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NEURAMINIDASE FROM INFLUENZA VIRUS A (H3N2)

SPECIFICITY TOWARDS SEVERAL SUBSTRATES AND PROCEDURE OF ACTIVITY DETERMINATION

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Summary

Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) from the influenza virus A/Hong Kong/68 (H3N2) was purified after treatment of the purified virus with sarcosyl (sodium laurylsarcosinate), centrifugation at 110 000 × *g*, and chromatography on DEAE-Sephadex and Sephadex G-200. It migrated as a single component during electrophoresis on polyacrylamide gel, and its molecular weight was estimated about 270 000.

The enzyme was thermolabile, the activity being reduced to 60% in 10 min at 50°C. The purified neuraminidase had an apparent K_m value of $4.1 \cdot 10^{-3}$ M for 5-*N*-acetyl-2-*O*-(3-methoxyphenyl)-α-D-neuraminic acid and was able to release sialic acid with linkages α2-3, α2-6 and α2-8 (with very different efficiency) from fetuin, gangliosides, colominic acid, and bovine and porcine submaxillary mucins.

The enzymic activity was measured by several procedures: (A) spectrophotometric determination at 340 nm of the NADH produced in the reaction catalysed by β-galactose dehydrogenase on β-galactose + NAD^+, this β-galactose was the product released from lactose by β-galactosidase and lactose was the product of the neuraminidase activity on *N*-acetylneuraminyl-lactose; (B) determination of the colored quinone yielded by the liberated methoxyphenol with 4-aminoantipyrine (Santer, U.V., Yee-Foon, J. and Glick, M.C. (1978) Bio-

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Abbreviations: MeOPhe-NeuAc, 5-*N*-acetyl-2-*O*-(3-methoxyphenyl)-α-D-neuraminic acid; Neu*N*Gc, *N*-glycolylneuraminic acid.

chim. Biophys. Acta 523, 435—442); (C) periodate-thiobarbiturate procedures (Warren, L. (1959) J. Biol. Chem. 234, 1971—1975 or Aminoff, D. (1961) Biochem. J. 81, 384—391). Some peculiarities of these methods are discussed.

Introduction

Neuraminidases (acylneuraminyl hydrolase, EC 3.2.1.18) from different virus influenza strains have different physicochemical, kinetic and immunological properties. On the other hand, their biological functions are not yet well understood [1—10]. Even the evidence for the origin of the Hong Kong neuraminidase rests on serology alone [11].

In 1973, some antigenic mutants of the strain A/NT60/68 (H3N2) were selected under pressure of antibody, starting with a strain isolated in 1968 [12]. A stabilized, dominant strain, was used as vaccine [13]; however, some problems (such as poor yielding when grown in eggs) have been found in the use of these strains as vaccines. Anyway, it seems that the production of 'prospective' viral variants in this manner offers, from an epidemiological point of view, few advantages over other methods [13].

Vaccines containing only haemagglutinin and neuraminidase should have some advantages on the whole vaccines which contain undesired antigens [14—16]. But there is some difficulty in obtaining neuraminidase from influenza virus in pure form.

It is interesting to study some peculiarities of the neuraminidase from the influenza virus A/Hong Kong (H3N2); this strain is mainly responsible for all the influenza in the world and it was circulating in 1979 under the name 'Texas 77' [9].

In this paper we describe the specificity of the purified enzyme from the A/NT60/68 (H3N2) strain, identical to the A/Hong Kong/68 (H3N2) strain, towards some natural substrates and a simple procedure of neuraminidase activity determination.

Materials and Methods

Materials. The strain of virus used was A/NT60/68 (H3N2), an isolate from Australia identical to the prototype A/Hong Kong/68 (H3N2). It was grown in the allantoic cavity of 11-day-old chick embryos and harvested after 48 h of incubation at 35°C and one night at 4°C. The pooled allantoic fluids were treated by a modification of the method described by Skehel and Schild [17] for virus purification. Briefly, the fluids were clarified by centrifugation at 10 000 × *g* for 20 min; the virus was pelleted at 40 000 × *g* for 3 h and the pellet was resuspended in buffer 1 (20 mM HCl-Tris/1 mM EDTA/0.1 M NaCl) pH 7.4. The suspension was then banded between 30 and 60% sucrose in buffer 1 at 150 000 × *g* for 90 min. The sharp band containing influenza virus was adjusted to 10% sucrose concentration, layered onto a 15—60% sucrose gradient in buffer 1 and centrifuged at 80 000 × *g* for 15 h. Purified virus is present in a single sharp band.

Acrylamide, bovine serum albumin, Tris(hydroxylmethyl)aminomethane,

sodium dodecyl sulphate (SDS), bovine submaxillary mucin, *N*-acetyl-neuraminyl-lactose and DEAE-cellulose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Blue Dextran, Sephadex G-200 and DEAE-Sephadex were obtained from Pharmacia (Uppsala, Sweden). Lactose/galactose Test-combination was from Boehringer (Mannheim, F.R.G.). Sarcosyl NL-97 (sodium lauryl sarcosinate) was from Ciba-Geigy Ltd. (Basle, Switzerland). CMP-*N*-acetyl[^{14}C]neuraminic acid was from The Radiochemical Centre (Amersham, U.K.). Fetuin, porcine submaxillary mucin and colominic acid were gifts from Dr. L. Warren (Philadelphia, PA, U.S.A.). 5-*N*-Acetyl-2-*O*-(3-methoxyphenyl)-α-D-neuraminic acid was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, NIH (Bethesda, MD, U.S.A.). Gangliosides of horse brain were prepared using the method of Reglero et al. [18]. All other reagents were of analytical grade. Generally, the purity of the substrates was checked by TLC and electrophoresis.

Purification procedure for neuraminidase. Purified virus (60 mg), suspended in 15 ml 0.1 Tris-HCl buffer (pH 8) containing 1 mM EDTA, was treated with sarcosyl (2.5% final concentration) for 1 h, and then dialyzed against the same buffer (pH 7.4) containing 0.05% sarcosyl. After centrifugation (1 h at (110 000 × *g*), the sample was applied to a DEAE-Sephadex A-50 column (2.5 × 20 cm) equilibrated with the dialysis buffer. Elution was performed using a linear NaCl (0—1.5 M) gradient in the same buffer.

Fractions containing either neuraminidase or hemagglutinin were pooled, concentrated by ultrafiltration on a PM10 Amicon membrane and layered on a (2.5 × 100 cm) Sephadex G-200 column equilibrated with 50 mM NH_4HCO_3 (pH 7.8) containing 0.05% sarcosyl.

Neuraminidase eluted in the first peak followed by the hemagglutinin, the latter was tested by the antibody blocking test [19].

Neuraminidase assays. Three procedures were employed:

(A) 25 μl enzyme preparation (containing 0.5 μg protein) were incubated at 37°C with 25 μl of a solution of *N*-acetylneuraminyl-lactose as substrate; this solution contained 6 mg substrate/ml, in 50 mM potassium phosphate buffer (pH 5.9). After 20 min incubation, the method for determination of lactose and galactose (commercialized by Boehringer Mannheim, catalogue No. 176303) was followed, employing half amounts of the solutions. Lactose (liberated by the neuraminidase activity) was the substrate for β-galactosidase activity [20], this enzyme released β-galactose. β-Galactose + NAD^+ yielded NADH, by β-galactose dehydrogenase activity [21]; NADH was measured by spectrophotometry at 340 nm. Generally, this method was employed to check the purity of the neuraminidase from the virus; the enzyme activity was determined in all steps of the purification procedure.

(B) In the kinetic and other assays, 5-*N*-acetyl-2-*O*-(3-methoxyphenyl)-α-D-neuraminic acid (MeOPhe-NeuAc) was used as a substrate. The methoxyphenol liberated by the enzyme (0.6 μg protein) was determined by the measure of the colored quinone formed by methoxyphenol with 4-aminoantipyrine in the presence of potassium ferricyanide, as described by Santer et al. [22].

(C) The activity of the enzyme on some glycoproteins, gangliosides and colominic acid was determined by the periodate-thiobarbiturate method of Warren [23] as modified by Aminoff [24]. The concentration of the liberated

sialic acid(s) was compared with the total amount of these acids, which was determined using the resorcinol method of Svennerholm [25] as modified by Miettinen and Takki-Luukkainen [26].

Protein determination. Protein was determined by the method of Lowry et al. [27] using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. Electrophoresis was performed employing essentially the method of Ornstein [28] and Davis [29] with 7, 5 and 3.75% acrylamide gels, run at room temperature for 60 min, using Tris-glycine buffer (pH 8.3). The proteins in the gels were stained with Coomassie brilliant blue according to Weber and Osborn [30]. In order to establish if the neuraminidase activity was located in the protein band, a duplicate gel run in parallel was sliced into 1-mm segments and each segment was incubated in 50 mM potassium phosphate buffer (pH 5.9), with 0.1 μmol MeOPhe-NeuAc as substrate. The total volume of reaction was 200 μl and the activity was determined as indicated above.

Estimation of molecular weight by gel filtration. A column (42 × 1.7 cm) of Sephadex G-200 was loaded with the sample of neuraminidase (1.2 mg protein in 1 ml), and equilibrated and eluted with 0.1 M phosphate buffer (pH 5.85), which was the same buffer employed in the enzyme reaction. Trypsin (M_r approx. 23 800), ovalbumin (45 000), bovine serum albumin (67 000) and γ-globulin (160 000) were used as column markers. Assays were performed on the eluted fractions to determine the neuraminidase position by the methoxyphenol procedure [22].

Assay of the glycosyl transferase activity. Several tests were carried out to determine whether or not the neuraminidase of this study could transfer the *N*-acetylneuraminic acid from CMP-*N*-acetyl[^{14}C]neuraminic acid to a glycoprotein acceptor (native fetuin and desialylated fetuin). The desialylation was performed in 100 mg fetuin by 10 ml of 0.05 M H_2SO_4 at 80°C for 60 min; after neutralization the fetuin was dialyzed overnight [31]. Enzyme (11.5 μg protein) was incubated with the radioactive substrate (70 000 cpm of CMP[^{14}C]NeuAc, specific activity 304 Ci/mol) for 60 min at 37°C. The incubation mixtures also contained controls with and without $MgCl_2$ and zero-time controls. The radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer model 3255.

Results

Purity of the isolated neuraminidase. After chromatography on Sephadex G-200, neuraminidase was eluted in the first peak and was completely separated from hemagglutinin. The pool of purified neuraminidase fractions showed one band by polyacrylamide gel electrophoresis (Fig. 1). This band and the neuraminidase activity were located at an identical position.

Molecular weight. The molecular weight of the purified neuraminidase, estimated by filtration through Sephadex G-200, was about 270 000.

Thermal stability of neuraminidase. The enzyme activity (considered as 100%), measured by the NADH procedure, was reduced to 70% after 5 min and to 60% after 10 min of previous incubation at 50°C; this value remains constant until 45 min at least.

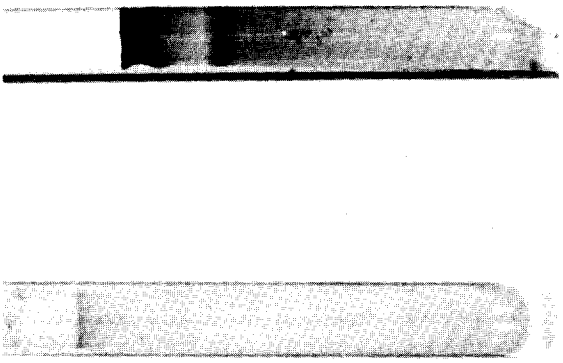

Fig. 1. Polyacrylamide gel electrophoresis of neuraminidase. Purified neuraminidase (25 μg) was applied to 5% (bottom) and 7% (top) polyacrylamide gels, subjected to electrophoresis and stained as described under Materials and Methods.

Kinetic study of enzyme activity. The concentration values of methoxyphenol released from MeOPhe-NeuAc at various incubation times, at 37°C, are shown in Fig. 2. The velocity was constant for the first 15 min and then decreased to reach a maximum at 90 min. At the initial reaction velocity, 9 μmol methoxyphenol/min per mg protein were liberated.

The variation of reaction velocity with enzyme concentration is shown in Fig. 3. Taking into consideration this result, 0.6 μg protein was the amount generally employed later.

The Michaelis constant (K_m) of the reaction was determined using concentrations of MeOPh-NeuAc as substrate ranging between 125 and 1000 μM, in 50 mM potassium phosphate buffer (pH 5.9), for 10 min at 37°C. The K_m value, obtained by Lineweaver-Burk plots, was $4.1 \cdot 10^{-3}$ M. This test was repeated five times, with coincident results. V was 11 μmol/min per mg protein.

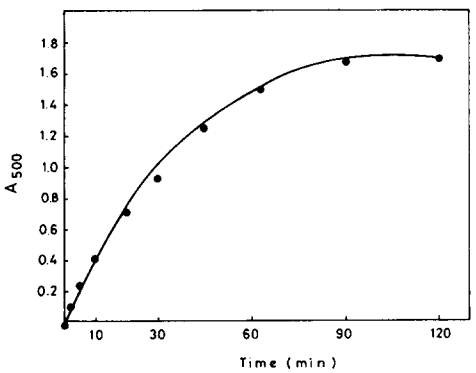

Fig. 2. Kinetics of release of methoxyphenyl by neuraminidase. Details of the assays are given in Materials and Methods.

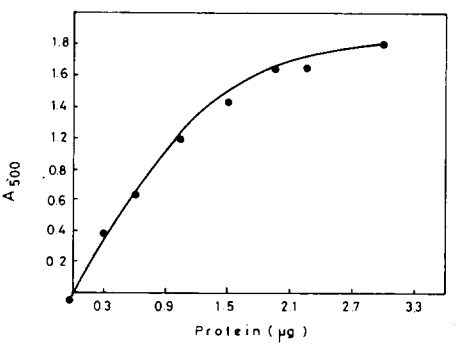

Fig. 3. Kinetics of release of methoxyphenyl by different enzyme concentrations. Aliquots of enzyme containing between 0.3 and 3.3 μg of protein were incubated with MeOPhe-NeuAc in the conditions indicated in Materials and Methods.

TABLE I

SUBSTRATE SPECIFICITY OF THE NEURAMINIDASE FROM VIRUS INFLUENZA A/HONG KONG/68 (H3N2) TOWARDS SEVERAL NATURAL SUBSTRATES

The concentrations of total sialic acids were determined in the substrates by the modified resorcinol procedure [25,26]. Substrates containing 20 μg total sialic acid(s) were employed in all the assays. Purified enzyme (1.1 μg protein) was incubated in 40 mM potassium phosphate buffer (pH 5.9), in a total volume of 0.25 ml, for 2 h at 37°C. The reaction was stopped by heating at 100°C for 5 min and the liberated sialic acid(s) was determined by the thiobarbituric acid method [24]. N.D., not determined.

Substrate	Linkage of the predominant sialic acid	Type of sialic acid (%)				Sialic acid liberated	
		NeuAc	Neu*N*Gc	N, O, Diacetyl-neuraminic acids	N, O, Triacetyl-neuraminic acids	μg	%
Fetuin	α 2—3	93 [a]	7 [a]	—	—	20	100
Bovine submaxillary mucin	α 2—6	20—25 [b]	15 [b]	40—50 [b]	5—10 [b]	1.4	7
Porcine submaxillary mucin	α 2—6	10 [c]	90 [c]	—	—	1	5
Colominic acid	α 2—8 [d]	100 [d]	—	—	—	2	10
Gangliosides [e]	N.D. [f]	97 [e]	3 [e]			3.7	18

[a] Ref. 32.
[b] Ref. 33.
[c] Ref. 34.
[d] Ref. 35. Other linkage(s) may be contained.
[e] A mixture of GD_{1a} (50%) + GM_1 (21%) + GD_{1b} (15%) + GT_1 (14%) from horse brain [18] was employed.
[f] Taking into consideration that α 2—3 is the unique linkage of the NeuAc found in GM_1, GD_{1a} and GD_{1b} (and α 2—3 and α 2—8 in GT_1) from human brain gangliosides [36], it may be supposed that the same linkage(s) could be found in horse brain gangliosides for NeuAc, which is the predominant sialic acid.

Hydrolysis of natural substrates. The activity of the purified neuraminidase was determined with MeOPhe-NeuAc. 1.1 μg enzyme protein released 10 nmol methoxyphenyl/min.

The purified enzyme was able to liberate sialic acids not only from glycoproteins such as fetuin, bovine submaxillary mucin and porcine submaxillary mucin, but also from colominic acid and from the studied gangliosides, at the assay conditions. Linkages in α2-3, α2-6 and α2-8 of the above mentioned substrates were split off, but not with the same efficiency (Table I).

Transferase activity. The radioactivity recovered in the acceptor glycoproteins was not significantly different from that of the controls (only about 1% higher). Although some hydrolases can also transfer glycosyl residues to several acceptors [37], it seems that this enzyme is devoid of glycosyl transferase activity, at the assay conditions at least.

Discussion

Purification of influenza virus neuraminidase as described in Material and Methods is an improvement of that described by Stanley et al. [38]. After gel filtration in the presence of sarcosyl, a single peak containing neuraminidase activity was detected, which gives a single band in SDS-polyacrylamide gel electrophoresis. Neuraminidase obtained by this procedure is extracted as a complete molecule from the membrane bilayer by contrast with the material obtained after bromelain treatment [39], or other proteases (for review see Ref. 4), which may cleave a short segment of the molecule; this segment is known to be rich in carbohydrates [40].

The molecular weight of this neuraminidase is in the same range as those previously reported for influenza virus strains (see Refs. 2 and 41).

The heat stability of neuraminidases from myxovirus is very variable [42]. Virus A/NT60/68 (H3N2) neuraminidase is thermolabile.

Several methods can be employed to determine the neuraminidase activity: (A) using natural substrates such as neuraminyl-lactose, fetuin, orosomucoid or mucins, by measure of the released sialic acid(s) with the reaction of the periodate-thiobarbiturate [23,24]; (B) using artificial substrates; the liberated product is determined by spectrophotometry, fluorimetry of radioisotopic techniques.

Some substrates can be either small molecules such as *N*-acetylneuraminyl-lactose (from a natural or synthetic source) or large molecules (mucins). Several types of linkages may be found even in small substrates. Thus, in bovine neuraminyl-lactose there are two types of α-ketosidic linkages: an α2-3, as the predominant and an α2-6 (about 8—14%). Neuraminidase from Newcastle disease virus or fowl plague virus cleaves the α2-3 linkage with much more efficiency than α2-6 linkage [43,44]. On the other hand, the ability and rate of neuraminidases for releasing sialic acids also depends: (i) on the type of sialic acid; (ii) on the source of the enzyme. Bacterial neuraminidases from *Vibrio cholerae (comma)* and *Clostridium perfringens* are better known in this respect (for reviews see Refs. 5, 8, 45—48) than virus neuraminidases. However, it has been reported that there are some differences between neuraminidases from bacteria and virus neuraminidases. Thus, the 5-*N*-acetyl-4-*O*-acetylneuraminic acid,

which is the major sialic acid component of the horse submaxillary mucin, is not cleaved by *V. cholerae* or *C. perfringens* neuraminidase [33], but it is released by the neuraminidase from influenza A2/Singapore/57 virus [49], which is a H2N2 virus [50].

The neuraminidase that we have studied shows a wide specificity. It was able to split off α2-3, α2-6 and α2-8 linkages from several natural substrates (Table I), but with varying efficiency, probably according to the type of sialic acid linkage.

On the other hand, it seems that this type of sialic acid, at least, has little or no influence on the ratio of sialic acid released, since similar results were obtained with bovine submaxillary mucin (which contains 15% Neu*N*Gc of total sialic acids amount) and porcine submaxillary mucin (in which Neu*N*Gc is the predominant, 90%). Probably, the type of the carbohydrate is of minor importance for the recognition of the viral neuraminidase; however, bulky substituents of these carbohydrates [43,44] or elimination of some carbon atoms such as 8 and 9 [51] decrease or destroy the neuraminidase activity of some viruses.

The periodate-thiobarbiturate technique [23,24] is a very sensitive method used for measuring free sialic acid(s). However, it must be kept in mind that the molar extinction coefficient of NeuAc given by this method is greater than that of Neu*N*Gc [23,24]; on the other hand, 5-*N*-acetyl-7-*O*-acetylneuraminic acid gives no reaction with periodate [24,33,52,53]. Besides, the occurrence of a dozens of different types of sialic acid has been reported [53—58]; but the molar extinction coefficient of only the predominant ones has been described [53]. Furthermore, sucrose [59] (usually employed in the gradients for fractionation procedures), L-fucose [23], deoxyribose [23,24], glycosides [60] and other substances [61,62] can interfere with some steps of this method.

The determination of the aglycone cleaved from several substrates has been introduced in the assays for neuraminidases, in a similar manner to that employed for other glycosidases. In this case, the aglycone could be the phenol released from 5-*N*-acetyl-2-*O*-phenyl-α-D-neuraminic acid, which could be determined by the Folin phenol procedure (for review, see Bucher and Palese, Ref. 3); or the 3-methoxyphenol (liberated from 5-*N*-acetyl-2-*O*-3-methoxyphenyl-α-D-neuraminic acid), which could be measured either by the Folin phenol assay [3], or by the chromogen given by combination with a diazonium salt [3], to avoid the interference with the product reaction of the proteins with the Folin reagent. The methoxyphenol method, as modified by Santer et al. [22], is a very simple procedure which avoids the interference with proteins; the substrate is now commercially available.

The determination of lactose, released from *N*-acetylneuraminyl-lactose by neuraminidases, has also been reported [63]. The *N*-acetylneuraminic acid and other sialic acids do not interfere with this assay. But this method requires the separation of lactose by adsorption, on a semi-micro ion-exchange column, and elution.

The determination of the neuraminidase activity by coupling with two other enzymic reactions, as described in Materials and Methods section, to finally measure NADH, appears satisfactory. If NADH is determined by spectrophotometry at 340 nm, its molar extinction coefficient is 6200. The sensitivity of

this procedure is about half that of the Santer et al. method [22] and a tenth of periodate-thiobarbiturate procedures [23,24]. Its sensitivity is also less than that of the resorcinol and orcinol methods [25,53,65] but similar to that of the alkali-Ehrlich technique [24]; the latter three procedures are usually employed for the determination of bound and total sialic acids.

Anyway, the sensitivity of the spectrophotometric NADH determination could be highly increased (about 100-fold [56]) by measuring the fluorescence change, as it has been reported for the NADH fluorimetric determination [66,67], instead of its spectrophotometric measure.

On the other hand, in the NADH determination (and in the Santer et al. procedure [22]) there is no difference due to the reactions with the different types of sialic acid. However, β-D-galactosides, α-L-arabinose and β-D-fucose could interfere with the measure of neuraminidase activity by the NADH procedure [20,21]; to avoid errors due to the presence of these substances, appropriate controls must be included.

The NADH produced by the neuraminidase activity could also be coupled with tetrazolium salts [68—70] to stain gels and to detect the enzyme in the fraction steps of the purification or characterization procedures.

Finally, other methods for neuraminidase activity determination have been reported: (i) using either radioisotopic substrates such as α-D-acetylneuraminyl-(2-3′)-lactit[^{3}H]ol [71], or [^{3}H]ganglioside G_{D1a} [72], or [^{14}C]gangliosides G_{D1a} and G_{M3} labeled in the sialic acid residue [73] (the nomenclature of the gangliosides is that of Svennerholm [74]) or the [^{3}H]*N*-acetylneuraminic acid analogs released from glycoproteins [75,76] and (ii) using fluorigenic substrates (methylumbelliferyl-derivatives) [77,78]. Only the former substrates are now commercially available.

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References

1 Drzeniek, R. (1974) in Symposium on Neuraminidase (Heide, K., Seiler, F.R. and Schwick, H.G., eds.), pp. 1—33, Behringwerke AG, Marburg/Lahn
2 Klenk, H.D. (1974) Curr. Top. Microbiol. Immunol 68, 29—58
3 Bucher, D. and Palese, P. (1975) in The Influenza Viruses and Influenza (Kilbourne, E.D., ed.), pp. 89—90, Academic Press, New York
4 Bottex, C. and Fontanges, R. (1976) Lyon Pharm. 27, 327—348
5 Rosenberg, A. and Schengrund, C.L. (1976) in Biological Roles of Sialic Acids (Rosenberg, A. and Schengrund, C.L., eds.), pp. 295—359, Plenum Press, New York
6 Hutchinson, D.W. and Kabayo, J.P. (1977) Trends Biochem. Sci. 2, 1—3
7 Rott, R. (1977) Med. Microbiol. Immunol. 164, 23—33
8 Schauer, R. (1977) Acta Histochem. 18, 25—44

9 Hannoun, C. (1979) La Recherche 96, 75—77
10 Rott, R. (1979) Arch. Virol. 59, 285—298
11 Laver, W.G. (1978) Virology 86, 78—87
12 Fazekas de St. Groth, S. and Hannoun, C. (1973) C.R. Acad. Sc. Pairs (Série D) 276, 1917—1920
13 Hannoun, C. (1976) in Influenza: Virus, Vaccines, and Strategy (Selby, P., ed.), pp. 217—225, Academic Press, New York
14 Bachmayer, H. (1976) in Influenza: Virus, Vaccines, and Strategy (Selby, P., ed.), pp. 149, 162, Academic Press, New York
15 Laver, W.G. (1976) in Influenza: Virus, Vaccines and Strategy (Selby, P., ed.), pp. 163—177, Academic Press, New York
16 Webster, R.G., Kassel, J.A., Cooch, R.B. and Laver, W.G. (1976) J. Infect. Dis. 134, 48—58
17 Skehel, J.J. and Schild, G.C. (1971) Virology 44, 396—408
18 Reglero, A., Garcia-Alonso, J. and Cabezas, J.A. (1980) J. Neurochem. 34, 744—746
19 Webster, R.G. and Darlington, R.W. (1969) Virology 4, 182—187
20 Kurz, G. and Wallenfels, K. (1974) in Methoden der Enzymathischem Analyse (Bergmeyer, H.U., ed.), Vol. 2, pp. 1225—1229, Verlag Chemie, Weinheim. (Also in english edition, Methods of Enzymatic Analysis, Vol. 3, pp. 1180—1184, Academic Press, New York)
21 Kurz, G. and Wallenfels, K. (1974) in Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 2, pp. 1324—1327, Verlag Chemie, Weinheim. (Also in english edition, Methods of Enzymatic Analysis, Vol. 3, pp. 1279—1282, Academic Press, New York)
22 Santer, U.V., Yee-Foon, J. and Glick, M.C. (1978) Biochim. Biophys. Acta 523, 435—442
23 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
24 Aminoff, D. (1961) Biochem. J. 81, 384—391
25 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 605—611
26 Miettinen, T.and Takki-Luukkainen, I.T. (1959) Acta Chem. Scand. 13, 856—857
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321—349
29 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
30 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
31 Ko, G.H.W. and Raghupathy, E. (1971) Biochim. Biophys. Acta 244, 369—409
32 Graham, E.R.B. (1972) in Glycoproteins: Their Composition Structure and Function (Gottschalk, A., ed.), Vol. 5A, p. 722, Elsevier, Amsterdam
33 Schauer, R. and Faillard, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 961—968
34 Gottschalk, A. and Bhargava, A.S. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), Vol. 5B, p. 823, Elsevier, Amsterdam
35 Gottschalk, A. and Drzeniek, R. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), Vol. 5A, pp. 393—394, Elsevier, Amsterdam
36 Marshall, R.D. and Neuberger, A. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), Vol. 5A, p. 342, Elsevier, Amsterdam
37 Committee of the International Union of Biochemistry (1979), Enzyme Nomenclature 1978, Recommendations, pp. 160, 274, Academic Press, New York
38 Stanley, P., Crook, N.E., Gail Streader, L. and Davidson, B.E. (1973) Virology 56, 640—645
39 Bachmayer, H. and Schmidt, G. (1972) Med. Microbiol. Immunol. 158, 91—94
40 Rott, R., Becht, H., Klenk, H.D. and Scholtissek, C. (1972) Z. Naturforsch. 27b, 227—233
41 Webster, R.G. (1972) Curr. Top. Microbiol. immunol. 59, 75—105
42 Drzeniek, R., Seto, J.T. and Rott, R. (1966) Biochim. Biophys. Acta 128, 547—558
43 Drzeniek, R. (1972) Curr. Top. Microbiol. Immunol. 59, 35—74
44 Drzeniek, R. (1973) Histochemistry J. 5, 271—290
45 Faillard, H. (1966) in Handbuch der physiologisch- und pathologisch-chemischen Analyse, (Hoppe Seyler/Thierfelder, eds.), Vol. 6B, pp. 1241—1259, Springer Verlag, Berlin
46 Gottschalk, A. and Drzeniek, R. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), Vol. 5A, pp. 381—402, Elsevier, Amsterdam
47 Müller, H.E. (1974) Dtsch. Med. Wschr. 99, 1933—1940
48 Holmquist, L. (1975) FOA Rep. 9, 1—20
49 Pepper, D.S. (1968) Biochim. Biophys. Acta 156, 317—326
50 WHO Bull. (1971) 45, 119—124
51 Suttajit, M. and Winzler, R.J. (1971) J. Biol. Chem. 246, 3398—3404
52 Paerels, G.B. and Schut, J. (1965) Biochem. J. 96, 787—792
53 Schauer, R. (1978) Methods Enzymol. 50, 64—89
54 Schauer, R. (1973) Angew. Chem. (Int. Edn.), 12, 127—138
55 Cabezas, J.A. (1973) Rev. Esp. Fisiol. 29, 307—322
56 Buscher, H.P., Casals-Stenzel, J. and Schauer, R. (1974) Eur. J.Biochem. 50, 71—82
57 Haverkamp, J., Schauer, R., Wember, M., Farriaux, J.P., Kamerling, J.P., Versluis, C. and Vliegenthart, F.G. (1976) Hoppe-Syeler's Z. Physiol. Chem. 357, 1699—1705

58 Kamerling, J.P., Haverkamp, J., Vliegenthart, J.F.G., Versluis, C. and Schauer, R. (1978) in Recent Developments in Mass Spectrometry in Biochemistry and Medicine (Frigerio, A., ed.), pp. 503—520, Plenum Pub. Corp., New York
59 Haslam, E.A., Hampson, A.W., Radiskevis, I. and White, D.O. (1970), Virology 42, 566—575
60 Kuwahara, S.S. (1979) Anal. Biochem. 101, 54—60
61 Cabezas, J.A. and Feo, F. (1969) Rev. Esp. Fisiol. 25, 153—156
62 Cabezas, M. (1978) Int. J. Biochem. 9, 47—49
63 Reference deleted.
64 Holmquist, L. (1969) Acta Chem. Scand. 23, 1045—1052
65 Faillard, H. and Cabezas, J.A. (1963) Hoppe-Seyler's Z. Physiol. Chem. 333, 266—271
66 Williamson, J.R. and Corkey, B.E. (1969) Methods Enzymol. 13, 435, 485—488
67 Klingenberg, M. (1974) in Methoden der Enzymatischen Analyse (Bergmeyer, H.U., ed.), Vol. 2, pp. 2094—2122, Verlag Chemie, Weinheim
68 Quastel, J.A. (1957) Methods Enzymol. 4, 334—336
69 Fine, I.H. and Costello, L.A. (1963) Methods Enzymol. 6, 962—972
70 Gabriel, O. (1971) Methods Enzymol. 22, 585—586
71 Bhavanandan, V.P., Yeh, A.K.and Carubelli, R. (1975) Anal. Biochem. 69, 385—394
72 Schraven, J., Cap, C., Nowoczek, G. and Sandhoff, K. (1977) Anal. Biochem. 78, 333—339
73 Tallman, J.F., Fishman, P.H. and Henneberry, R.C. (1977) Arch. Biochem. Biophys. 182, 556—562
74 Svennerholm, L. (1963) J. Neurochem. 10, 613—623
75 Van Lenten, L. and Ashwell, G. (1971) J. Biol. Chem. 246, 1889—1894
76 Schauer, R., Veh, R.W. and Wember, M. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 559—566
77 Thomas, J.J., Folger, E.C., Nist, D.L., Thomas, B.J. and Jones, R.H. (1978) Anal. Biochem. 88, 461—467
78 Warner, T.G. and O'Brien, J.S. (1979) Biochemistry 18, 2783—2787