### *Chemicals and other products*

Commercial chemicals were of analytical or of the highest available grade. Solvents were redistilled before use; the routinely used water was freshly redistilled in a glass apparatus. Triton X-100, *N*-acetylneuraminic acid (NeuAc) and crystalline bovine serum albumin were obtained from Sigma Chemical Co.

Dowex 2-X8 resin, 200–400 mesh (Bio-Rad) was prepared in acetate form according to Svennerholm [11]. Silica gel H and precoated silica gel thin-layer plates were provided by Merck.

### *Human fibroblast lines*

Skin biopsies were obtained by the punch technique from normal adult individuals and fibroblast cultures were initiated and maintained according to Leroy et al. [12] using 72-cm<sup>2</sup> Falcon plastic flasks. Fibroblasts, which had been grown for at least three weeks, and of the same number of subcultures, were collected by trypsinization, washed three times with 10 ml 0.15 M NaCl and pelleted (1000  $\times$  *g*, 15 min). If necessary, the pellets were stored at  $-80^{\circ}\text{C}$ . Prior to use, each pellet (containing 2–4 mg of protein) was suspended in 0.5 ml distilled water and sonicated (MSE sonicator, five treatments to 15  $\mu\text{m}$ , 15 s each, with a 30-s interval in between, in an ice bath). In some cases the treated fibroblast suspensions were pooled together and homogenized with a glass-Teflon homogenizer.

TABLE I

CHEMICAL AND PHYSICO-CHEMICAL CHARACTERISTICS OF THE SIALYLGLYCOCONJUGATES EMPLOYED FOR THE NEURAMINIDASE ASSAY

Sialylglycoconjugate	Sialyl linkage *	Physico-chemical properties
<b>Sialyloligosaccharides</b>		
$\alpha(2 \rightarrow 3)$ Sialyllactose	$\alpha(2 \rightarrow 3)$ Gal	Soluble free molecule: 633 daltons
$\alpha(2 \rightarrow 6)$ Sialyllactose	$\alpha(2 \rightarrow 6)$ Gal	Soluble free molecule: 633 daltons
Disialyllactose	$\alpha(2 \rightarrow 3)$ Gal	Soluble free molecule: 924 daltons
	$\alpha(2 \rightarrow 8)$ NeuAc	
<b>Sialylglycolipids</b>		
Disialoganglioside GD1a	$\alpha(2 \rightarrow 3)$ Gal	as monomers: insoluble(?) free molecules: 1800 daltons
Disialoganglioside GD1b	$\alpha(2 \rightarrow 8)$ NeuAc $\alpha(2 \rightarrow 3)$ Gal	as micelles: soluble multimolecular aggregates: 200 000–300 000 daltons
<b>Sialylglycoproteins</b>		
Ovine submaxillary glyco-protein	$\alpha(2 \rightarrow 6)$ GalNAc	Soluble free molecule: 394 000 daltons
Pronase-digested ovine sub-maxillary glycoprotein	$\alpha(2 \rightarrow 6)$ GalNAc	Soluble free molecule: 5000–6000 daltons

\* Gal, galactose; NeuAc, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine.

### Preparation of substrates

Three different series of sialylglycoconjugates were used as neuraminidase substrates: sialyloligosaccharides, sialylglycolipids (gangliosides), sialylglycoproteins and sialylglycopeptides.

(a) *Sialyloligosaccharides*. Monosialyllactose,  $\alpha(2 \rightarrow 3)$ - and  $\alpha(2 \rightarrow 6)$ isomers, and disialyllactose were prepared from cow colostrum according to Ohman and Hygsted [13]. The purity of the used sialyloligosaccharides, assessed by chromatographic and chemical methods (see Ghidoni et al. [16]) was over 95%.

(b) *Sialylglycolipids*. Disialogangliosides GD1a and GD1b \* were prepared as the potassium salts from bovine brain according to Tettamanti et al. [15]. Their identification and purity (over 97%) was assessed by the criteria specified by Ghidoni et al. [16].

(c) *Sialylglycoproteins and sialylglycopeptides*. Ovine submaxillary glycoprotein (mucin) (30% bound NeuAc) was prepared according to Tettamanti and Pigman [17]. The pronase glycopeptides (40% bound NeuAc) were prepared from the same ovine submaxillary glycoprotein as described by Carubelli et al. [18].

Some chemical and physicochemical characteristics of the used sialylglycoconjugates are schematically presented in Table I.

### Neuraminidase assay

The incubation mixtures contained aliquots of sonicated fibroblast preparation, given amounts (see later) of substrates, 0.05 M sodium acetate/acetic acid buffer, of a given pH, in a final volume of 0.2 ml. All substrates and Triton X-100 (when present) were added in aqueous solution. The incubation

\* Ganglioside nomenclature according to Svennerholm [14].

mixtures, prepared at 0°C in an ice bath, were placed in an incubator shaker, and incubated at 37°C. The enzyme reaction was stopped by immersing the tube in an acetone/solid CO<sub>2</sub> bath. The liberated NeuAc was determined by the chromatographic method of Preti et al. [19], with some modifications. Briefly, 0.8 ml of 0.05 M H<sub>2</sub>SO<sub>4</sub> was added to each frozen sample and the thawed mixture poured on a 0.5 × 1.0 cm Dowex 2-X8 column, provided with a sintered glass disc and adaptable to a plastic centrifuge tube. The column was centrifuged to dryness (1000 × *g*, 5 min), washed with 10 ml of distilled water and centrifuged again. NeuAc was eluted by addition of 2.0 ml of 2 M sodium acetate/acetic acid buffer, pH 4.6, and centrifugation as above. 0.4 ml of Warren's [20] periodate reagent was added and the reaction carried on according to the original procedure [20]. The yield, checked each time with pure standard NeuAc, resulted to be higher than 92% and reproducibility good (±2%) for a NeuAc range of 3–50 nmol. The absorbance of 0.100 (as corrected absorbance, see Warren [21]) corresponded to 14–14.5 nmol free NeuAc. The control incubation mixtures (blanks) were set up using complete incubation mixtures using a boiled (20 min) enzyme preparation. Nonspecific chemical hydrolysis of the sialylglycoconjugate used as substrate, was always checked and found to be practically negligible (less than 0.1% of the total bound NeuAc) within a 2-h incubation period.

The effect of neuraminidase on endogenous substrates was assessed with incubation mixtures lacking of any added substrates, with or without Triton X-100. No interference of NeuAc aldolase, assessed as reported elsewhere [22], could be detected in the assay system. One unit (U) of neuraminidase is defined as the amount of enzyme which liberates, under the assay conditions, 1 nmol NeuAc/min.

#### *Other analytical methods*

Bound NeuAc was determined by the method of Svennerholm [11]. The protein content of the enzyme preparation was determined by the method of Lowry et al. [23], bovine serum albumin being used as the reference standard.

### **Results**

#### *(1) Action of fibroblast neuraminidase on endogenous substrates*

Human fibroblasts contain sialylglycoconjugates (an average of 5 nmol totally bound NeuAc/mg protein, as reported by Cantz et al. [3]); therefore in the fibroblast preparation we used, substances were present which might act as endogenous substrates for neuraminidase. Upon incubation for 2 h at pH 4.0 (the pH optimum for the enzyme, see later) under standard assay conditions, without added substrates, the fibroblast preparation (0.5 mg protein) released a product which, by direct assay with Warren's reaction [20], gave origin to a chromogen characterized by a single and sharp absorption peak at 532 nm, not corresponding to NeuAc. If the reaction mixture was run on a Dowex 2-X8 column before treatment with Warren's reaction no chromogen at all was formed, thus indicating that no appreciable liberation of NeuAc occurred from endogenous sialylglycoconjugates upon incubation.

Other experiments showed that in the pH range from 3.5–6.5 and for

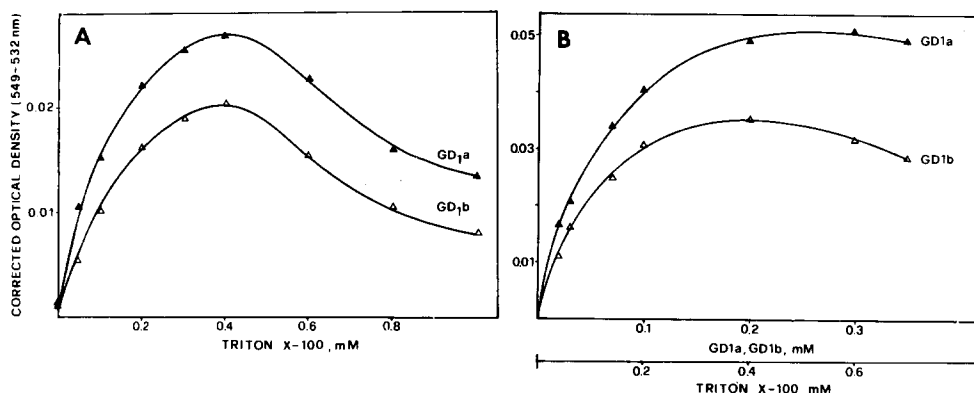


Fig. 1. Effect of increasing concentrations of Triton X-100 on the activity of fibroblast neuraminidase using ganglioside GD1a and GD1b as substrates: (A) with a fixed (0.2 mM) concentration of ganglioside; (B) with a fixed ganglioside/Triton X-100 molar ratio (1 : 2). Experimental conditions: pH 4.0, incubation time: 2 h; enzyme protein: 0.4 mg (A) and 0.5 mg (B).

incubation periods up to 6 h, with or without Triton X-100 (final concentration 0.05–0.8 mM), no measurable activity or endogenous sialylglycoconjugates could be detected.

## (2) Action of fibroblast neuraminidase on different sialylglycoconjugates

(a) *Requirements for activity.* Using  $\alpha(2 \rightarrow 3)$ monosialyllactose (2 mM) as a substrate in standard incubation conditions the enzyme released a product which, after colorimetric reaction, provided a nice peak at 549 nm. Substantial activities were also measured using  $\alpha(2 \rightarrow 3)$ monosialyllactose and disialyllactose, under standard assay conditions. In order to act on gangliosides GD1a and GD1b fibroblast neuraminidase required the presence of Triton X-100 and exhibited apparent maximum activity at a given and optimal ganglioside/Triton X-100 ratio (1 : 2 on a molar ratio) (See Fig. 1a). Maintaining fixed this optimum ganglioside/Triton X-100 ratio, for both GD1a and GD1b, the  $v/s$  curve resulted to be hyperbolic (see Fig. 1b), indicating regular Michaelis-Menten enzyme-substrate interactions. Triton X-100 at the used concentrations did not affect the enzyme activity on  $\alpha(2 \rightarrow 3)$ monosialyllactose. The activity of fibroblast neuraminidase on ovine submaxillary glycoprotein and ovine submaxillary glycopeptides was very poor under a wide range of experimental conditions. Measurable enzyme activities were displayed only after prolonged (more than 12 h) incubation. Because these conditions do not grant for enough precision, no quantitative and kinetic studies on sialylglycoproteins and sialylglycopeptides were undertaken.

(b) *pH optimum,  $v/s$ ,  $v/t$ ,  $v/\text{enzyme}$  relationships.* All experiments on gangliosides were performed in the presence of 0.4 mM Triton X-100. As shown in Fig. 2 the optimum pH (as the pH of the added buffer \*) was 4.0 for all substrates; the shape of the  $v/\text{pH}$  curves was similar with all substrates except for  $\alpha(2 \rightarrow 3)$ monosialyllactose. All  $v/s$  curves followed regular hyper-

\* The actual pH, in the incubation mixture, was 0.2–0.3 units greater and remained unchanged through the incubation.

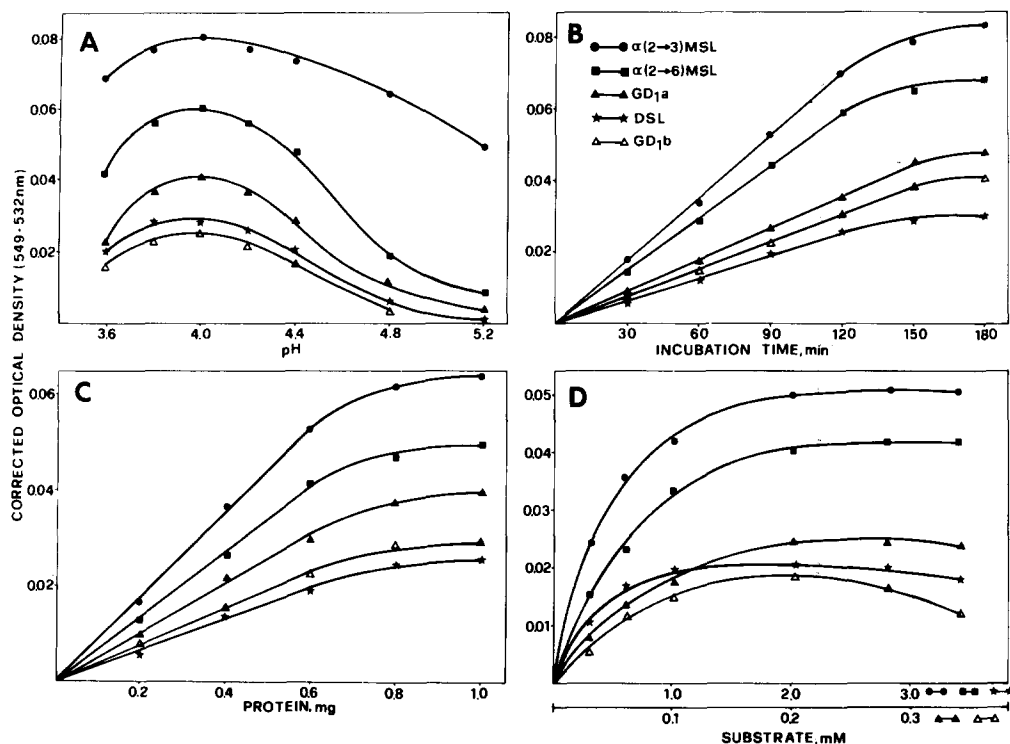


Fig. 2. Activity of fibroblast neuraminidase on different substrates versus: (A) pH; (B) incubation time; (C) enzyme protein; (D) substrate concentration. With gangliosides the enzyme activity was determined in the presence of 0.4 mM Triton X-100. Experimental conditions: (A) with  $\alpha(2 \rightarrow 3)$ monosialyllactose 1 h incubation and 0.4 mg enzyme protein, for other substrates 2 h incubation and 0.6 mg enzyme protein; (B) pH 4.0, with  $\alpha(2 \rightarrow 3)$ monosialyllactose 0.19 mg enzyme protein, with the other substrates 0.59 mg enzyme protein; (C) pH 4.0  $\alpha(2 \rightarrow 3)$ monosialyllactose, incubation time 30 min, with the other substrates 2 h; (D) pH 4.0, with  $\alpha(2 \rightarrow 3)$ monosialyllactose, 1 h incubation and 0.26 mg enzyme protein, with the other substrates 2 h incubation and 0.45 mg enzyme protein. MSL, monosialyllactose; DSL, disialyllactose.

bolic kinetics and exhibited, in the case of gangliosides, apparent inhibition by excess substrate. The enzyme activity was linear with time for up to 2 h with all substrates and a linear activity response was obtained from 0.1–0.6 mg of protein.

Of the various tested buffers (acetic acid/sodium acetate; citric acid/sodium citrate; citric acid/disodium phosphate; Tris-HCl) acetic acid/sodium acetate gave, with all substrates, the highest activity. The optimal molarity was in the range 0.03–0.07 M.

(c) *Apparent maximum hydrolysis rates of different sialylglycoconjugates.* The values of apparent  $K_m$  and maximum hydrolysis rate ( $V$ ) of the various substrates by fibroblast neuraminidase are reported in Table II. The affinity of the enzyme appeared to be higher for gangliosidic ( $K_m = 10^{-4}$  M) than for oligosaccharidic ( $K_m = 10^{-3}$  M) substrates; conversely the highest rates of hydrolysis were recorded on sialyloligosaccharides, i.e.,  $\alpha(2 \rightarrow 3)$ monosialyllactose. No appreciable difference in both  $K_m$  and  $V$  was observed using fresh or stored (at  $-80^\circ\text{C}$ ) fibroblasts.

TABLE II

APPARENT  $K_m$  VALUES AND MAXIMUM RATES OF HYDROLYSIS OF DIFFERENT SIALYLGLYCOCONJUGATES BY FIBROBLASTS NEURAMINIDASE

The hydrolysis rates are expressed in mU/mg protein.

Substrate	Apparent $K_m$ (M)	Apparent maximum hydrolysis rate
Sialyloligosaccharides		
$\alpha(2 \rightarrow 3)$ monosialyllactose	$0.43 \cdot 10^{-3}$	400
$\alpha(2 \rightarrow 6)$ monosialyllactose	$0.95 \cdot 10^{-3}$	100
disialyllactose	$0.45 \cdot 10^{-3}$	40
Sialylglycolipids *		
GD1a	$0.54 \cdot 10^{-4}$	60
GD1b	$0.96 \cdot 10^{-4}$	50

\* Assayed in the presence of 0.4 mM Triton X-100.

TABLE III

MEAN VALUES  $\pm$  S.E. OF THE FIBROBLASTS NEURAMINIDASE ACTIVITY ASSAYED, UNDER OPTIMIZED CONDITIONS, ON DIFFERENT SUBSTRATES

A pool of fibroblasts was used and 15 determinations on each substrate were performed using enzyme concentrations (as protein) ranging from 0.2 to 0.5 mg.

Substrate	Neuraminidase activity (mU/mg protein $\pm$ S.E.)
Sialyloligosaccharides	
$\alpha(2 \rightarrow 3)$ monosialyllactose	$400 \pm 6$
$\alpha(2 \rightarrow 6)$ monosialyllactose	$100 \pm 2$
disialyllactose	$40 \pm 0.30$
Sialylglycolipids	
GD1a	$60 \pm 0.40$
GD1b	$50 \pm 0.31$

### (3) Reproducibility of the assay procedure of fibroblast neuraminidase

Using optimal assay conditions, the procedure for the assay of fibroblast neuraminidase proved to be quite reproducible, as indicated by the data

TABLE IV

LEVELS OF NEURAMINIDASE ACTIVITY, MEASURED WITH VARIOUS SUBSTRATES, IN FIBROBLASTS OF DIFFERENT LINES

Number of examined lines; 16. The values are means  $\pm$  S.D. Minimum and maximum values are given in brackets

Substrate	Neuraminidase activity (mU/mg protein)
Sialyloligosaccharides	
$\alpha(2 \rightarrow 3)$ monosialyllactose	$397 \pm 79$ (270—530)
$\alpha(2 \rightarrow 6)$ monosialyllactose	$109 \pm 36$ (70—210)
disialyllactose	$41 \pm 8$ (26— 55)
Sialylglycolipids	
GD1a	$59 \pm 9$ (44— 75)
GD1b	$51 \pm 11$ (32— 70)

reported in Table III. But as expected, a marked variation of the enzyme activity with all substrates was observed using different fibroblast lines (see Table IV).

#### *(4) Suggested optimized method for the assay of fibroblast neuraminidase*

Mixtures containing in a final volume of 0.2 ml 0.05 M sodium acetate/acetic acid buffer (pH 4.0) aliquots of sonicated fibroblast preparation (0.15–0.6 mg protein), 2 mM  $\alpha(2 \rightarrow 3)$ monosialyllactose; or 2 mM  $\alpha(2 \rightarrow 6)$ monosialyllactose; or 2 mM disialyllactose; or 0.2 mM ganglioside GD1a or GD1b in the presence of 0.4 mM Triton X-100 are incubated at 37°C for 2 h in an incubator shaker. The enzyme reactions are stopped and released NeuAc determined by the chromatographic procedure described in Materials and Methods.

## Discussion

Neuraminidase (sialidases) in human cultured fibroblasts have recently become of special interest since a number of investigators [1–9] have reported a severe deficiency of these (or one of these) enzymes in fibroblasts and leukocytes of patients with mucopolidoses. Since the results obtained in different laboratories were partially at variance, a reliable method for the enzyme determination in fibroblasts is mandatory.

The present work was aimed at setting up a reproducible, precise and reasonably sensitive method for the determination of neuraminidase(s) in fibroblasts from human skin explants. For this purpose we employed, as enzyme substrates, different naturally occurring sialylglycoconjugates (sialyloligosaccharides, sialylglycolipids, sialylglycoproteins, sialylglycopeptides), carrying one or two of the sialyl linkages present in natural compounds (NeuAc  $\alpha(2 \rightarrow 3)$ Gal; NeuAc  $\alpha(2 \rightarrow 6)$  Gal; NeuAc  $\alpha(2 \rightarrow 8)$ NeuAc; NeuAc  $\alpha(2 \rightarrow 3)$ -GalNAc). For technical convenience the assay method was based on the determination, by the thiobarbituric acid reaction of Warren [20], of released NeuAc.

Fibroblast neuraminidase was shown to split sialic acid from sialyloligosaccharides ( $\alpha(2 \rightarrow 3)$ sialyllactose,  $\alpha(2 \rightarrow 3)$ sialyllactose, disialyllactose) and from sialylglycolipids (ganglioside GD1a and GD1b), while no appreciable activity was displayed with the sialylglycoproteins (ovine submaxillary glycoprotein) and sialylglycopeptides (ovine submaxillary glycopeptides) used. For the enzyme action on gangliosides the presence of Triton X-100 in a ganglioside/detergent ratio (1 : 2 in molar terms) was required for maximal activity. Since the enzyme did not appreciably split gangliosides in either the monomeric and micellar forms, and Triton X-100 did not influence the enzyme action on sialyloligosaccharides, it is likely that the best substrate for the neuraminidase is a mixed micelle formed by ganglioside and detergent with a given molar ratio between these components. In this respect fibroblast neuraminidase resembles the neuraminidases from other mammalian tissues. For practical reasons, however, we suggest to maintain Triton X-100 at a fixed concentration (for instance 0.4 mM) and to choose the ganglioside concentration yielding maximal activity (0.2 mM).

The enzymic liberation of NeuAc from all the sialylglycoconjugates (in the



presence of 0.4 mM Triton X-100 with gangliosides) had optimum pH 4.0, followed apparently regular Michaelis-Menten kinetics at varying substrate concentration, and had a linear (up to a certain level) relationship with time of incubation and with enzymic protein concentration. The apparent rate of hydrolysis varied with the different substrates and was greatest with  $\alpha(2 \rightarrow 3)$ -sialyllactose.

The minimum amount of sialic acid which could be detected by the procedure was 3–4 nmols: this means that the method is acceptably sensitive. In order to get fairly measurable extinction readings, one should use at least 0.2–0.25 mg of fibroblast preparation (as protein) with an incubation time of 1 h or 0.1–0.15 mg of protein with an incubation time of 2 h. Finally the reproducibility of the procedure as shown in Table III was good.

Thus all the fundamental requirements for reliability were fulfilled for a neuraminidase assay in fibroblasts. We strongly recommend to remove all interfering materials by column chromatography on Dowex 2-X8 column, prior to determination of released NeuAc. The procedure described here based on facilitated elution by centrifugation renders the method reasonably simple and accurate.

With regard to the procedures of neuraminidase assay in fibroblasts which were employed until now [1–5], the method described by us is at least as sensitive (in terms of minimum amounts of enzyme protein needed for the assay) and surely more accurate. It has also the advantage of using the same procedure for determination of the reaction products obtained from different substrates. Thus it may be helpful in investigating the properties, subcellular location, and possible multiplicity of fibroblasts neuraminidase in normal and in mucopolipidotic patients. In the future radiolabelled unmodified natural substances may be found that could be employed for more sensitive, yet acceptably simple methods of neuraminidase assay in fibroblasts. Work on these lines is at present in progress in our laboratory.

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## References

- 1 Strecker, G., Michalski, J.C., Montreuil, J. and Farriaux, J.P. (1976) *Biomedicine* 25, 235–239
- 2 Thomas, G.H., Tiller, G.E., Reynolds, L.W., Miller, C.S. and Bace, J.W. (1976) *Biochem. Biophys. Res. Commun.* 74, 188–191
- 3 Cantz, M., Gehler, J. and Spranger, J. (1977) *Biochem. Biophys. Res. Commun.* 74, 732–733
- 4 Durand, P., Catti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C. and Strecker, G. (1977) *Helv. Paediatr. Acta* 32, 391–400
- 5 O'Brien, J.S. (1977) *Biochem. Biophys. Res. Commun.* 79, 1136–1141
- 6 Spranger, J., Gehler, J. and Cantz, M. (1977) *Am. J. Med. Genet.* 1, 21–29
- 7 Kelly, T.E. and Graetz, G. (1977) *Am. J. Med. Genet.* 1, 31–46
- 8 O'Brien, J.S. (1978) *Am. J. Med. Genet.* 14, 55–60
- 9 Strecker, G. and Michalski, J.C. (1978) *FEBS Lett.* 85, 20–24
- 10 Santer, U.V., Yee-Foon, J. and Glick, M.C. (1978) *Biochim. Biophys. Acta* 523, 435–442
- 11 Svennerholm, L. (1957) *Biochim. Biophys. Acta* 24, 604–611

- 12 Leroy, J.G., Ho, M.W., MacBrinn, M.C., Zielke, K., Jacob, J. and O'Brien, J. (1972) *Pediatr. Res.* 6, 752—757
- 13 Ohman, R. and Hygstedt, O. (1968) *Anal. Biochem.* 23, 391—402
- 14 Svennerholm, L. (1970) in *Comprehensive Biochemistry* (Florkin, M. and Stotz, E.M., eds.), Vol. 18, pp. 201—215, Pergamon Press, Oxford
- 15 Tettamanti, G., Bonali, F., Marchesini, S. and Zambotti, V. (1973) *Biochim. Biophys. Acta* 296, 160—170
- 16 Ghidoni, R., Sonnino, S., Tettamanti, G., Wiegandt, H. and Zambotti, V. (1976) *J. Neurochem.* 27, 511—515
- 17 Tettamanti, G. and Pigman, U.W. (1968) *Arch. Biochem. Biophys.* 124, 41—53
- 18 Carubelli, R., Bhavanandan, V.P. and Gottschalk, A. (1965) *Biochim. Biophys. Acta* 101, 67—82
- 19 Preti, A., Lombardo, A. and Tettamanti, G. (1970) *Ital. J. Biochem.* 19, 371—385
- 20 Warren, L. (1959) *J. Biol. Chem.* 234, 1971—1974
- 21 Warren, L. (1963) in *Methods in Enzymology* (Colowich, S.P. and Kaplan, N.O., eds.), Vol. 6, p. 465, Academic Press, New York, NY
- 22 Tettamanti, G., Preti, A., Lombardo, A., Suman, T. and Zambotti, V. (1975) *J. Neurochem.* 25, 451—456
- 23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265—275
- 24 Lowden, J.A. and O'Brien, J.S. (1979) *Am. J. Hum. Genet.* 31, 1—18