A CHROMOGENIC SUBSTRATE FOR THE INVESTIGATION OF NEURAMINIDASES

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1. Introduction

Neuraminidases (EC 3.2.1.18) have been detected in viruses [1], bacteria [2] and mammalian tissues [3] and appear to be involved in biological processes such as the infection cycle of certain viruses, the metabolism of gangliosides and the inactivation of *N*-acylneuraminic acid containing glycoproteins. In the course of studies on the purification and characterisation of a neuraminidase from pig kidney [4] a chromogenic substrate was synthesized and employed for the localization of the enzyme on electropherograms. The preparation of the new substrate, MPN *, and its use to distinguish neuraminidases of different origin is the subject of this communication.

2. Materials and methods

2.1. *2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid*

2-Chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid was prepared from *N*-acetylneuraminic acid as previously described [5]. The following procedure is similar to that used in the synthesis of 2-phenyl-*N*-acetyl-α-neuraminic acid [6]. 7.4 g 2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid, 5.4 g silver carbonate and 35 g freshly distilled 3-methoxyphenol were kept for 2 hr at 40°C. The reaction mixture was dissolved in 150 ml acetone and filtered through a layer of Hyflo Supercel. After washing with a mixture of 100 ml acetone and 50 ml distilled water the combined filtrates were applied to a column of 500 ml Dowex 1 X 8 (acetate form). The column was washed with 2 l of acetone/water (2:1, v/v) and the adsorbed product was subsequently eluted with 4 N acetic acid/acetone (1:1, v/v). The *N*-acetylneuraminic acid containing fractions were concentrated *in vacuo*. When triturated with 30 ml water about half of the yellow solid remained undissolved. 30 ml water and acetone just enough to dissolve the solid were added to this residue. When the clear solution was left overnight in an open beaker, crystals separated (2.1 g), which were twice recrystallized from acetone/water. 2-(3'-methoxyphenyl)-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-α-neuraminic acid thus obtained (1.53 g) was colourless, chromatographically homogeneous and free of its β-anomer. 0.51 g were mixed with 35 ml absolute methanol and 6 ml 1 N NaOH in absolute methanol. After 20 min 3 g Amberlyst-15 (H^+ form) were added to the solution. The mixture was filtered and subsequently concentrated *in vacuo*. The dry residue was dissolved in 7 ml methanol and the solution mixed with 100 ml ethyl ether and 40 ml petroleum ether. After standing for 24 hr another 40 to 80 ml petroleum ether were added. From this solution white crystals of MPN were obtained (0.26 g). For analytical data see table 1.

2.2. *Neuraminidases*

Purified influenza A virus, strain Melbourne, which was used as a source for viral neuraminidase, was kindly provided by Dr. P.Meindl, Arzneimittelforschung GmbH, Vienna. A solution of *Vibrio cholerae* neuraminidase was purchased from Behringwerke AG (Marburg, GFR). Pig kidney neuraminidase was prepared as described recently [4], omitting the chromatography on Bio-gel and SE-cellulose.

* Abbreviations: MPN, 2-(3'-methoxyphenyl)-*N*-acetyl-α-neuraminic acid; DS, diazonium salt of 4-amino-2,5-dimethoxy-4'-nitroazobenzene.

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Table 1
3-Methoxyphenyl-α-ketoside of *N*-acetylneuraminic acid and its 4,7,8,9-tetraacetyl derivative.

	2-(3′-methoxyphenyl)-4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-α-D-neuraminic acid	2-(3′-methoxyphenyl)-*N*-acetyl-α-D-neuraminic acid
Formula	$C_{26}H_{33}O_{14}N$	$C_{18}H_{25}O_{10}N.\frac{1}{2}H_2O$
Found (%), C	53.7	50.7
H	5.6	6.1
N	2.4	3.5
Required (%), C	53.5	51.0
H	5.7	6.2
N	2.4	3.3
Melting point (°C)	169–175	127–129
Optical rotation $[\alpha]_D^{24}$	+69°	−31°
g/l	4.2	4.5
solvent	acetic acid	dimethyl sulfoxide
R_f *	0.42	0.37

* Thin-layer chromatography [6]

2.3. *Electrophoresis*

Gel electrophoresis was performed for 1–2 hr at 4°C in the apparatus described by Kaschnitz [7] at 5 mA per tube. 7.5% and 3.75% polyacrylamide gels were used at a pH of 7.5 [8] or 4.3 [9]. Neither spacer nor sample gel was layered over the running gel. Polymerization was achieved using riboflavin (0.001%). 50 μl of a pig kidney neuraminidase preparation (1.4 enzyme units [4], 100 μg protein) or 25 μl of the commercial preparation from *V. cholerae* (previously dialyzed against water) were applied to the gels in an aqueous solution containing 30% (w/v) sucrose.

Cellulose acetate electrophoresis of the viral enzyme was carried out at a running pH of 8.9 in the buffer described by Aronsson and Grönwall [10] which was diluted 1:4 with water and contained 0.5% (w/v) sodium deoxycholate [11]. 1 ml of the virus suspension was centrifuged at 105,000 × *g* for 1 hr. The pellet was resuspended in 50 μl buffer containing 2% (w/v) sodium deoxycholate. After sonication for 3 min in a MSE "Ultrasonic Disintegrator" a 10 μl aliquot of this mixture was applied as a line to a 3.9 cm wide strip of cellulose acetate (Sartorius Membranfilter GmbH, Göttingen, GFR) and run at room temperature for 1.5 hr at approx. 8 V/cm. For the electrophoresis of *V. cholerae* neuraminidase 0.036 M sodium barbital buffer pH 8.5 was employed. 20 μl of the commercial solution, which had been dialyzed before, was applied to a 3.9 cm wide strip of cellulose acetate. Bromophenol blue was used as a marker for the buffer front moving towards the anode.

2.4. *Specific staining of the neuraminidases*

After disc electrophoresis the bands of neuraminidase activity were detected by incubating the gels for at least 1 hr at 37°C in 0.1 M sodium acetate buffer pH 4.9, which contained 0.002 M MPN and 0.1% (w/v) of the diazonium salt (DS) of 4-amino-2,5-dimethoxy-4′-nitroazobenzene (Koch & Light, Colnbrooke, England).

In order to detect neuraminidase activity in cellulose acetate strips they were incubated for 15–20 min at 37°C with a filter paper pressed against them which was soaked with 0.01 M solution of MPN in 0.1 M phosphate pH 6.4. Subsequently the stips were immersed in a 0.01% (w/v) solution of DS in 0.1 M sodium barbital buffer pH 8.5. Dark red bands developed at the sites of neuraminidase activity.

3. Results and discussion

The advantage of MPN as a chromogenic substrate for neuraminidase resides in the fact that methoxyphenol enzymatically released readily couples with DS, even in the acid pH region, to form an insoluble dark red dye. Proteins are not deeply stained by DS unless they are present in large amounts. Since the unspecific staining of proteins by diazo coupling is not dependent on the presence of the neuraminidase substrate, MPN, and gives a different colour, it can be readily distinguished from the specific action of neuraminidase.

The commercial sample of *V. cholarae* neuraminidase invariably produced a single dark red band when incubated with MPN and DS after disc electrophoresis in 7.5% polyacrylamide at either pH 7.5 (fig. 1, gel A) or pH 4.3 (fig. 2, gel D) or in 3.75% polyacrylamide at pH 4.3 (fig. 3, gel D) or after cellulose acetate elec-

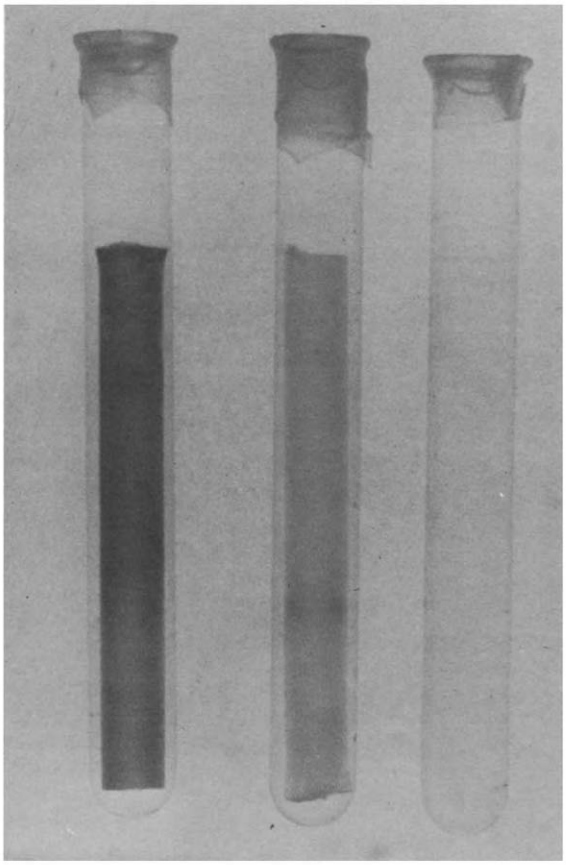

Fig. 1. Disc electrophoresis of *V. cholerae* neuraminidase in 7.5% polyacrylamide (anode at the bottom). "Running pH" of 7.5. Gels A and B: incubation with DS in the presence (A) and absence (B) of MPN. The faint band in B is due to bromophenol blue used as a marker. Gel C: staining with amino black 10B.

trophoresis at pH 8.5 (fig. 4, strip A). The protein content of the *V. cholerae* enzyme preparation was too low to be detected either by the unspecific diazo staining (fig. 1, gel B; fig. 2, gel E) or by amido black (fig. 1, gel C). In contrast, a crude preparation of pig kidney neuraminidase contained a large amount of contaminating proteins which after disc electrophoresis and subsequent incubation with DS produced one major and several minor bands (fig. 2, gel A; fig. 3, gel A). When the neuraminidase substrate, MPN, was included in the incubation mixture, there appeared a single additional band due to enzyme action. After electrophoresis at pH 4.3 in 7.5% polyacrylamide the neuraminidase band was found on the very top of the

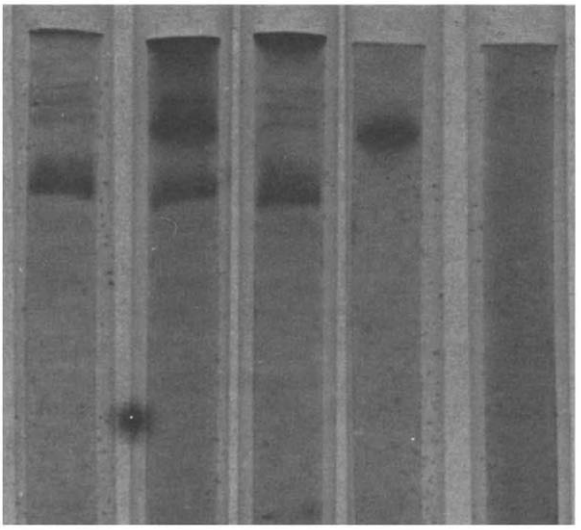

Fig. 2. Disc electrophoresis of pig kidney and *V. cholerae* neuraminidases in 7.5% polyacrylamide (cathode at the bottom). "Running pH" of 4.3. Gels A and C: crude kidney neuraminidase preparation; incubation with DS in the absence (A) and presence (C) of MPN. Gels D and E: *V. cholerae* neuraminidase; incubation with DS in the presence (D) and absence (E) of MPN. Gel B: mixture of kidney and *V. cholerae* neuraminidase preparations; treatment with MPN and DS.

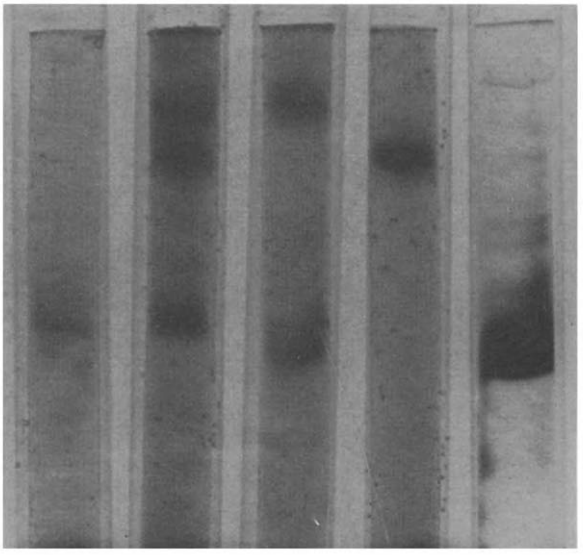

Fig. 3. Disc electrophoresis of pig kidney and *V. cholerae* neuraminidases in 3.75% polyacrylamide (cathode at the bottom). "Running pH" of 4.3. Gels A and C: crude kidney neuraminidase preparation; incubation with DS in the absence (A) and presence (C) of MPN. Gel D: *V. cholerae* neuraminidase; incubation with MPN and DS. Gels B and E: mixture of kidney and *V. cholerae* neuraminidase preparations; staining with MPN plus DS (B) and with amido black (E).

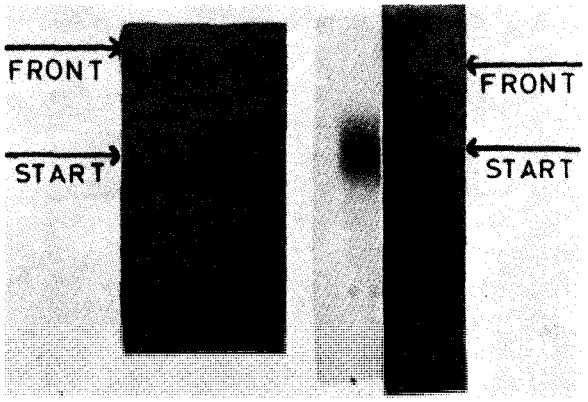

Fig. 4. Cellulose acetate electrophoresis of *V. cholerae* neuraminidase (A) at pH 8.5 and deoxycholate-solubilized viral neuraminidase (B1 and B2) at pH 8.9 (cathode at the bottom). Strips A and B2: incubation with MPN followed by treatment with DS. Strip B1: treatment with amido black 10B to stain viral proteins.

tube, thus indicating that the enzyme had failed to penetrate into the small-pore gel (fig. 2, gel C). 3.75% polyacrylamide, on the other hand, allowed the enzyme to enter into the gel (fig. 3, gel C). As shown in fig. 2 (gel B) and fig. 3 (gel B) the neuraminidases from *V. cholerae* and pig kidney could be readily separated from each other and distinguished electrophoretically.

Electrophoresis of influenza A virus neuraminidase on cellulose acetate at pH 8.9 required the presence of deoxycholate in the medium. Under these conditions neuraminidase activity had a mobility different from that of the bulk of viral protein (fig. 4, strips B1 and B2). The enzyme activity, however, formed a rather broad band indicating that, despite the deoxycholate treatment, the viral neuraminidase was not completely solubilized.

The results so far obtained concerning the electrophoretic identification and separation of neuraminidases using the chromogenic substrate, MPN, as an analytical tool suggest that this method may prove valuable in further investigations on mammalian, bacterial and viral neuraminidase isozymes.

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References

[1] F.M.Burnet and J.D.Stone, Austr. J. Exp. Biol. Med. Sci. 25 (1947) 227.
[2] J.F.McCrea, Austr. J. Exp. Biol. Med. Sci. 25 (1947) 127.
[3] L.Warren and C.W.Spearing, Biochem. Biophys. Res. Commun. 3 (1960) 489.
[4] H.Tuppy and P.Palese, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 1169.
[5] P.Meindl and H.Tuppy, Mh. Chem. 96 (1965) 802.
[6] P.Meindl and H.Tuppy, Mh. Chem. 98 (1967) 53.
[7] R.Kaschnitz, Z. klin. Chemie klin. Biochem. 5 (1967) 126,
[8] D.E.Williams and R.A.Reisfeld, Ann. N.Y. Acad. Sci. 121 (1964) 373.
[9] R.A.Reisfeld, U.J.Lewis and D.E.Williams, Nature 195 (1962) 281.
[10] T.Aronsson and A.Grönwall, Scand. J. Clin. Lab. Invest. 9 (1957) 338.
[11] W.G.Laver, Virology 20 (1963) 251.