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A RAPID ASSAY FOR NEURAMINIDASE

THE DETECTION OF TWO DIFFERENCES IN ACTIVITY ASSOCIATED WITH VIRUS TRANSFORMATION

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Summary

Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) activity in fibroblast homogenates was measured by a rapid and simple assay with a synthetic substrate. The activity of neuraminidase in virus transformed hamster fibroblasts was increased over the normal counterpart. In addition, the differential activity seen using the synthetic substrate and fetuin made it possible to detect an enzyme activity hitherto not described. The advantages of this assay for metabolic screening are discussed.

Introduction

Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18), purified from a variety of sources, is used to hydrolyze α-ketosidically linked sialic acids in glycoproteins and glycolipids. Although the role of the enzyme during infection by certain viruses is well established [1], the quantitation of activity in biological material is not often reported since the assays are both lengthy and tedious. The finding of deficiency of neuraminidase in human mucolipidoses [2] has made it imperative to have a rapid, simple assay that can be used for screening in metabolic laboratories. In addition, a simple assay makes feasible the further examination of the role of this enzyme in virus transformation and the regulation of cell functions [3] and in the distribution of glycoproteins within the organism [4].

Some of the methods currently in use to assay neuraminidase activity in complex biological systems require the measurement of the released sialic acid by a complicated colorimetric assay after 2—24 h incubation with natural substrates such as neuraminlactose or fetuin [5]. Other methods, using natural

substrates radiolabelled with borotritide, are more sensitive but require additional procedures such as column chromatography or dialysis and depend on substrates which are not available commercially [6—10].

We have combined several observations by others and devised a method for quantitating neuraminidase activity in the presence of large amounts of protein. The reactions which occur are shown in Fig. 1. Compound I, 2-(3-methoxyphenyl)-*N*-acetyl-α-D-neuraminic acid (methoxyphenyl-NeuAc) originally synthesized by Tuppy and Palese [11] was used as the substrate and 4-aminoantipyrine (compound IV) in the presence of an oxidizing agent was used to measure the enzymatically released methoxyphenol, compound III. Methoxyphenyl-NeuAc (I) was employed to measure the activity of purified viral neuraminidases, localize foci of viruses in tissue culture and to detect neuraminidase on polyacrylamide gels [12,13]. The method of color development which was used to assay purified neuraminidase quantitatively was based on the Folin-Ciocalteau reagent and cannot be used in the presence of high concentrations of protein [12]. To circumvent this, we used 4-aminoantipyrine (IV) which forms a colored quinone (V) with para unsubstituted phenols under conditions similar to those reported by Asp [14] for phenol. The standardization of this procedure and the results of measuring the activities of neuraminidase in homogenates of cultured fibroblasts, virus transformed and the normal counterpart, are reported.

Methods and Materials

Neuraminidase assay

Unless otherwise specified, the incubation mixture contained 150 μg methoxyphenyl-NeuAc (Boehringer Mannheim Biochemicals) in 100 μl 0.1 M phosphate buffer (pH 5.9) and enzyme in a final volume of 200 μl, and was incubated at 37° C for 60 min. In some experiments, 0.05 M sodium acetate buffer (pH 4.5) was substituted for the phosphate buffer. A substrate control at the appropriate pH was incubated without enzyme. The source of the purified enzyme was *Vibrio cholerae* or influenza virus (Calbiochem) which was diluted in 0.1 M phosphate buffer (pH 5.9) to the desired activity. Cell homogenates were prepared in Triton X-100 (Packard Instrument Co.) from a clone of baby hamster kidney cells (BHK_{21}/C_{13}) or this clone transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4), both in confluent phase of growth and within one passage of each other. In no experiments were the cells used beyond passage 12. The growth, harvesting and homogenization have been described in detail [15,16]. Proteins were measured by the method of Lowry et al. [17]. Incubation was as described for the purified enzymes with the exception that the mixture was brought to the final volume with Triton X-100. All assays were performed in duplicate and the cell homogenates were examined at three protein concentrations.

Detection of released methoxyphenol

Purified neuraminidase or cell homogenates were incubated with methoxyphenyl-NeuAc and the reaction was stopped by the addition of 150 μl 1.3 mM 4-aminoantipyrine (Aldrich Chemical Co.) in 1 M Tris · HCl buffer (pH 8.5)

containing 1.33% ethanol (v/v) followed by the addition of 50 µl 6 mM potassium ferricyanide. The precipitate which formed in the presence of a protein content greater than 150 µg was centrifuged at 730 × g for 15 min. The supernatant solutions or the incubation mixtures which did not form precipitates were read at 500 nm in a Zeiss spectrophotometer. The substrate and enzyme controls were treated similarly and subtracted from the test samples. Authentic methoxyphenol (Eastman Kodak) served as standard.

Natural substrate

Fetuin (GIBCO, No. 918S) 1.0 mg, containing 200 nmol sialic acid, was incubated with diluted *V. cholerae* or influenza virus neuraminidase, in 0.1 M phosphate buffer (pH 5.9) 0.08 mM $CaCl_2$ in a final volume of 200 µl for the specified time. For detection of neuraminidase activity toward fetuin in the

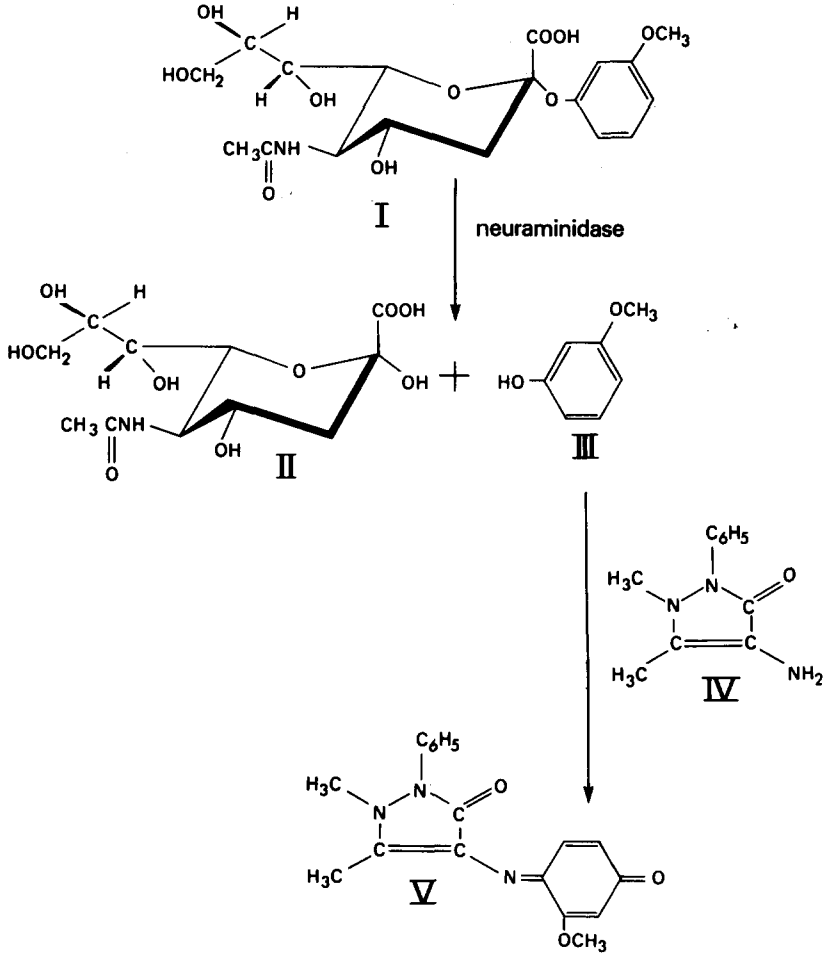

Fig. 1. Reactions involved in the assay of neuraminidase. 2-(3-Methoxyphenyl)-*N*-acetyl-α-D-neuraminic acid (I) is hydrolyzed by neuraminidase to yield NeuAc (II) and methoxyphenol (III). III and aminoantipyrine (IV) in buffer, pH 8.5, in the presence of an oxidizing agent, potassium ferricyanide, will yield a colored quinone (V).

fibroblasts, the cell homogenates (300 μg protein) were incubated similarly or in 0.05 M acetate buffer (pH 4.5) without $CaCl_2$. The released *N*-acetylneuraminic acid was assayed by the thiobarbituric acid assay modified to detect less than 1 nmol [18] after eluting from Dowex 1-X8 (formate form) with 0.3 M formic acid.

Results

Sensitivity of the assay

Methoxyphenol and 4-aminoantipyrine form a colored complex which has an absorption maximum at 500 nm. Under the assay conditions described in Methods and Materials, the amount of absorption of the colored complex (Fig. 1, compound V) was directly proportional to the quantity of methoxyphenol present up to 48 nmol (Fig. 2a). The molar extinction coefficient at 500 nm of compound V (Fig. 1) was 11,000 and was similar to that reported by Asp [14] for phenol (ϵ_{508} = 14 000). The effect of the oxidizing agent on the color development is shown in Fig. 2b. In the presence of 200 μg potassium ferricyanide, an orange colored complex was formed with authentic methoxyphenol instead of the pink complex formed with 100 μg or less. Since in the presence of high amounts of oxidizing agent, the fibroblast homogenates gave an interfering orange color, 100 μg potassium ferricyanide was used, limiting the amount of methoxyphenol which can be quantitated to less than 45 nmol.

Parameters of the assay

Using methoxyphenyl-NeuAc as substrate for *V. cholerae* or influenza virus

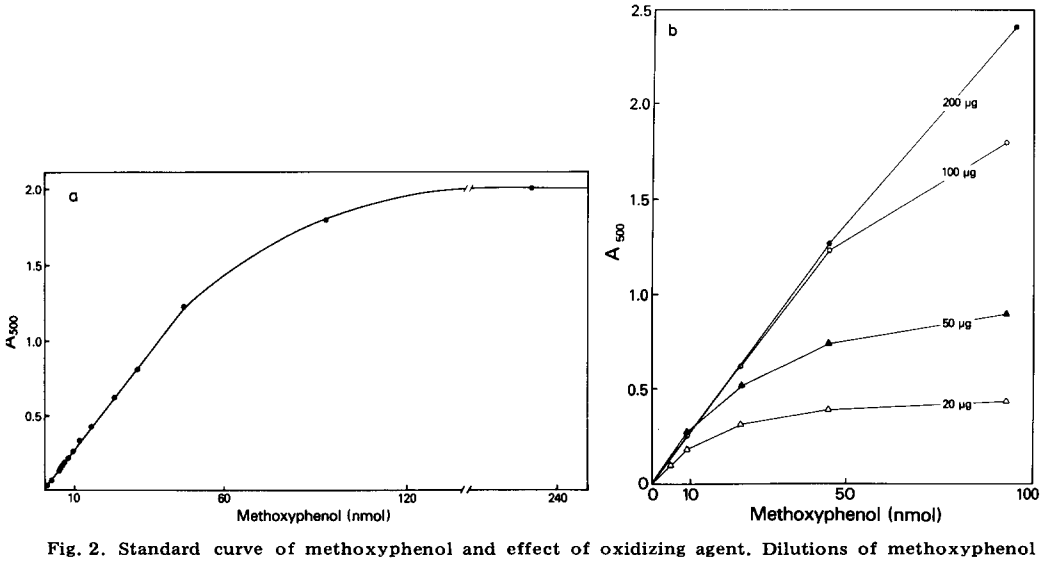

Fig. 2. Standard curve of methoxyphenol and effect of oxidizing agent. Dilutions of methoxyphenol (50 μl) in 10% ethanol, 50 μl of 0.1 M phosphate buffer, pH 5.9, and 100 μl of 0.1% Triton X-100 were assayed as described in Methods and Materials. A measured density of 1.15 g/ml was used to calculate the concentration of methoxyphenol. (a) Standard curve for the assay using 100 μg of potassium ferricyanide. (b) Potassium ferricyanide, 20 to 200 μg, in 50 μl was added to increasing concentrations of methoxyphenol following the addition of aminoantipyrine.

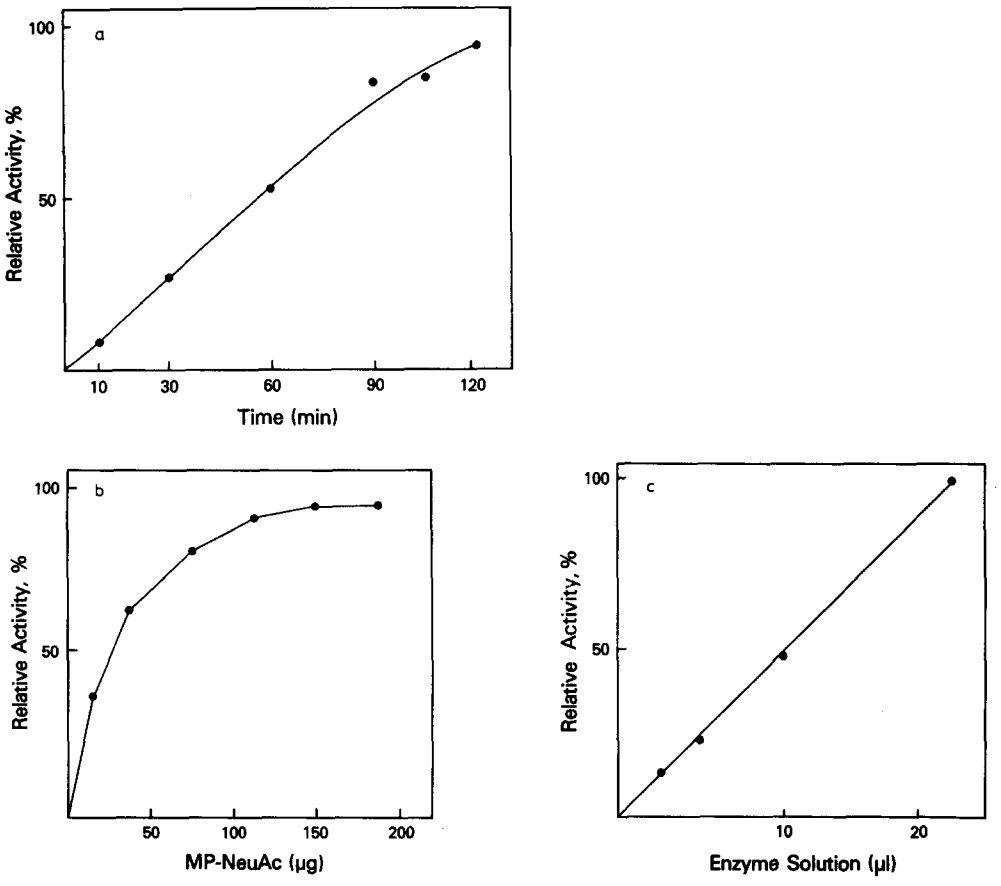

Fig. 3. Kinetics of influenza virus neuraminidase measured with methoxyphenyl-NeuAc. (a) Time course of the reaction obtained using 150 µg of methoxyphenyl-NeuAc in 0.1 M phosphate buffer, pH 5.9, with or without calcium; (b) Effect of increasing methoxyphenyl-NeuAc concentration. Methoxyphenyl-NeuAc in 0.1 M phosphate, pH 5.9, was incubated with a constant amount of neuraminidase. A substrate blank for each concentration was similarly incubated and the value obtained subtracted from each point. (c) Dependence of methoxyphenol release on enzyme concentration. Dilutions of enzyme were made in phosphate buffer, pH 5.9. A relative activity of 100% represented 35 nmol of methoxyphenol released in (a) and (c) and 25 nmol in (b).

neuraminidase, diluted to the approximate activity expected of cell homogenates, the standard parameters of enzyme kinetics can be readily shown. The release of methoxyphenol is directly proportional to amount of enzyme added or time of incubation, and the initial velocity is saturated by higher substrate concentration (Fig. 3). Methoxyphenyl-NeuAc saturated the reaction at approximately 150 µg at an enzyme concentration which hydrolyzes 7% of the substrate per hour (Fig. 3b). The hydrolysis of methoxyphenyl-NeuAc proceeded in a linear manner to 120 min with the total release of 35 nmol methoxyphenol (Fig. 3a). Increasing concentrations of neuraminidase resulted in a proportional increase in absorbance (Fig. 3c). The pH optima for *V. cholerae* neuraminidase was pH 5.0 while that of influenza virus neuraminidase was from pH 5.8 to pH 6.5.

The activities of *V. cholerae* or influenza virus neuraminidase on fetuin and

TABLE I

NEURAMINIDASE ACTIVITY IN HAMSTER CELL HOMOGENATES

Cell line	Methoxyphenyl-NeuAc (nmol methoxyphenol/mg protein/h)		Fetuin (nmol NeuAc/mg protein/15 h)	
	pH 4.5 *	pH 5.9 **	pH 4.5 *	pH 5.9 **
C_{13}/B_4	69	65	71	23
BHK_{21}/C_{13}	42	20	38	10
$\frac{C_{13}/B_4}{BHK_{21}/C_{13}}$	164%	329%	187%	230%

* 0.05 M acetate buffer, pH 4.5.
** 0.1 M phosphate buffer, pH 5.9.

methoxyphenyl-NeuAc were compared. Using the same concentration of the enzymes, the activity toward the synthetic and natural substrates at 1 h verified the rapidity of the reaction with methoxyphenyl-NeuAc. It required increased concentrations of the enzymes and incubation for 24 h to obtain a release of sialic acid from fetuin comparable to that from methoxyphenyl-NeuAc.

Neuraminidase activity in fibroblast homogenates

Homogenates of hamster kidney fibroblasts, transformed (C_{13}/B_4) and non-transformed (BHK_{21}/C_{13}), were assayed for neuraminidase activity. Table I shows that neuraminidase activity was increased after virus transformation. In 0.05 M acetate buffer (pH 4.5) when either methoxyphenyl-NeuAc or fetuin served as substrate similar increases were seen, 164% and 187% of the control. In contrast, in 0.1 M phosphate buffer (pH 5.9) using methoxyphenyl-NeuAc as substrate the virus transformed cells maintained the level of activity similar to that at pH 4.5 while the activity of the normal counterpart dropped by 50%. Making this more striking is the fact that the activities of both homogenates toward fetuin showed decreases which were similar to each other in phosphate buffer (pH 5.9). Thus it appears from the differential activity toward methoxyphenyl-NeuAc and fetuin, that a neuraminidase activity, not detectable with fetuin, increases with virus transformation in these hamster fibroblasts.

Using methoxyphenyl-NeuAc as substrate, increasing activity was found with increasing protein content of the homogenates and was linear to 40 nmol of released methoxyphenol. Homogenates retained full activity toward the synthetic substrate after one freezing and thawing, but on subsequent thawing 10—20% of the activity was lost.

Discussion

A rapid and relatively simple assay for neuraminidase has been described using a synthetic substrate, methoxyphenyl-NeuAc. The method has advantages over the existing procedures for detecting neuraminidase activity. It is (1) simple — the assay measures the aglycone, methoxyphenol, by the addition of only two reagents; (2) rapid — the enzyme readily hydrolyzes the substrate within 30 to 60 min; (3) sensitive — 2.5 nmol can be detected; (4) functional —

high protein concentrations do not interfere; (5) routine — only $5–10 \cdot 10^5$ fibroblasts are required and the total time is less than 90 min; and (6) the color is read in the visible range, requiring no special spectrophotometer. In addition, the synthetic substrate, methoxyphenyl-NeuAc, is available commercially and stable when dessicated or in 0.1 M phosphate buffer (pH 5.9) at −40°C. The fact that it can be used with fibroblast cultures makes it of value for metabolic screening in a manner similar to the *p*-nitrophenyl-glycosides.

Using either methoxyphenyl-NeuAc or fetuin as substrate the activity of neuraminidase in virus transformed hamster fibroblasts was increased over that of the normal counterpart. In addition to the increased activity with both substrates, a further increase (to 330%) was found with methoxyphenyl-NeuAc in phosphate buffer (pH 5.9) which was not apparent in 0.05 M acetate buffer (pH 4.5) or with fetuin (Table I). Thus, with the synthetic substrate it was possible to detect a specific increase in enzyme activity in transformed cells which was hitherto not described. The relationship of these neuraminidase activities to the highly branched oligosaccharides found in the transformed cells [19] is under investigation. The effect of transformation on acid neuraminidase and other glycosidases [20] has been reviewed.

The activity of neuraminidase was higher with methoxyphenyl-NeuAc than toward the glycoprotein, fetuin. The units of activity measured with various substrates, however, are not directly comparable since neuraminidase preparations have been reported to express a spectrum of activities toward different glycoproteins [3]. Although with methoxyphenyl-NeuAc as synthetic substrate we detected additional enzyme activity, the opposite has been reported in detecting differences between Hurler and Scheie syndromes [21]. Using the natural substrates, a difference was found between the two disorders which was not evident when α-L-phenyliduronide was the substrate.

The sialic acid content of cells in culture has been reported to change throughout the cell cycle [22]. It is now possible to determine if neuraminidase activity is associated with these events, perhaps as a controlling mechanism for growth. Our preliminary results indicate that there are changes in neuraminidase activity with cell growth and warrant further examination of this enzyme throughout the cell cycle.

Neuraminidase activities in homogenates from control human fibroblasts have been compared with those from individuals with cystic fibrosis [23], and from a patient with mucolipidosis type II (Spritz, R. and Glick, M.C., unpublished data). These studies show that it is feasible to use the assay in screening for genetic disorders. Thus, this simple assay not only makes possible studies on the role of neuraminidase in biological phenomena, but also opens new avenues of clinical testing.

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