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Purification of the Influenza Hemagglutinin Glycoprotein and Characterization of Its Carbohydrate Components

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Hemagglutinin from influenza A/PR8 virus was purified after treatment of the virus with sodium deoxycholate followed by extraction with tri-*n*-butyl phosphate. This fully disrupted the virus while preserving hemagglutinating activity. The hemagglutinin was obtained in the form of small aggregates that could be separated from other viral components. Purified hemagglutinin was hydrolyzed to determine carbohydrate composition and digested with Pronase to analyze oligosaccharide structures. Sugars present in the hemagglutinin were galactose, mannose, fucose, and glucosamine in molar rates of about 6:11:2:5, and these comprised 16% of the hemagglutinin glycoprotein. Oligosaccharides obtained from virus included a major component of a molecular weight of 2,800, composed of glucosamine, galactose, mannose, and fucose, and a minor heterogenous component of a molecular weight of 1,500 to 2,000, containing predominantly mannose. The 2,800-molecular-weight oligosaccharide was a constituent of the hemagglutinin, and treatment of this large oligosaccharide with specific exo-glycosidases demonstrated the presence of terminal galactose and fucose and allowed the deduction of a general structure for this component.

The influenza virus hemagglutinin (HA) glycoprotein can be purified after disruption of the virus with detergents (7, 18, 32, 35, 52) or after disruption with organic solvents (22, 42, 45). Recovery of hemagglutinating activity, which is dependent on aggregation of the HA spikes (35) is possible by using the mild ionic detergents deoxycholate (DOC) (37) or cetyl trimethyl ammonium bromide (3), but the large aggregates obtained contain both HA and neuraminidase (NA) glycoproteins. Similar aggregates or rosettes are obtained by disruption of the virus with organic solvents such as ether (22, 45) or tri-*n*-butyl phosphate (TNBP) (42). Treatment with the strong ionic detergent, sodium dodecyl sulfate, enables separation of HA and NA glycoproteins, but leads to denaturation of the HA of several strains (34). Combined treatment with Sarkosyl and ether has enabled separation of the spikes while preserving their activity, but the HA preparations contained some NA (52). The present communication describes purification of the HA from influenza A/PR8 (HON1), a strain whose HA is sensitive to denaturation, after treatment of the virus with DOC followed by extraction with TNBP. This procedure enabled recovery of the HA in highly active form, free of other viral components.

The carbohydrates present in the influenza virion include mannose, galactose, fucose, and glucosamine (1, 14), and these sugars are found on the HA from A/Bel (33), A/Memphis (54), and the fowl plague strain (47). The carbohydrate components of the HA may be involved in several viral functions. Inhibition of glycosylation (15, 25, 27, 46) prevents infectious particle formation and may severely limit release of virus from cells. Host antigenic determinants may be carbohydrate (16, 17, 20, 21, 36), and carbohydrate may also be involved in hemagglutinating activity (5, 27). Analysis of the carbohydrates of the HA of influenza A/PR8 is reported here.

MATERIALS AND METHODS

Cell and virus growth. Madin-Darby canine kidney cells were grown in 32-ounce (ca. 960-ml) prescription bottles with Dulbecco-modified Eagle medium containing 1% fetal calf serum. For virus growth, bottles were seeded with 1×10^7 to 2×10^7 cells that were allowed to attach at 37°C for 4 to 5 h and then infected with virus at low multiplicity (~0.01). The A/PR8 strain that had been passaged many times in embryonated chicken eggs was adapted to the MDCK cells by three successive passages by using Dulbecco-modified Eagle medium containing 2% dog serum. This was followed by three plaque purifications. Virus was grown in the presence of 2% dog serum for 48 to 55 h at 37°C.

Labeling of virus. For radioactive labeling of the virus, cells were plated in 10-cm dishes at 6×10^6 to 8×10^6 cells per dish, allowed to attach for 4 to 5 h, then infected with PR8 at a multiplicity of infection of 0.01. Virus was allowed to adsorb for 1 h at 37°C, and the

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inoculum was replaced with 10 ml of Dulbecco-modified Eagle medium containing 2% dog serum. For labeling with mannose and galactose, the medium was changed at 5 h to Dulbecco-modified Eagle medium in which the glucose concentration had been lowered to 4.4 mM and which contained the radioactive precursor. For labeling with fucose and glucosamine, label was added at 12 to 14 h to the unchanged medium. The following labeled compounds were used at a final concentration of 10 μCi/ml: [1,5,6-^{3}H]fucose, specific activity 2.78 Ci/mmol; [6-^{3}H]glucosamine, 10.7 Ci/mmol; [1-^{3}H]mannose, 13.2 Ci/mmol; and [1-^{3}H]-galactose, 14.2 Ci/mmol. [*U*-^{14}C]glucosamine, 56.6 Ci/mmol, was used at a final concentration of 2 μCi/ml.

Virus purification. Virus-infected fluids were frozen and thawed followed by clarification to remove cells and debris. Virus was then pelleted at 8,000 × *g* for 16 h and resuspended in standard buffer (0.02 M sodium phosphate buffer [pH 7.2]–0.1 M sodium chloride–1 mM EDTA). The virus was next layered on a discontinuous 20 to 65% D_2O-buffered sucrose density gradient and centrifuged at 36,000 rpm in an SW41 rotor for 90 min. This was followed by collecting the virus band, diluting 1:3, and layering on a preformed 20 to 65% buffered sucrose density gradient. After centrifugation in the SW41 rotor at 36,000 rpm for 4 h, the virus was harvested in the density range 1.220 to 1.250 g/cm^3 and then dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) to remove sucrose. For carbohydrate analysis of the virus, the sucrose density gradient-purified virus was further purified on 20 to 40% sodium-potassium tartrate gradients in standard buffer. The virus band was collected and dialyzed to remove tartrate.

HA assay. HA was titrated by combining 0.1 ml of serial twofold dilutions of virus in 0.85% saline (in microtiter dishes) with 0.1 ml of 0.5% chicken erythrocytes. After 1 h at room temperature, the end point was estimated visually, and the reciprocal of the end-point dilution was defined as the titer.

NA assay. Twofold dilutions of the virus or sample to be assayed were made in 0.1 ml of 0.85% saline. To each dilution was added 0.1 ml of neuramine lactose (Sigma Chemical Co.) at 0.8 mg/ml in 0.1 M sodium phosphate buffer (pH 5.9)–2 mM $CaCl_2$. After incubation at 37°C for 1 h, the released *N*-acetylneuraminic acid was determined by the method of Warren (55) as recommended by Aymard-Henry et al. (2). A unit of activity was defined as the amount of enzyme giving an absorbancy of 1.0 at 549 nm.

Polyacrylamide gel electrophoresis. Slab polyacrylamide gel electrophoresis was performed with a 1-cm 3% stacking gel and a 10-cm 10% resolving gel prepared by the method of Laemmli (31). Electrophoresis was performed at 15 mA of constant current for 1 to 1.5 h, then at 25 mA of current until the tracking dye reached 0.5 cm from the bottom. The slab was stained with Coomassie brilliant blue by the method of Weber and Osborn (56). The following marker proteins were used to determine molecular weight: β-lactoperoxidase, 92,000 (Worthington Biochemicals Corp.); bovine serum albumin, 68,000 (Armour Pharmaceutical, Inc.); catalase subunit, 60,000 (Worthington); pyruvate kinase, 57,000 (Sigma); ovalbumin, 43,000 (Worthington); aldolase, 40,000 (Worthington); papain, 23,000 (Sigma); and RNase, 11,700 (Worthington).

Gel filtration with Sephadex G-150. Sephadex G-150 was equilibrated with 0.02 M Tris (pH 8.0). Gel filtration of disrupted virus was carried out on a column (1.4 by 90 cm) under a hydrostatic head of 20 cm. Fractions (5 ml) were collected and analyzed for absorbancy at 280 nm, hemagglutinating activity, and hemolyzing activity.

Hydrolysis. Acid hydrolysis was carried out with the resin hydrolysis procedure of Lehnhardt and Winzler (38), a technique that minimizes destructive carbohydrate-amino acid reactions. After dialysis to remove salts, samples of purified HA or whole virus containing 2 to 50 nmol of carbohydrate were dried under reduced pressure at 50°C. The samples were then resuspended in 50 μl of water and 50 μl of a 40% (wt/vol) suspension of AG 50-X2 200- to 400-mesh (H^+) resin in 0.02 M HCl. The mixture was sealed under nitrogen in glass vials and put at 100 to 105°C. After 36 h the vials were cooled, the liquid was centrifuged from the walls, and the contents were analyzed for neutral and amino sugar as described below.

Separation of neutral sugar and amino sugar. Separation of these two components was achieved by a modification of the Boas procedure (6) as follows. After hydrolysis the entire contents of the vial were transferred to a premade column containing 0.2 ml of the same AG-50 resin equilibrated with 0.02 M HCl. The vial and the column were then washed twice with 0.4 ml of 50% methanol. The eluate was allowed to pass on to a 0.2-ml column of a 20% (wt/vol) suspension of AG-1 X8 200 to 400 mesh (formate). The first column quantitatively bound amino sugar, which was recovered by elution with 0.5 ml of 2 N HCl. The 50% methanol eluates from the first and second columns were combined with a further 0.4-ml 50% methanol wash of the second column to obtain the neutral sugar fraction.

Assay of the sugar fractions. The eluates from the above columns were dried in vacuo, resuspended in distilled water, and assayed by the Park-Johnson ferricyanide-reducing test by the method of Spiro (50). Quantitative results were obtained by determination of reducing equivalent per microgram from calibration curves for each of the individual sugars. Average values for the neutral sugars and for glucosamine were used to quantify the two classes of sugars.

Thin-layer chromatography. Precoated plastic sheets of Silica Gel G (0.25-mm thick; Brinkmann Instruments Inc.) were equilibrated with 0.1 M sodium phosphate buffer (pH 5.0). After thoroughly drying, the neutral sugars were delivered with a 10-μl applicator, developed with butanol/acetone/water (4:5:1), and visualized with anisaldehyde (39). Galactose, glucose, mannose, and fucose were all separated by this system. Amino sugars were separated on cellulose layers (Brinkmann) with ethyl acetate/acetic acid/pyridine/water (5:1:5:3) (26). Complete separation of glucosamine and galactosamine required two ascents of the solvent. Visualization was with silver nitrate. For quantitative analysis of the sugars, the adsorbant material adjacent to a visualized standard was removed, eluted with 50% methanol on a short

column, dried, and assayed by the Park-Johnson method. To control for recovery, a second set of standards was included in the elution and assay procedure. Recovery was always greater than 95%.

Pronase digestion of the virus and the HA and gel filtration of oligosaccharides. Purified virus of HA samples to be digested were dialyzed against water, then adjusted to 0.1 M Tris (pH 8.0) and 1 mM $CaCl_2$ in a volume of 1 ml. Pronase was dissolved at a concentration of 10 mg/ml in 0.1 M Tris (pH 8.0) containing 1 mM $CaCl_2$. This was added to virus or HA in five 25-μl portions at 0, 24, 48, 60, and 72 h. Digestion was at 37°C for 60 h, then at 55°C for 48 h. The digested samples were clarified at 80,000 × *g* for 20 min, then chromatographed on a Bio-Gel P-6 (100- to 200-mesh) column (1.2 by 110 cm) equilibrated with 0.1 M ammonium acetate. Fractions (1.1 ml) were collected at a flow rate of 5.5 ml/h.

Preparation of standard oligosaccharides. Oligosaccharides from fetuin (49) and from ovalbumin (23) were prepared by Pronase digestion. The glycoproteins were dissolved in 0.1 M Tris (pH 8.0)–1 mM $CaCl_2$ and digested with Pronase at 55°C for 60 h at an initial enzyme/substrate ratio of 1:100. Subsequent additions of Pronase at 24 and 48 h brought the ratio up to 1:25. After digestion, insoluble material was removed by centrifugation, and the mixture was applied to a Bio-Gel P-6 column as above. The oligosaccharides were detected by the phenol-sulfuric acid method for neutral sugar (12) and the thiobarbituric acid assay for sialic acid-containing components (55). After the peaks were identified, the appropriate fractions were pooled and lyophilized, and the entire digestion procedure was repeated.

Glycosidase digestion of the oligosaccharides. Oligosaccharides were prepared from pooled fractions of the P-6 column. The material was lyophilized, resuspended in distilled water, passed over a short Bio-Gel P-2 column (10 by 0.6 cm) to remove salts, and finally relyophilized. Digestion with glycosidases was carried out in 1 ml of 0.15 M citrate-phosphate buffer at 37°C in the presence of 0.02% toluene for up to 120 h with additions of enzyme every 24 h. Undigested oligosaccharides were simultaneously incubated to check for degradation. β-Galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase were purified from jack bean meal by the method of Li and Li (40). Glycosidase activities were determined by using the appropriate *p*-nitrophenyl glycosides as substrates and measuring the amount of *p*-nitrophenol released by its absorbance at 400 nm. A 0.2-ml amount of the *p*-nitrophenyl glycoside at 0.6 mg/ml was incubated with the enzyme to be tested for 5 to 30 min, depending on the activity, and the reaction was stopped by adding 1 ml of 2% sodium carbonate. One unit of glycosidase activity was defined as the amount which released 1 mol of *p*-nitrophenol per min at 37°C using an extinction coefficient of $2 \times 10^4\ M^{-1}\ cm^{-1}$.

α-Fucosidase was purified by the method of Bahl (4) from Rohm and Haas Rhozyme HP-150, a glycosidase mixture form *Aspergillus niger*. This enzyme was assayed by using canine submaxillary mucin by the method of Bahl (4), and released fucose was assayed with the Dische-Shettles reaction according to Spiro (50).

RESULTS

Disruption of the virus. To determine the optimal conditions for virus disruption, several concentrations of DOC were used, and the amount of HA activity recoverable was assayed after removal of undissociated virus by centrifugation. Virus at 100 μg/ml in 0.02 M Tris buffer (pH 8.0) was mixed with DOC and stirred at room temperature for 30 min. The reaction mixture was then centrifuged at 80,000 × *g* for 30 min, and the resuspended pellet and supernatant fluid were assayed for HA activity. At DOC concentrations of 3 to 4%, 20 to 50% of the HA activity originally associated with the virus was found in the supernatant fluid. The amount of DOC could be reduced substantially with an increased recovery of HA activity if, after disruption, the mixture was extracted with an equal volume of TNBP by rapid mixing for 30 min. After extraction, the phases were separated by centrifugation. Virus was pelleted from the aqueous phase, and the supernatant was then assayed for HA activity. Optimal recovery of HA activity was found after treatment of virus with 1% DOC and subsequent TNBP extraction (Fig. 1). The HA did not pellet at 80,000 × *g* and was found to be considerably smaller in size than whole virus upon density gradient sedimentation.

Separation from other viral components. After disruption of the virus by the combined DOC-TNBP procedure, excess detergent and TNBP were removed from the mixture. The bulk of the TNBP was removed by centrifugation at 14,000 × *g* for 10 min, which separated the aqueous and organic phases. The organic phase was again mixed with buffer and centrifuged, and the two aqueous phases were combined. The aqueous mixture contained all the viral components except some of the viral lipids and the "M," or membrane protein, that re-

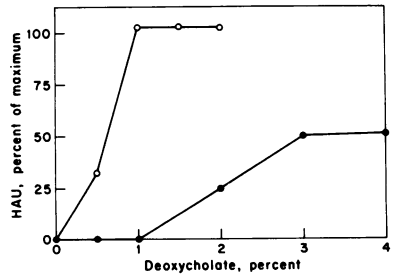


FIG. 1. *Effect of DOC on release of hemagglutinin (HAU) from the virus. Virus was disrupted with DOC at pH 8.0 followed by centrifugation to remove undissociated virus. Activity remaining in the supernatant fluid after DOC treatment alone* (●) *or after combined DOC and TNBP treatments* (○).

mained at the interface between the two phases. The M protein could be recovered from the interface where it formed an insoluble precipitate.

Excess DOC present in micellar form and residual TNBP present as mixed micelles with DOC were removed simultaneously from the aqueous phase by gel filtration with Sephadex G-150. The viral protein and HA activity eluted with the void volume while the DOC eluted in a second peak detected by the ability of these fractions to lyse erythrocytes.

Separation of the HA from the proteins present in the void fractions of the Sephadex column was accomplished by using a DEAE-cellulose ion-exchange column. The elution pattern is shown in Fig. 2. Several protein peaks were identified from their absorbance at 280 nm, and a broadly distributed peak of HA activity was found to elute with the salt gradient. The fractions of each peak were pooled, and portions were precipitated with 70% ethanol and then analyzed on polyacrylamide gels (Fig. 3). Pool 2 represented the HA glycoproteins, HA_1 and HA_2 (tract 4, Fig. 3). The purified HA contained a trace of NA that showed up on gels as a very faint band above the HA_1 band. NA activity could not be measured, as it was completely destroyed by the combined DOC-TNBP treatment. Determination of the amount of NA from densitometer tracings of the polyacrylamide gels showed it to be less than 3% of the total recovered HA glycoprotein.

Polyacrylamide gel analysis of the other viral proteins showed the partially purified M protein from the DOC-TNBP interface (track 2, Fig. 3) and the NA from pool 1 of the DEAE-cellulose column (tract 3, Fig. 3). NA was distinguished from the nucleoprotein by running the samples containing NA on polyacrylamide gels under

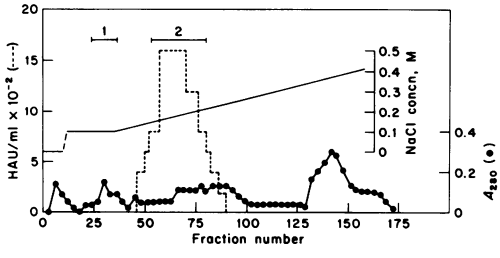


FIG. 2. *DEAE-cellulose chromatography of disrupted influenza virus. Fractions from the Sephadex G-150 column that showed HA activity were applied to a column (1.6 by 18 cm) of DEAE-cellulose equilibrated with 0.02 M Tris (pH 8.0). The column was washed with 0.1 M NaCl and then with a continuous gradient from 0.1 M to 0.5 M NaCl. Fractions (4 ml) were collected and analyzed for HA activity and absorbance at 280 nm (A_{280}).*

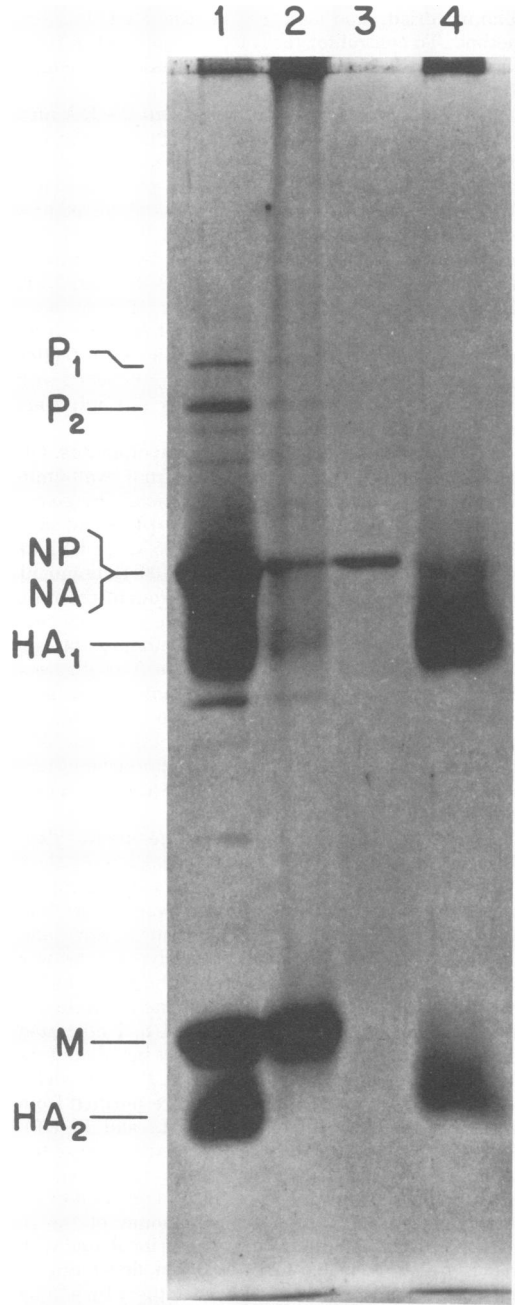


FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel analysis of viral proteins. Track 1, purified influenza virus; tract 2, proteins collected from the TNBP aqueous interface; tract 3, proteins contained in pool 1 from the DEAE-cellulose column as shown in Fig. 2; and tract 4, proteins in pool 2 from the same column.*

nonreducing conditions. Pool 1 material migrated as a high-molecular-weight aggregate greater than 200,000 and was thus identified as

NA (34). The NP protein was found in other experiments to elute last from the column in the region corresponding to 0.35 M sodium chloride.

Carbohydrate composition. The carbohydrate present on the virus and HA was determined by using techniques applicable to microgram amounts of material. After hydrolysis, amino sugars were separated from neutral sugars; quantitative recovery of these sugars was accomplished based on experiments with monosaccharides. To determine individual neutral sugars, the neutral sugar fraction was subjected to thin-layer chromatography, and separated sugars were eluted, assayed, and converted to amount present by using the standard curves shown in Fig. 4. The composition determined with these techniques is shown in Table 1. The virus contained 5.5% carbohydrate, consisting of galactose, mannose, fucose, and glucosamine. No galactosamine or sialic acid could be detected. The values found for neutral and amino sugar present in the HA were 12.8 and 2.6%, respectively, whereas the individual monosaccharides included galactose, mannose, fucose, and glucosamine in the molar ratios of approximately 6:11:2:5. Protein was determined by the Lowry procedure. All of the values reported were readily reproducible with the exception of glucose, in which wide variations were found, especially if the virus was not purified by an additional tartrate density gradient. For this reason, and because glucose contamination can readily occur via cellulosic material (10, 13), no value is reported for glucose.

Oligosaccharides from virus and purified HA. Oligosaccharides were obtained from the labeled virus or purified HA by exhaustive Pronase digestion followed by gel filtration with Bio-Gel P-6. The HA activity was lowered by over 99.9%, and the protein was hydrolyzed to small peptides and amino acids.

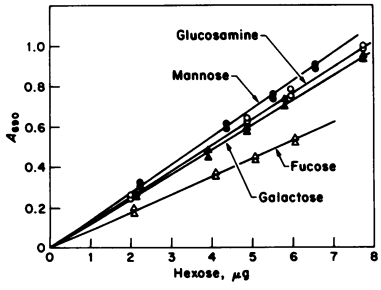


FIG. 4. *Standard curves for quantitation of carbohydrates. The amount of each sugar present was quantitated by comparing its reduction of ferricyanide to that of known standards. Ferricyanide was reduced to ferrocyanide by the sugar assayed, and this was measured as ferric ferrocyanide at 690 nm (A_{690}).*

In Fig. 5 the elution pattern of oligosaccharides derived from intact virus and from the purified HA are compared. Size was estimated for each by comparison with the standards. Similar size distribution patterns were observed for oligosaccharides derived from virus and from the purified HA when these were labeled with glucosamine or fucose as shown in panels a, b, d, and e of Fig. 5. Oligosaccharides from both HA and virus showed a peak at a molecular weight of 2,800. In addition, oligosaccharides from virus labeled with galactose had an identical size distribution to those labeled with fucose (data not shown). Oligosaccharides derived from the purified HA that had been labeled with galactose are shown in panel f of Fig. 5. Galactose-labeled HA oligosaccharides showed a sharp peak at a molecular weight of 2,800, and there was usually an additional small peak at the void volume of the column. The nature of this material was not investigated further. When mannose was used to label virus, the oligosaccharides showed a different pattern. The 2,800-molecular-weight peak was present, but a broad shoulder of labeled material of a molecular weight ranging from 1,500 to 2,000 was also apparent (panel c, Fig. 5).

Components labeled with radioactive precursors. To determine which carbohydrate components in the virus became labeled when these various precursors were used during virus synthesis and how much of the incorporated label was in carbohydrate, labeled virus and HA were hydrolyzed, and the neutral and amino sugars were separated and subjected to thin-layer chromatography. Both glucosamine and fucose remained in the same chemical form in the virus as shown in Fig. 6, and greater than 95% of the incorporated label was contained in its original form. However, both mannose and galactose were converted to other compounds because only approximately 40 to 50% of the incorporated label could be recovered as carbohydrate (Table 2). Also, a galactose-labeled virus contained some carbohydrate label as glucose, possibly in glycolipid, and virus labeled with mannose showed incorporation into both mannose and fucose. The incorporation of label into fucose from labeled mannose may have occurred through conversion of GDP-mannose to GDP-fucose (11).

From the amount of carbohydrate on the HA, approximately 16%, and an estimate of the molecular weight of the glycoprotein, 75,000, the carbohydrate can be calculated to represent a molecular weight of approximately 11,500. The size of the HA was determined by sodium dodecyl sulfate-gel electrophoresis under nonreducing conditions by comparison with the migration of HA with proteins of known molecular

weight. Because the sizes of the oligosaccharide chains are smaller than the total complement of carbohydrate, there may be several chains of each type. However, due to the incorporation of some labeled mannose into fucose, the relative numbers were not easily determined.

Glycosidase digestion of the oligosaccharides. Because the viral oligosaccharides labeled with each of the carbohydrate components present on the oligosaccharide chains could be readily obtained and consisted of only one predominant size class, data were sought on the organization of the individual monosaccharides present. To do this, glycosidase enzymes with specific cleavage properties were isolated and used to digest the oligosaccharides. These included β-galactosidase, β-*N*-acetylglucosaminidase, and α-mannose from jack beans and α-fucosidase from *A. niger*. Purification and assay of these enzymes were essentially by published procedures (4, 40). Oligosaccharides used for glycosidase digestion were from pooled P-6 column fractions shown in Fig. 5.

TABLE 1. *Carbohydrate composition of influenza A/PR8 and its HA*

Determination	PR8 (μg/100 μg)[a]	HA (μg/100 μg)[a]
Neutral hexose	5.5 ± 0.6	12.8 ± 0.9
Galactose	1.2 ± 0.2	4.6 ± 0.1
Mannose	3.5 ± 0.7	8.2 ± 0.2
Fucose	1.1 ± 0.6	1.5 ± 0.7
Glucosamine	1.7 ± 0.3	2.6 ± 0.4

[a] Values represent the mean of determinations on three HA or virus samples, expressed with one standard deviation. Values are based on protein determined by the Lowry assay.

To determine whether galactose was a terminal monosaccharide, a [^{3}H]galactose-labeled oligosaccharide obtained from P-6 fractionation of Pronase-digested virus was digested with β-galactosidase. The oligosaccharide was digested for 24, 72, and 120 h, with additions of β-galactosidase each 24 h. Release of [^{3}H]galactose from the oligosaccharide occurred at each of these time intervals (data not shown) up to a maximum of approximately 30% (panel b, Fig. 7).

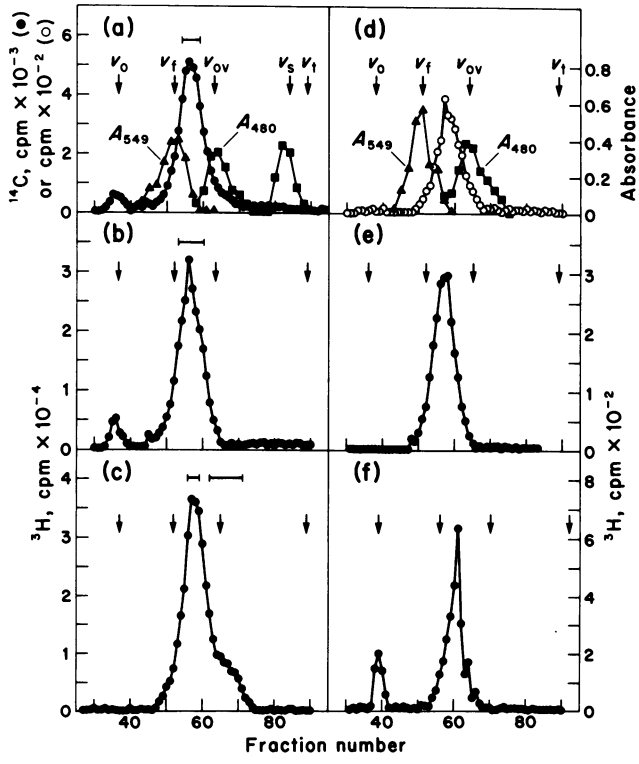


FIG. 5. *Bio-Gel P-6 gel filtration of oligosaccharides from virus and purified HA. Pronase digestion was carried out with virus labeled with [^{14}C]glucosamine (a); [^{3}H]fucose (b); and [^{3}H]mannose (c). Purified HA was prepared from virus labeled with [^{14}C]glucosamine (d); [^{3}H]fucose (e); and [^{3}H]galactose (f). Markers run with the [^{14}C]glucosamine-labeled oligosaccharides included the fetuin oligosaccharide (▲) and the ovalbumin and stachyose oligosaccharides (■). The elution volumes of these standards are designated as V_f, V_{ov}, and V_s, respectively, whereas the void volume and included volume are indicated by V_o and V_t, respectively.*

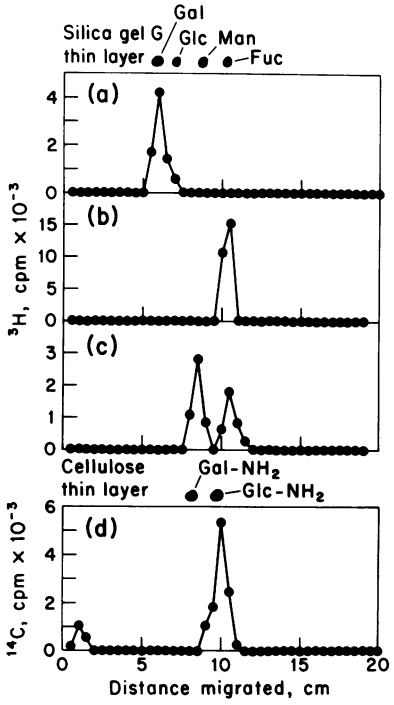


FIG. 6. *Thin-layer chromatography of labeled carbohydrates from hydrolyzed virus. The virus was hydrolyzed, and the carbohydrates were separated into neutral and amino sugar fractions. Neutral sugars were run on Silica Gel G; cellulose layers were used for amino sugars. Virus was labeled with (a) [^{3}H]galactose; (b) [^{3}H]fucose; (c) [^{3}H]mannose; and (d) [^{14}C]glucosamine.*

TABLE 2. *Incorporation of labeled sugars into influenza A/PR8*

Determination	Amt incorporated as carbohydrate	Chemical form
Glucosamine	95%	Glucosamine
Fucose	95%	Fucose
Mannose	40–50%	Mannose, fucose[a]
Galactose	40–50%	Galactose, (glucose)[b]

[a] Approximately equally distributed.
[b] Only trace amount of glucose.

To determine whether other carbohydrates were terminal, digestion was carried out with other specific exo-glycosidases. The α-fucosidase from *A. niger*, specific for the α1-2 linkage between fucose and galactose, was purified and, as shown (panel c, Fig. 7), this enzyme released [^{3}H]fucose from the viral oligosaccharides, suggesting that fucose was terminal and may be linked in an α1-2 fashion to galactose. However, only a portion of the fucose was released, even after 120 h of incubation. Possibly fucose is linked also to glucosamine as in oligosaccharides found in serum glycoproteins.

Because fucose could be released with α-fu-

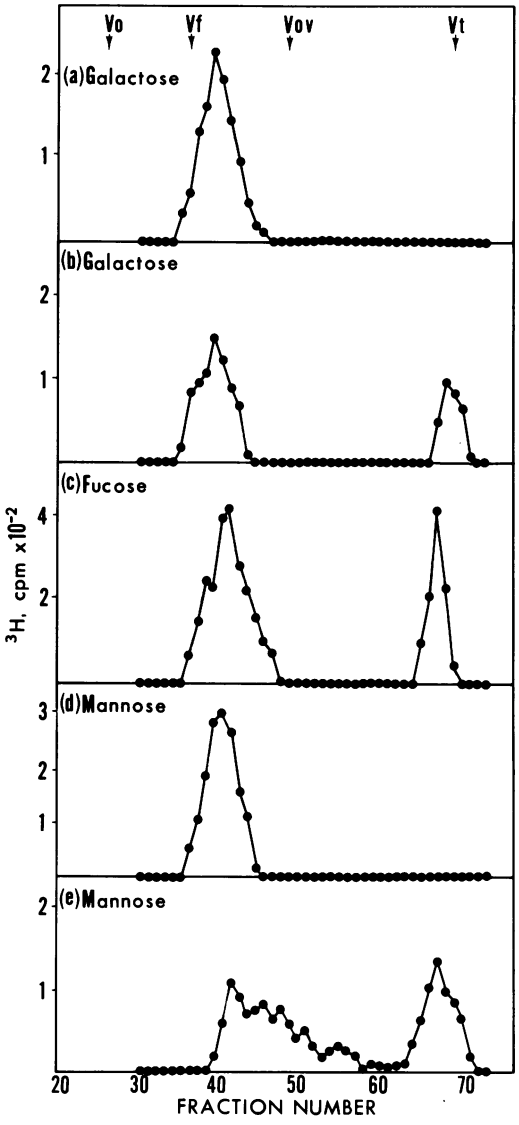


FIG. 7. *Digestion of labeled viral oligosaccharides with glycosidases. Pooled, labeled oligosaccharides were digested at 37°C in 0.15 M citrate buffer for 120 h with one unit of enzyme added each 24 h. After digestion, the mixture was run on a Bio-Gel P-6 column, and fractions were counted for radioactivity. (a) [^{3}H]galactose-labeled oligosaccharide with no digestion; (b) [^{3}H]galactose-labeled oligosaccharide digested with β-galactosidase; (c) [^{3}H]fucose-labeled oligosaccharide digested with α-fucosidase; (d) 2,800-molecular-weight [^{3}H]mannose-labeled oligosaccharide digested with α-mannosidase; and (e) 1,500- to 2,000-molecular-weight [^{3}H]mannose-labeled oligosaccharide digested with α-mannosidase.*

cosidase from *A. niger*, combined treatment with this enzyme and β-galactosidase was attempted to increase the release of galactose. A pool of galactose-labeled oligosaccharide was divided into three portions. One was digested with β-galactosidase alone, whereas another was digested with α-fucosidase and β-galactosidase. Table 3 shows that only slightly more galactose is released, 58% of the label compared to 43%, when α-fucosidase is combined with β-galactosidase to digest the oligosaccharide.

Attempts were also made to release mannose and glucosamine from the oligosaccharides. After 120 h of incubation with β-*N*-acetylglucosaminidase, no glucosamine was released, suggesting that the sugar may be internally linked in the chain. Two pools of oligosaccharide were prepared from mannose-labeled chains, and each was digested with α-mannosidase from jack bean. The pools consisted of the 2,800-molecular-weight [^{3}H]mannose-labeled oligosaccharide and the region of the P-6 elution pattern that corresponded to a molecular weight of 1,500 to 2,000. No mannose could be released from the large 2,800-molecular-weight oligosaccharide even with incubation up to 120 h (panel d, Fig. 7). However, the smaller mannose-containing oligosaccharides were degraded by this enzyme, releasing free mannose and giving heterogeneous degradation products (panel e, Fig. 7). This suggests that these small oligosaccharides may contain several terminal α-mannose residues that are susceptible to α-mannosidase digestion. Again, the reaction did not accomplish complete removal of the label from the oligosaccharides. This could be due to incomplete reaction or to the presence of mannose residues that are resistant to removal, possibly in β-linkage, or at internal positions.

DISCUSSION

A procedure for the purification of influenza virus HA has been developed that offers several advantages over previous procedures. Virus was disrupted under mild conditions which enabled recovery of the HA in nondenatured form. The HA was obtained in active form from A/PR8, a strain whose HA is sensitive to denaturation by sodium dodecyl sulfate and, thus, cannot be purified by the technique of Laver (32). The membrane protein was readily removed, and the viral lipids and the detergent, which was used in excess to disrupt the virus, were removed by gel filtration on Sephadex G-150. At pH 8.0, which was used to disrupt the virus, a DOC is aggregated into very small micelles (53). These were small enough to equilibrate with the included volume of the Sephadex gel and, thus, separated from the HA. Mixed DOC-lipid micelles probably also separated because these would be considerably smaller than a molecular weight of 150,000. Binding of some lipid to the hydrophobic regions of the HA spikes could not be completely ruled out. The broad elution profile of the HA from the DEAE column suggests that the disruption procedure may give rise to aggregates of HA with different numbers of spikes. Although the technique was not optimized for isolation of other viral proteins, the membrane protein, nucleoprotein, and NA glycoprotein could all be recovered from some preparations. The procedure, although multistep and involving several manipulations, permits the isolation of highly purified, active HA.

TABLE 3. *Release of [^{3}H]galactose from [^{3}H]-galactose-labeled oligosaccharides by β-galactosidase and α-fucosidase*

Enzyme	[^{3}H]galactose remaining with large oligosaccharide (cpm)	[^{3}H]galactose released as galactose (cpm)	Total (cpm)	% Released
None	1,880	32	1,912	0
β-Galactosidase	818	658	1,476	43
β-Galactosidase and α-fucosidase	604	822	1,426	58

The separation of NA from HA was most likely due to the lack of formation of mixed aggregates of the glycoprotein spikes that occur with most other disruption procedures. Disruption with TNBP and Tween 80 leads to mixed aggregates (42), as does disruption with DOC alone (37). The combined DOC-TNBP treatment may solubilize the NA glycoprotein to a monomer spike. This could explain its elution from the column as a sharp peak and the lack of inclusion in HA aggregates. A trace amount of NA eluted from the column with the HA; therefore, minor mixed-aggregate formation cannot be completely ruled out. The separation of these glycoproteins is similar to results with another virus, Semliki forest virus, with which DOC disruption enabled complete separation of two different glycoproteins due to monomer spike formation (19).

Although NA could be removed from the HA, it was not easily recovered in sufficient quantity to enable characterization. Tentative estimates of the NA content of the virus were less than 3% of the protein, based on microdensitometer tracings of the stained gels, whereas HA comprised approximately 22%. Much more virus is needed to measure NA activity than to measure HA activity. In addition, NA activity was apparently abolished by DOC-TNBP treatment, making its

detection possible only on polyacrylamide gels.

The PR8 HA was found to contain about 16% carbohydrate (13% neutral sugar and 3% amino sugar). This is similar to the values previously reported for A/Memphis (H2) and A/Bel (HO) (33, 54) in which determinations were made on HA_1. The sugars present on the PR8 HA include fucose, galactose, mannose, and glucosamine in molar ratios of about 2:6:11:5. The possible presence of glucose cannot be ruled out, but this is unlikely because glucose is rare in oligosaccharides and contamination can easily occur. Other viral glycoproteins contain these same sugars, including small amounts of glucose, but also contain sialic acid (8, 13). Influenza HA lacks sialic acid, possibly because it is cleaved off by the viral NA.

By using the value 75,000 for the molecular weight of HA, the carbohydrate moiety can be calculated to be a molecular weight of approximately 11,500. The predominant oligosaccharides present on the HA have a molecular weight of about 2,800. Assuming that there is at least one amino acid represented by this figure and that only a small part of the HA carbohydrate is present as small mannose-rich chains, it could be estimated that there could be three or four large oligosaccharide chains present in the HA monomer. The relatively large amounts of fucose and galactose present suggests that there are more large chains than small high-mannose chains, but this possibility cannot be completely substantiated due to conversion of labeled mannose to fucose.

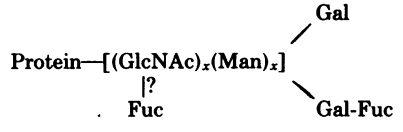
The presence of one major oligosaccharide size class on the HA contrasts with the heterogeneous nature of the oligosaccharides from the glycoproteins of vesicular stomatitis virus (41, 44), Sindbis virus (48), and Rous sarcoma virus (30). However, this heterogeneity has been attributed in part to the presence or absence of sialic acid. Analysis of the carbohydrates of the glycoproteins of another influenza viral strain, the fowl phage virus strain (47), also shows this same predominant size class. The NA and HA_1 and HA_2 glycoproteins have a large oligosaccharide side chain of 2,600 molecular weight when the virus is grown in chicken embryo fibroblasts. Two additional smaller-sized chains are present on the NA and the HA_2, one of which may correspond to the mannose-rich chain found on the HA (HA_1 + HA_2) of PR8.

Partial analysis of the sugar sequences on the chains indicates several structural features. Galactose and fucose are both at nonreducing termini because they can be released by specific exo-glycosidases. This resembles the structure of oligosaccharides from other viral glycoproteins and is consistent with the observation that sialic acid may be attached to the HA when NA activity is inhibited (43), because this sugar appears always to be terminally linked to galactose in glycoproteins (29). Digestion with the α-fucosidase from *A. niger* suggests a fuc-α(1,2) gal linkage, because this enzyme has a strict specificity for this linkage (4). Fucose that is not released may be linked in another fashion, and possibly other fucosidases (9) would release it.

The incomplete nature of the digestion of the oligosaccharides with the glycosidases used implies either that the conditions may not have been optimal or that the oligosaccharides are heterogeneous in sequence. Other factors may be relevant in the present study. The concentrations of the labeled oligosaccharides (i.e., substrates) may have been below the K_m of the glycosidase enzymes and the enzymes, which were highly active on the *p*-nitrophenyl substrates, may have had less affinity for the influenza oligosaccharides.

Enzymatic treatment of the mannose-labeled oligosaccharides suggests that mannose occupies an internal position on the large oligosaccharide, whereas it is terminal on the lower-molecular-weight mannose-containing oligosaccharides. Failure to remove the sugar cannot be taken as proof that it is not terminal because the linkage may be resistant to the enzyme. However, the enzyme hydrolyzed mannose from the smaller chains, and an internal position is consistent with the results of analysis of other large complex oligosaccharides (29). Therefore, it is unlikely that mannose is terminal on the large chains.

From the glycosidase digestion experiments the following composite structure is suggested:



where GlcNAc is *N*-acetylglucosamine, Fuc is fucose, Man is mannose, and Gal is galactose. This structure is consistent with several possible detailed structures derived for complex oligosaccharides found in a variety of serum glycoproteins such as fetuin (51), α_1-acid glycoprotein (24), and the immunoglobulins (28), except for the occurrence of sialic acid in these structures. To completely sequence these oligosaccharides additional experiments are necessary, possibly involving sequential digestions.

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