APPLIED MICROBIOLOGY, Feb. 1973, p. 195-201
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Vol. 25, No. 2
Printed in U.S.A.

Applications of a Synthetic Neuraminidase Substrate

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Received for publication 10 November 1972

A rapid and precise assay for neuraminidase using 2-(3'-methoxyphenyl)-*N*-acetyl-α-neuraminic acid (MPN) is described. It is proposed that this substrate be used for the standardization of activity of neuraminidases from viral, bacterial, and mammalian sources. MPN is also used as a chromogenic substrate to localize influenza and parainfluenza virus foci in tissue culture. This technique permits the recovery of infective virus from these stained "plaques." It has also been demonstrated that immunoprecipitin lines containing neuraminidase complexes with antibody in the Ouchterlony test can be observed by a similar staining procedure. No enzyme inhibition occurs in the presence of anti-neuraminidase antibodies or concanavalin A when MPN is used as a substrate in contrast to the results with high-molecular-weight substrates such as fetuin.

Neuraminidases (EC 3.2.1.18) hydrolyze substrates containing α-ketosidically bound *N*-acylneuraminic acids. There are primarily two ways of determining neuraminidase activity. By one method only the "free", i.e., unbound *N*-acylneuraminic acid, is measured by chemical means after enzymatic hydrolysis. In most laboratories the thiobarbituric acid assay (1, 20) is used to determine *N*-acylneuraminic acid released from either a high-molecular-weight substrate such as fetuin (45,000) or a small compound such as *N*-acetylneuraminosyl-lactose (633). After treatment with an oxidizing reagent, *N*-acylneuraminic acids undergo a color reaction with thiobarbituric acid, which can be readily followed spectrophotometrically. The coupled enzyme assay (21) is an alternate method for measuring free *N*-acylneuraminic acid. *N*-acylneuraminic acid liberated in the neuraminidase assay is hydrolyzed by an aldolase, and the pyruvate generated by this reaction is measured in a standard pyruvate assay involving lactic acid dehydrogenase and reduced nicotinamide adenine dinucleotide.

An alternate approach to the assay of neuraminidase activity has the following principle. After enzymatic hydrolysis of the neuraminic acid-containing substrate, the aglycon rather than the liberated *N*-acylneuraminic acid is determined. For example, after hydrolysis of the natural substrate of *N*-acetylneuraminosyllactose, the lactose released from the substrate can be measured; however, the assay for lactose is rather complicated and this method is not widely accepted (5). Meindl and Tuppy (12) first described the synthesis of 2-phenyl-*N*-acetyl-α-neuraminic acid, a neruaminidase substrate in which phenol is the aglycon determined after enzymatic hydrolysis. Priwalowa and Chorlin synthesized similar chromogenic neuraminidase substrates (15).

In this report we describe some further applications of another synthetic substrate 2-(3'-methoxyphenyl)-*N*-acetyl-α-neuraminic acid (MPN) which had been previously used to demonstrate viral, bacterial, and mammalian neuraminidases in polyacrylamide gels (2, 19) and to localize neuraminidase-active foci of viruses in tissue cultures (14). Each MPN molecule split by neuraminidase generates stoichiometrically one 3-methoxyphenol and one *N*-acetylneuraminic acid molecule. Conditions are given for the standardized quantitative determination of neuraminidase measuring the release of 3-methoxyphenol. Experiments are also described using MPN as a chromogenic substrate with the liberated 3-methoxyphenol precipitated by a diazonium salt. This application is used in tissue culture work with influenza and parainfluenza viruses and as a general means for observation of neuraminidase activity, e.g., in the Ouchterlony technique.

MATERIALS AND METHODS

MPN and enzyme assay. MPN (MPN for chromogenic use can be obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014) was synthesized as described earlier (19). The chromatographically pure product was stored at room temperature in air-tight vials wrapped in aluminum foil for extended periods of time (over 1 year). Special care was taken to minimize exposure of the substrate to humidity. The acid nature of the substrate facilitates autohydrolysis, but the substrate is very stable in buffered solutions. For most purposes a 10^{-2} M solution of MPN (4.23 mg/ml) in 0.1 M sodium-phosphate buffer, *p*H 5.9, was used. This solution was kept in the freezer (−15 C) for months, thawed when used, and refrozen. Under these conditions, the maximal autohydrolysis observed was less than 5%.

The enzyme determination was performed by methods similar to the assay developed by Meindl and Tuppy for 2-phenyl-*N*-acetyl-α-neuraminic acid (13). Enzyme assays for viral neuraminidase were performed in a total volume of 0.2 ml, containing 1.5×10^{-3} M MPN, 0.05 or 0.1 M sodium phosphate buffer (*p*H 5.9), and varying amounts of enzyme at 37 C. At appropriate times of incubation, the reaction was stopped by the addition of 1.5 ml of 10% Na_2CO_3 and 0.2 ml of Folin-Ciocalteau reagent (2 N) was added. After 20 min, the color development of the solution was read at 750 nm. For determination of anti-neuraminidase antibody in sera from rabbits, virus and sera at different dilutions were preincubated for 30 min at 20 C, followed by neuraminidase assay using either fetuin, *N*-acetylneuraminosyllactose, or MPN as substrate. The *N*-acetylneuraminic acid concentration for these particular substrates used in the assay mixture was 10^{-3} M, 10^{-3} M, and 1.5×10^{-3} M, respectively. All protein determinations were performed by the method of Lowry et al. (10).

Viruses. Influenza viruses X-7 [A/NWS(HO)-RI/5+/57(N2)](9), X-15 [A/equine 1/56(Heql)-X-1L-(N2)](6), X-31 [A/HK/Aichi/2/68(H3N2) - PR8/34], A/WSN/33[HON1](17), and A/equine 1/56[Heql-Neql](6) were grown and purified when necessary as described earlier. Sendai virus and parainfluenza viruses of the strain 3HA-1 and 4A were obtained from the Research Resources Branch of the National Institutes of Health, Bethesda, Md. The SV-5 strain of parainfluenza was kindly provided by Purnell Choppin of Rockefeller University, New York. Clone 1-5C-4 conjunctival cells and bovine kidney (MDBK) cells were propagated as reported earlier (7, 17).

Observation of neuraminidase-containing viral foci in tissue culture and virus recovery from stained plaques. Using a modification of a method described previously (14), we grew monolayers of different cell systems in Falcon tissue culture dishes (diameter 6 cm). After inoculation with serial dilutions of virus, a thin layer of 0.8% agar overlay medium (2 ml) and a thick layer of 0.35% agar (6 ml) were applied on top of the monolayers instead of the usual 0.6% agar overlay medium (10 ml). After appropriate incubation periods, the upper layer (0.35% agar) of the overlay medium was removed, and 0.6 ml of a solution containing 600 μg of MPN and 500 μg of the diazonium salt of 4-amino-2,5-dimethoxy-4-nitrozaobenzene (Black K salt) (Koch and Light, Colnbrook, England) was applied on top of the remaining agar layer. Care was taken to spread the liquid over the entire surface. The solution of the diazonium salt was filtered before mixing with the substrate. After 5 to 10 min in an incubator at 37 C, red foci began to develop, indicating the sites of actual neuraminidase activity. For some experiments, dishes were preincubated for 15 min with substrate alone. The neuraminidase of the virus hydrolyzes the substrate (MPN) which diffuses through the thin agar layer, and the liberated methoxyphenol forms an insoluble red azo dye in the presence of the diazonium salt. After localization, virus was removed from the red focus ("plaque") by a pipette, and various dilutions of the virus were inoculated into eggs for demonstration of infective virus.

Immonodiffusion. Influenza virus X-7 (5 mg/ml) was disrupted with 1% sodium dodecyl sulfate and 0.01 M dithiothreitol in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.4) for 30 min at 37 C and used as an antigen in the Ouchterlony technique to detect antibodies directed against X-7. Antisera were prepared by immunization of rabbits with 100 μg of viral protein intradermally in Freund adjuvant using NWS (HON1) and X-7 (HON2) purified virus preparations. Antiserum was also prepared to X-7 neuraminidase which had been eluted from polyacrylamide gels. A boosting dose of the same quantity was given after 6 weeks, and the animals were bled 1 week later. Immunodiffusion plates were prepared by using 3-cm diameter plates containing 2 ml of 0.75% agarose (Bio-Rad) and 0.05% sodium azide in 0.02 M Tris-hydrochloride buffer, *p*H 7.4. Disrupted virus was applied to the center well, and heat-inactivated antiserum (30 min at 56 C) was applied to the outer wells.

After 2 or 3 days, the plates were overlaid with several changes of 0.02 M Tris-hydrochloride buffer to remove sodium azide and unprecipitated protein. MPN (0.002 M) and $CaCl_2$ (0.005 M) each in 0.5 ml of 0.02 M Tris-hydrochloride buffer (*p*H 7.4) were then added to the plates and incubated at 37 C for 5 to 10 min. The neuraminidase bands were observed after the addition of 0.1 ml of a 0.5% solution of Black K salt to the reaction mixture and incubation for another 5 to 10 min at 37 C. The supernatant fluid was decanted, and the unreacted Black K salt was removed by several changes of Tris-hydrochloride buffer over a 24-hr period. Under these conditions, the pattern was quite stable for days. The addition of acetic acid (7%) further stabilized the immunoprecipitin lines. Concanavalin A (purchased from Pharmacia, Sweden) was used at a concentration of 30 mg/ml to replace antiserum in some experiments.

RESULTS

Neuraminidase assay. Figure 1 shows the reaction of MPN hydrolyzed by neuraminidase with methoxyphenol and *N*-acetylneuraminic acid as the end products. With this assay the neuraminidase test is reduced to a simple analytical determination of 3-methoxyphenol, a one-step reaction. The standard curve in Fig. 2 demonstrates the linearity of absorption of 3-methoxyphenol at 750 nm. Also shown is the absorption of protein alone produced by the reaction of tyrosine residues with this reagent. A final concentration of 0.5 mg of protein/ml in the reaction mixture results in an absorption of 0.15 optical density units. Under the conditions of the thiobarbituric acid assay, partial hydrolysis of MPN occurs, and therefore this test cannot be used with MPN as substrate. Purified virus preparations and commercially available purified bacterial neuraminidases do not show significant background absorption for protein in the assay system. However, we are not able to use MPN as a substrate to measure neuraminidase activity of viruses in chicken embryo allantoic fluids because of the high protein concentration present.

Using the present assay system, the unit of neuraminidase activity can be defined according to the rules of the Commission on Enzymes of the International Union of Biochemists as follows. One neuraminidase unit is the amount of enzyme which splits at 37 C 1 μmole of MPN per minute, the substrate concentration being 1.5×10^{-3} M.

Localization of foci of myxovirus replication in cell cultures. Another application of MPN is the direct observation of sites of myxovirus replication in cell cultures by the localization of the viral neuraminidase activity. We sought to recover the virus located in monolayers by this method and thus to isolate noncytopathic viruses. X-7, X-31, and A/WSN virus readily develop red foci in the respective cell systems (see Table 1). All three viruses were recovered from the foci, demonstrating that infective virus remained after the staining procedure with MPN and diazonium salt. After one passage through eggs, the viruses showed identical behavior with respect to staining for neuraminidase (MPN) and cytopathic effects (CPE). In cell-virus systems which presumably did not show any CPE (X-15 and A/equi 1 inoculated into clone 1-5C-4 cells), monolayers were stained for neuraminidase activity. At a low final dilution of virus (10^{-3}), red plaques specific for neuraminidase could be detected on the third and fourth day postinfection but no CPE as demonstrated by crystal violet staining was seen. However, later experiments showed that with longer incubation periods (5 to 6 days) small cytolytic plaques did appear, and, at all dilutions where neuraminidase-positive plaques could be demonstrated, CPE were subsequently detected.

In another instance where viruses had been observed to show no or very little CPE in a given cell system, we found that foci stained for neuraminidase in those cell systems only where some CPE was demonstrable (Table 1). Parainfluenza virus SV-5 produced small plaques in our MDBK cells, and small but clear neuraminidase foci were detected.

Our present data indicate that neuraminidase foci can be detected under circumstances in which myxoviruses replicate in cell monolayers and produce some degree of grossly discernible cellular damage. Thus far we have not identified such foci in the absence of coincident CPE. Whether this result reflects the necessity

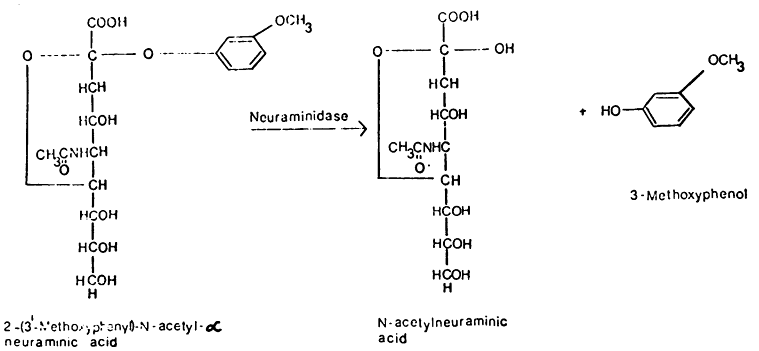

FIG. 1. *Enzymatic hydrolysis of 2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN). The enzyme hydrolyses the ketosidic linkage at C-2 of N-acetyl-neuraminic acid. The enzymatically released 3-methoxyphenol is either determined by a Folin test or coupled to a diazonium salt to give an insoluble dye.*

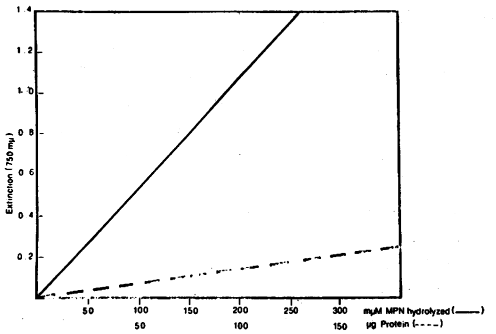

FIG. 2. *Standard curve for neuraminidase activity using MPN as substrate. The ordinate shows the absorption of enzymatically released 3-methoxyphenol (MPN hydrolysed) at 750 nm as obtained under standard assay conditions using the Folin reagent. The assay mixture of 0.2 ml contains 1.5 × 10^{-3} M MPN. The graph also shows the background absorption of protein, which interferes with this test when present in high concentration.*

TABLE 1. *Virus-cell systems used for the localization of viral neuraminidase foci*

Virus	Cells	Neuraminidase-active foci[a]	Cytolytic plaques[a]	Infective virus
X-7	1-5C-4	+ (4)	+ (4)	+
A/WSN	MDBK	+ (4–6)	+ (4–6)	+
X-31	1-5C-4	+ (4–6)	+ (4–6)	+
X-15	1-5C-4	+[b] (3)	+[b] (5)	+
A/equi 1	1-5C-4	+[b] (4)	+[b] (6)	ND
SV-5	MDBK	+ (5)	+ (5)	ND
SV-5	1-5C-4	–	–	
Sendai	MDBK	±	±	ND
3 HA-1	1-5C-4, MDBK	–	–	
4 A	1-5C-4, MDBK	–	–	

[a] Numbers in parentheses indicate day postinfection when neuraminidase-active foci or cytolytic plaques were first detected.

[b] Only at very low dilutions of inoculating virus (10^{-3}).

[c] ND, Not done.

for cellular destruction so that the enzymatic foci can be observed or merely the fact that we have not yet examined a truly "non-cytopathic" virus, remains to be determined. In any event, the system is valuable for finding plaques at the "CPE threshold" where CPE is minimal and plaques are not readily detected.

Demonstration of active viral neuraminidase in antigen-antibody precipitin lines. It had been shown by Fazekas that in the neuraminidase inhibition test for determination of antibodies to this enzyme, fetuin, a large molecule of about 45,000 daltons, was an excellent substrate since increased inhibition of neuraminidase activity occurred with increased concentration of anti-neuraminidase antibody (3). However, the small substrate *N*-acetylneuraminosyl-lactose (MW-633) was unsuitable for this test since no inhibition of neuraminidase activity could be demonstrated in the presence of anti-neruaminidase antibodies (3). It was concluded that the antibodies formed against neuraminidase are not directed to the active site, but to another site. On combination of the antibody with neuraminidase, small substrates such as *N*-acetylneuraminosyl-lactose are allowed access to the active site and are cleaved; large substrates such as fetuin are sterically blocked from the active site by the antibody and are not cleaved, resulting in neuraminidase inhibition.

Anti-neuraminidase antiserum with a 50% inhibition titer of 1:1,000 with fetuin as a substrate did not show any inhibitory activity when *N*-acetyl-neuraminosyl-lactose or MPN was used as substrate, even at serum dilutions as low as 1:40 or 1:20, respectively. It should be noted, however, that enzyme inhibition tests using MPN with serum dilutions as low as 1:40 to 1:20 are difficult to measure because of the high background absorption of the protein present in serum.

Neuraminidase inhibition was also titrated using large and small substrates with concanavalin A substituted for antiserum. Evidence has suggested that concanavalin A reacts with the neuraminidase component of the influenza virion (16). When concanavalin A was substituted for antiserum in an enzyme inhibition test identical to that used for anti-neuraminidase antibody titration, similar results were obtained as for the antiserum. Concanavalin A, at a concentration of 123 μg/ml, caused a 50% inhibition of neuraminidase activity with fetuin as a substrate. When MPN was used as a substrate, no inhibitory effect could be observed even with concanavalin A at 50 times higher concentration, 6.15 mg/ml.

From this evidence we inferred that MPN is readily accessible to the active site of neuraminidase even when anti-neuraminidase antibodies or concanavalin A are complexed with the enzyme. Therefore, MPN should serve as a reagent for the detection of neuraminidase precipitin lines in immunodiffusion tests.

Figure 3 shows the immunodiffusion pattern of disrupted X-7 influenza virus against antibodies to three viral preparations including A/NWS (H0N1), wells 1 and 4; X-7 (H0N2), wells 2 and 5; and neuraminidase from X-7, wells 3 and 6. After incubation with MPN,

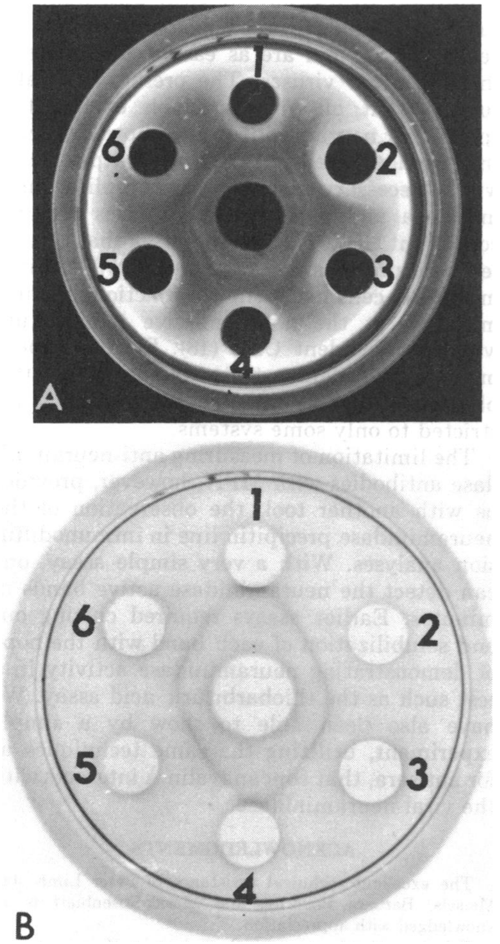

FIG. 3. Immunodiffusion patterns are shown for X-7 virus (center well) against three different antisera (outer wells). The X-7 virus was disrupted by heating with 1% sodium dodecyl sulfate and 0.01 M dithiothreitol at 37 C for 30 min. Wells 1 and 4 contained antiserum against A/NWS (HON1), wells 2 and 5 contained antiserum against X-7 (HON2) virus, and wells 3 and 6 contained antiserum against X-7 neuraminidase. The patterns were photographed before (top) and after (bottom) incubation with MPN and diazonium salts. The lines in the bottom pattern are bright red.

followed by the addition of diazonium salt, one precipitin line developed the characteristic red color indicative of neuraminidase activity.

By a simple radial diffusion test, we were able to identify one major band when disrupted X-7 virus was diffused against concanavalin A (wells 1 and 4 in Fig. 4). Wells 2, 3, 5, and 6 contained the same reagents as for Fig. 3. Under the same conditions of incubation with MPN used above, we were able to demonstrate neuraminidase activity in the concanavalin A precipitin band (shown by arrows) and verify that concanavalin A interacts with the neuraminidase antigen. However, the incubation

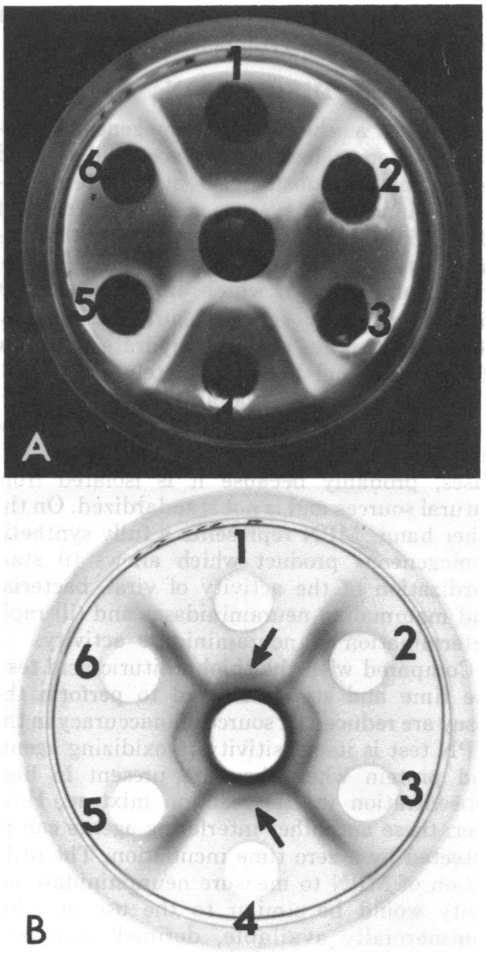

FIG. 4. Conditions for immunodiffusion were as given in Fig. 3. The center well contained disrupted X-7 virus, wells 1 and 4 contained concanavalin A (30 mg/ml), wells 2 and 5 contained antiserum against X-7 virus, and wells 3 and 6 contained antiserum against X-7 neuraminidase. The axial lines radiating from the center in Fig. 4 top and bottom were heavy precipitates of concanavalin A and serum protein. These lines absorbed some diazonium salt in the staining procedure and thus are evident in Fig. 4, bottom. However these lines were stained light brown, not red, showing complexing to protein rather than neuraminidase activity. The radial bands, both to concanavalin A (see arrows) and the antisera in wells 2 and 3, were red, indicating neuraminidase activity.

times required for the degree of color development were approximately twice as long for the concanavalin A-neuraminidase complex as for the antibody-neuraminidase complex. The neuraminidase precipitin line does not appear to be contiguous between wells 1, 2, and 3 in Fig. 4 because of the heavy axial lines formed between the outer wells by the precipitation of concanavalin A with components in the serum.

DISCUSSION

MPN is a chemically homogeneous substrate, whereas fetuin, the most widely used neuraminidase substrate, is quite heterogeneous. Fetuin has an average molecular weight of about 45,000 and contains two different neuraminic acid derivatives, *N*-acetyl and *N*-glycolyl-neuraminic acid, which are hydrolyzed at different rates by various neuraminidases (4, 11). Furthermore, the carbohydrate structure of fetuin has not been completely elucidated. The commercially available low-molecular-weight substrate *N*-acetylneuraminosyl-lactose also shows variable reactions with neuraminidases, probably because it is isolated from natural sources and is not standardized. On the other hand, MPN represents a fully synthetic homogeneous product which allows (i) standardization of the activity of viral, bacterial, and mammalian neuraminidases and (ii) rapid determination of neuraminidase activity.

Compared with the thiobarbituric acid test, the time and steps involved to perform the assay are reduced. A source of inaccuracy in the MPN test is its sensitivity to oxidizing agents and protein when they are present in high concentration in the reaction mixture. However, these and other interfering agents can be detected by a zero time incubation. The utilization of MPN to measure neuraminidase activity would be similar to the use of other commercially available, defined, synthetic substrates for the determination of hydrolases such as glycosidases and phosphatases. Therefore it is desirable to introduce a chemically defined substrate for a standard neuraminidase assay. It should be noted, however, that the low-molecular-weight substrate MPN can not be used to measure neuraminidase inhibition activity of antineuraminidase antibodies as this test requires a large neuraminidase substrate such as fetuin.

The specific staining for neuraminidase with MPN in different virus-cell systems is promising for defining viral neuraminidase activity during the course of infection. It is interesting that A/WSN virus, the neuraminidase of which is difficult to measure in vitro with fetuin as substrate, produces red plaques which develop rapidly and are as easily discernible as those of other viruses. The present data also suggest that all viruses which exhibit foci specific for neuraminidase also showed CPE in that particular cell system. Future experiments will be necessary to show whether replication of influenza and parainfluenza viruses requires coincident CPE. It is worth noting that certain temperature-sensitive mutants of WSN virus in MDBK cells never show a functional neuraminidase at the nonpermissive temperature without coincident CPE (18). Further experiments should demonstrate whether or not this phenomenon of asymmetric covariation is restricted to only some systems.

The limitation of measuring anti-neuraminidase antibodies with MPN, however, provides us with another tool, the observation of the neuraminidase precipitin line in immunodiffusion analyses. With a very simple assay, one can detect the neuraminidase active bands in minutes. Earlier assays required cutting out and solubilization of each band with the hope of demonstrating neuraminidase activity in a test such as the thiobarbituric acid assay. We have also been able to show by a simple experiment, utilizing the same techniques as for antisera, that concanavalin A interacts with the viral neuraminidase.

ACKNOWLEDGMENTS

The excellent technical assistance of Peter Lima, Jan Meisels, Barbara Pokorny, and Mark Rosenblatt is acknowledged with appreciation.

This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by research contract no. DA-49-193-MD-1795 from the U.S. Army Medical Research and Development Command, by Public Health Service research grant no. A1-09304 from the National Institute of Allergy and Infectious Diseases, and by contract no. U-1023 from the Health Research Council of the City of New York.

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