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THE CARBOHYDRATE CHAINS OF INFLUENZA VIRUS HEMAGGLUTININ*

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ABSTRACT

The major surface antigen of influenza virus A/Leningrad/385/80 (H3N2), H3 hemagglutinin, as well as its heavy and light subunits were obtained by bromelain treatment, followed by gel chromatography. Carbohydrate chains were split off from both subunits by lithium borohydride–lithium hydroxide in aqueous 2-methyl-2-propanol, and individual oligosaccharides isolated. The main oligosaccharides, whose structure was determined by ^{1}H-n.m.r. spectroscopy and chemical methods, are of the ordinary oligomannoside and complex types. It was found that, in spite of the great difference in number of glycosylation sites in heavy and light subunits, the amount and even relative abundance of variants of carbohydrate chains in both subunits are very similar.

INTRODUCTION

It is well known that the immunological properties of hemagglutinin (HA), the major surface antigen of influenza virus, are mainly conditioned by the structure of its polypeptide chain, and changes in the primary amino acid sequence result in the variance of the antigenic status of HA and appearance of new virus strain[1,2]. The carbohydrate chains that take part in the formation of the spatial structure of a glycoprotein and play an important role in stabilizing the polypeptide chain and preventing its proteolysis[3,4] may also be of significance for exhibiting antigenic activity and specificity of HA[5]. However little is known of the primary structures of the oligosaccharide fragments of HA, as well as its dependence on virus strain and host-cell and their effect on HA activity. In addition, knowledge of the functional role of carbohydrates is necessary for the preparation of natural and synthetic vaccines.

We have started a systematic study of the structures of the carbohydrate chains of hemagglutinins from different strains of influenza virus. In the present

*Dedicated to Professor Walter T. J. Morgan.

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paper, we describe the results obtained for the H3 hemagglutinin of influenza virus A/Leningrad/385/80 (H3N2). By use of this virus, methods of isolation of HA and its heavy (HA1) and light (HA2) subunits were elaborated, as well as methods for splitting off the carbohydrate chains from the polypeptide backbone, the isolation of individual oligosaccharides, and the elucidation of their structure. The distribution of different types of carbohydrate fragments in HA1 and HA2 are also presented.

RESULTS AND DISCUSSION

The isolation of HA by use of a detergent is complicated owing to the similarity of its molecular size to that of the other surface antigen, neuraminidase, and their tendency to form mixed aggregates after solubilization. More useful was the method based on splitting off HA from the virion with the proteolytic enzyme, bromelain[6]. The trimer of HA obtained lost a small part of the HA2 polypeptide chain but still contained all the carbohydrate components and conserved its antigenicity. Ultracentrifugation in sucrose or guanidinium chloride density gradient is usually employed for the isolation of such HA[6,7]. We elaborated[8] a simpler method for the preparation of HA, HA1, and HA2.

The suspension of virions was incubated with bromelain, centrifuged off, and the supernatant was chromatographed on a Sepharose CL-6B column to give the HA fraction (Fig. 1). On electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (SDS–PAGE), a single band was present prior to, and two bands after, reduction with 2-mercaptoethanol; neuraminidase activity and phosphorus were absent[8]. According to the amino acid and sugar content, the yield of HA was typically of 70–80%, based on a 30% content of HA in the virus.

Treatment of HA with sodium dodecyl sulfate and dithiothreitol resulted in formation of HA1 and HA2 subunits, which were separated on Ultrogel AcA-34 column (Fig. 2) under SDS–PAGE control. Analyses of HA1 and HA2 showed

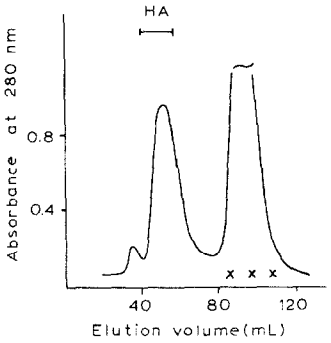


Fig. 1. Isolation of HA by gel chromatography on Sepharose CL-6B; (× × ×) protease activity.

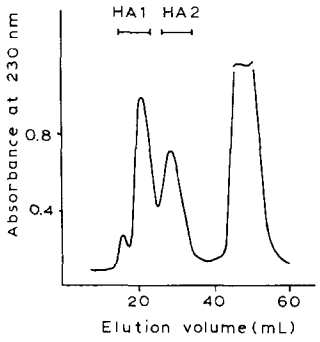


Fig. 2. Separation of HA1 and HA2 on Ultrogel AcA-34.

their similarity in amino acid composition, but a considerable difference in sugar content (~21 and 9 mol per cent, respectively) was found.

The release of the carbohydrate chains from HA1 and HA2 glycoproteins was performed by a new method[9]. It consists in the reductive cleavage of the *N*-glycosylamine carbohydrate–peptide linkage (as well as peptide linkages) upon treatment with lithium borohydride–lithium hydroxide in aqueous 2-methyl-2-propanol, followed by hydrolysis of the glycosylamine at the reducing end of the oligosaccharide chain to give an aldose, subsequently reduced with sodium borohydride. Control experiments showed that the presence of sodium dodecyl sulfate (up to 1%) did not affect the course of the reaction.

The mixture of reduced oligosaccharides, peptides, and glycopeptides obtained after the aforementioned treatment of HA1 and HA2, followed by desalting on Sephadex G-15 column and reduction with sodium borohydride (see Experimental section), was separated on a column of AG 50 (H^+) cation-exchange resin. Elution with water and then with ammonia gave oligosaccharide and peptide–glycopeptide fractions, respectively. According to sugar analysis, the neutral oligosaccharide fractions obtained from both HA1 and HA2 contained ~70% of the total amount of carbohydrates present in the starting glycoproteins. The molar ratio of glucosamine, mannose, galactose, fucose, and glucosaminitol was close to 4:4:2:1:1 in both cases.

The oligosaccharide mixtures isolated from HA1 and HA2 were further fractionated on a Bio-Gel P-6 column and similar distribution patterns of oligosaccharides of different size were obtained (Fig. 3). The elution volumes of the fractions corresponded well to the known data[10,11] on gel chromatographic behavior of Asn-linked oligosaccharide fragments of glycoproteins. This, together with the monosaccharide composition of these fractions (Table I), suggested that Fraction I was a mixture of oligomannoside and mostly low-molecular-weight complex oligosaccharides, and Fractions II and III contained presumably some tri- and tetra-antennary complex chains.

It is to be noted that the glycopeptide fraction contained a negligible amount of amino acids, except for aspartic acid, and exhibited practically the same distribu-

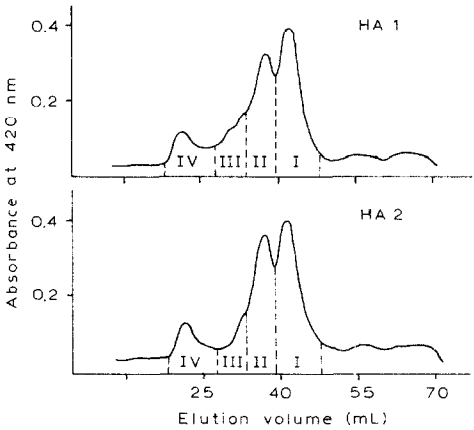


Fig. 3. Bio-Gel P-6 chromatography of oligosaccharide mixtures from HA1 and HA2. Absorbance given by the reaction with orcinol and sulfuric acid.

tion of components according to molecular size upon gel chromatography as did the oligosaccharide fraction. The studies on these glycopeptides may give additional information on the carbohydrate chains of glycoprotein remaining after reductive splitting. Fractions I and II containing a total of ~80% of all the carbohydrates of the oligosaccharide fraction were further investigated. The isolation of individual oligosaccharide chains of glycoproteins is difficult because of the extensive heterogeneity and existence of isomeric structures. This problem was solved by liquid chromatography under pressure (l.c.)[12]. Thus, the oligosaccharide mixtures were fractionated as follows. In a first step, the components of Fractions I and II were separated on Lichrosorb RP-18 and RP-8 columns, respectively, with water as the eluent (Fig. 4). To separate larger quantities of mixtures and to achieve a better resolution, two columns connected in succession were used in some cases. Subsequently, each of the isolated components of Fractions I and II was chromatographed on an Amino column with 75% aqueous methanol as the eluent (Figs. 5

TABLE I

RELATIVE PERCENTAGE OF MONOSACCHARIDES IN FRACTIONS I–IV (FROM HA) AND THEIR RELATIVE MOLAR CARBOHYDRATE COMPOSITION

Fraction	*Relative percentage of monosaccharides*				*Molar ratio of monosaccharides*			
	GlcNAc	*Man*	*Gal*	*Fuc*	*GlcNAc*	*Man*	*Gal*	*Fuc*
I	11	57	19	18	1.5	3	0.6	0.3
II	43	29	45	41	3.4	3	2.5	1.3
III	30	9	21	23	5.5	3	4.1	2.3
IV	16	5	15	18	9.1	3	6.5	4.9
Total	100	100	100	100				

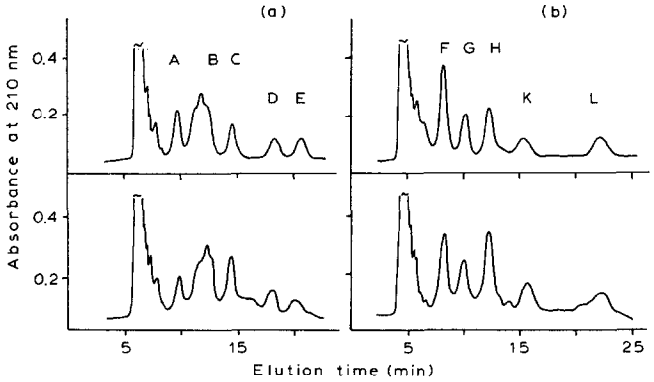


Fig. 4. L.c. of oligosaccharide fractions obtained from HA1 and HA2 (from top to bottom) on reverse-phase columns: (a) Fraction I on Lichrosorb RP-18; (b) Fraction II on Lichrosorb RP-8.

and 6). In this way, 21 oligosaccharides were obtained, and the oligosaccharides having identical chromatographic characteristics obtained from HA1 and HA2 were pooled.

The analysis of monosaccharide composition of the oligosaccharides thus obtained was carried out by anion-exchange chromatography of the hydrolyzates monitored with the copper bicinchoninate reagent[13]. Athough this reagent has been known for more than ten years, it is seldom used in carbohydrate analysis of glyco-

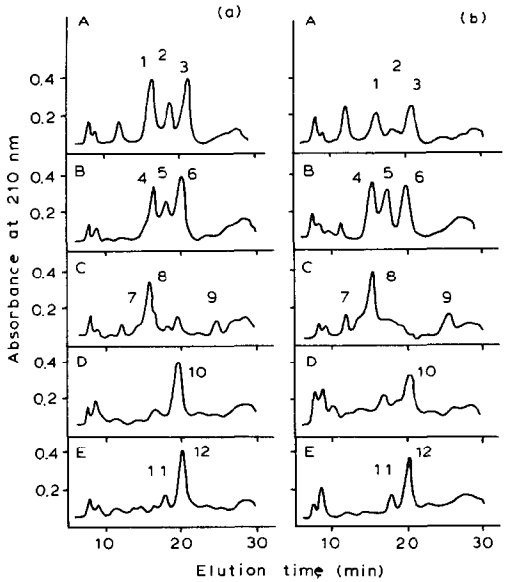


Fig. 5. L.c. of the components **A–E** of Fraction I (see Fig. 4) obtained from HA1 (a) and HA2 (b) on an Alltech-NH_2 column. The oligosaccharides were isolated on a preparative scale.

TABLE II

MONOSACCHARIDE COMPOSITION OF THE OLIGOSACCHARIDES FROM FRACTIONS I AND II

No.[a]	*GlcN*	*Man*	*Gal*	*Fuc*	*GlcNol*
Fraction I					
1	1	6.3	0.2	[b]	[c]
2	1	8.2	[b]	0	[c]
3	1	6.3	0.1	0	[c]
4	1	7.1	[b]	0	0.8
5	1	7.8	0	0	[c]
6	1	9.3	0	0	0.7
7	1	5.4	0.1	[b]	[c]
8	1	5.7	[b]	0	[c]
9	1	3.3	0.5	0.3	[c]
10	1	5.1	0.4	0.1	[c]
11	1	2.0	0.5	0.1	[c]
12	1	1.8	0.5	[b]	[c]
Fraction II					
13	4.0	3	2.1	[b]	0.8
14	4.1	3	2.3	2.2	0.7
15	4.3	3	2.0	1.0	[c]
16	3.7	3	2.1	1.8	0.7
17	4.5	3	2.3	2.1	[c]
18	4.4	3	2.2	0.9	[c]
19	3.6	3	2.1	1.0	0.6
20	4.8	3	2.3	1.2	[c]
21	5.1	3	2.2	1.3	[c]

[a]See Figs. 5 and 6 for numbering. [b]Traces. [c]Not determined.

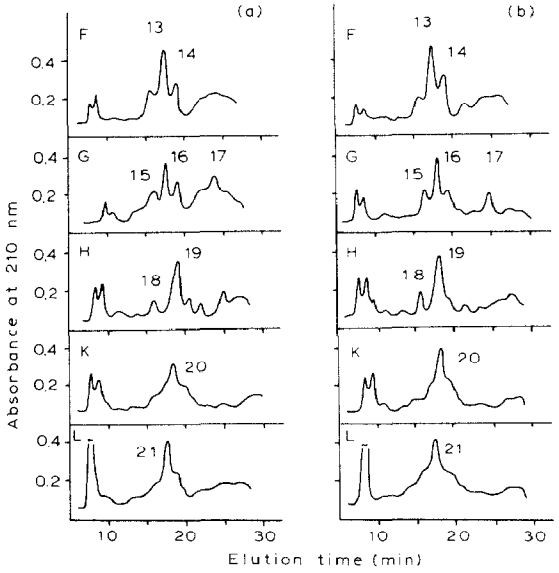


Fig. 6. L.c. of the components **F–L** of Fraction II (see Fig. 4) obtained from HA1 (a) and HA2 (b) on an Alltech-NH_2 column. The oligosaccharides numbered were isolated on a preparative scale.

proteins. We found this method to be rapid and sensitive. Quantitative determination of glucosamine, mannose, fucose, and galactose required 2–5 nmol of an oligosaccharide and took about 40 min. Glucosamine and glucosaminitol were determined on a separate aliquot with an amino acid analyzer, and good correlation between the results of both methods was found. The relative sugar composition of the oligosaccharides, presented in Table II, showed that Fraction I contained mainly oligomannosidic chains, and Fraction II complex chains. The most interesting observation was that each chromatographic procedure gave very similar distributions of the fractions and components (Figs. 3–6) for HA1 and HA2. So, the two subunits of HA contains the same number of different oligosaccharide chains and similar distributions of the chains.

Influenza virus A (H3N2) is known[14,15] to have six or seven glycosylation sites in HA1 and only one in HA2. Thus, it is surprising that, in spite of such a great difference in the number of sites for HA1 and HA2, we were able to isolate a great number of oligomannoside as well as complex chains from both subunits[16]. Although the possible existence of more than one unique oligosaccharide structure at a single site agrees with earlier data[14,17,18], such a great heterogeneity of carbohydrate chains in HA and especially in HA2 subunit was found for the first time. This observation may lead to a revision of our views about the glycosylation of influenza virus hemagglutinin.

The structure of the carbohydrate chains of influenza virus HA is known in a general outline only. Matsumoto *et al.*[18] investigated in more detail the X-31 variant of A2/Hong Kong/68 virus, and found that oligomannoside chains are the major type of carbohydrate fragments of HA. Four main oligomannoside (**1–4**) and four complex oligosaccharides (**5–8**) were isolated (numbers 8, 4, 5, 6, in Fig. 5 and 13, 14, 16, 19, in Fig. 6 correspondingly) in amounts (0.1–0.2 μmol) sufficient for structural studies. In total, they comprise more than 70% of the whole oligosaccharide fraction, and therefore represent the principal carbohydrate chains of hemagglutinin.

The monosaccharide composition of **1–4** showed that they differ from each other only in the number of mannose residues (Table II). The composition, as well as the homogeneity of the oligosaccharides was confirmed by ^{1}H-n.m.r. spectroscopy. The signals of six protons for the two *N*-acetyl groups of 2-acetamido-2-deoxy-glucose and -glucitol residues were present at δ ~2.05–2.10. The downfield region showed seven, eight, nine, and ten signals of anomeric protons of mannose and 2-acetamido-2-deoxyglucose residues. Moreover, the characteristic patterns of the H-1 chemical shifts of mannose residues allowed the elucidation of the structure of these oligosaccharides. Thus, the experimental values of H-1 chemical shifts of oligosaccharides **1–4** were compared with the known ones[19] for the series of isomers having the same number of mannose residues. By use of this fingerprint approach, we were able to select the most preferable variant of structure in each case[20]. For example, from the spectra of several variants (**9–12**) of the structure for the oligosaccharide having seven mannose residues (some of which are presented in Fig. 7), **9** and **12** could be rejected without hesitation and **10** exhibited the pattern most similar to that of **2**. The structures of **1**, **3**, and **4** were identified in the same way.

α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-R
6
↑
1
α-D-Man*p*-(1→3)-α-D-Man*p*
6
↑
1 1
α-D-Man*p*

α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-R
6
↑
1
α-D-Man*p*-(1→3)-α-D-Man*p*
6
↑
2 1
α-D-Man*p*-(1→2)-α-D-Man*p*

α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-R
6
↑
1
α-D-Man*p*-(1→3)-α-D-Man*p*
6
↑
3 1
α-D-Man*p*-(1→2)-α-D-Man*p*

α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-R
6
↑
1
α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Man*p*
6
↑
4 1
α-D-Man*p*-(1→2)-α-D-Man*p*

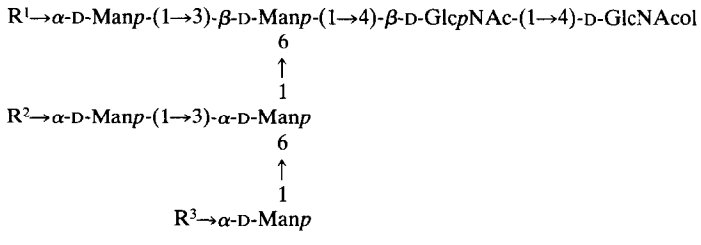
R = (1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAcol

R′→β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAcol
2 6 6
↑ ↑
1 R
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc
1
β-D-Glc*p*NAc-(1→2)-α-D-Man*p*

5 R = R′ = H

6 R = α-L-Fuc*p*, R′ = H

7, 8 R = R′ = α-L-Fuc*p*

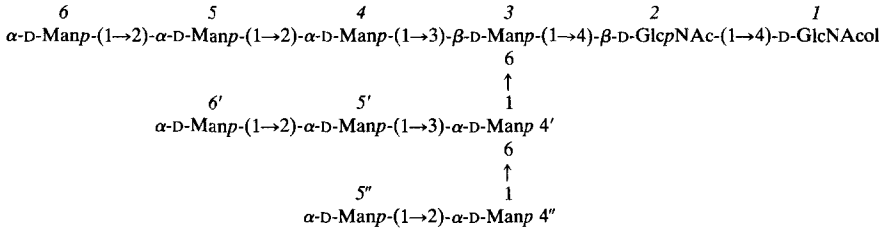


9 $R^1 = R^2 = \alpha$-D-Man*p*-(1→2), $R^3 = H$

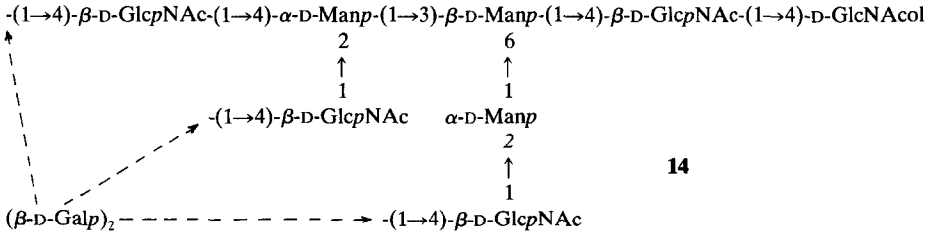
10 $R^1 = R^3 = \alpha$-D-Man*p*-(1→2), $R^2 = H$

11 $R^1 = H$, $R^2 = R^3 = \alpha$-D-Man*p*-(1→2)

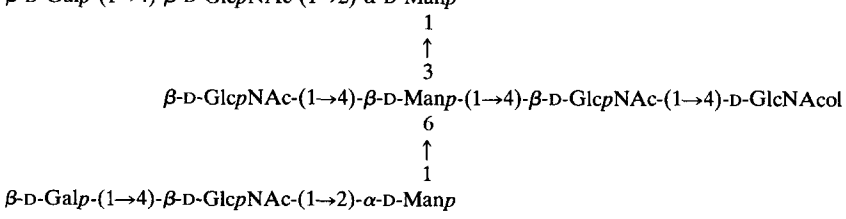
12 $R^1 = \alpha$-D-Man*p*-(1→2)-α-D-Man*p*-(1→2), $R^2 = R^3 = H$



13



14



15

This finding suggested that transformation of the oligomannoside into the complex chain during biosynthesis of a glycoprotein is a rather selective process. At first, $Man^{6'}$ (see structure **13**) is split off under α-D-mannosidase I action, then Man^{6} and $Man^{5''}$ in succession. An analogous sequence of removal of α-(1→2)-

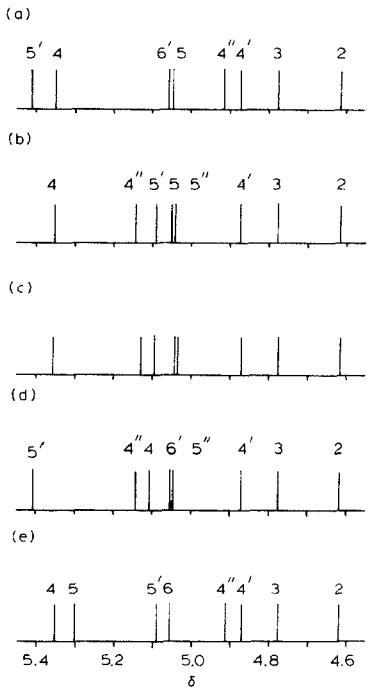


Fig. 7. Positions of chemical shifts of anomeric protons in the ^{1}H-n.m.r. spectra of oligosaccharide **2** and various oligosaccharides (**9–12**) containing seven mannose residues. The values for reference structures are taken from ref. 19. For numbering of residues, see structure **13**: (a) Compound **9**, (b) **10**, (c) **2**, (d) **11**, and (e) **12**.

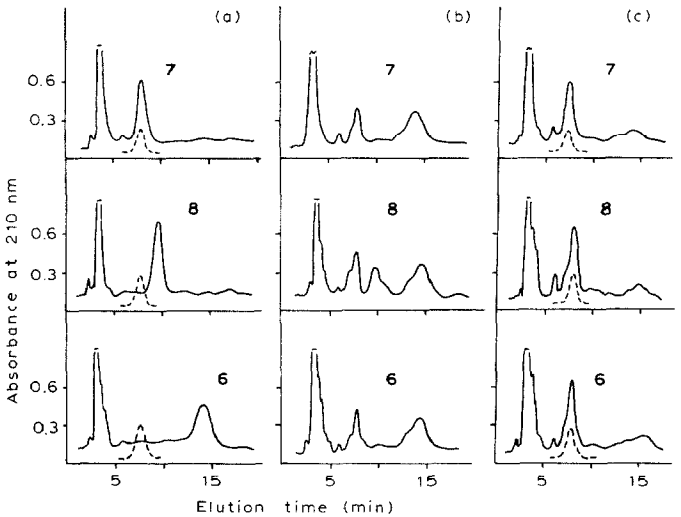


Fig. 8. Lichrosorb RP-8 chromatography of starting oligosaccharides **7, 8,** and **6** (a), and the products of their mild hydrolysis with 0.05M trifluoroacetic acid for 1 h (b) and 4 h (c). The position of fucose-free oligosaccharide **5** is shown with a dotted line.

linked D-mannose residues follows from the structure of oligomannoside chains of other glycoproteins[21–23], although an alternative pathway is not excluded[22,24]. It is of interest to note that, as for many other glycoproteins[25], we have not found any considerable amount of short intermediate carbohydrate chains resulting from the biochemical "processing", such as $Man_5GlcNAc_3$, $Man_3GlcNAc_3$, etc. This may mean that the action of α-D-mannosidase I is both selective and a rate-limiting step of carbohydrate-chain processing.

The four main complex oligosaccharides (**5–8**) contained an equal number of 2-acetamido-2-deoxyglucose, mannose, galactose, and 2-acetamido-2-deoxyglucitol residues, but differed in the number of fucose residues (Table II, numbers 13, 14, 16, and 19). As can be seen from Fig. 8, oligosaccharides **6–8** having one, two, and two fucose residues, respectively, were transformed upon mild acid hydrolysis[26] into the same fucose-free oligosaccharide **5**. This led to the suggestion that all the oligosaccharides have an identical backbone to which various numbers of fucose residues are attached.

The composition of the oligosaccharides and their elution volumes upon Bio-Gel P-6 gel chromatography correspond to triantennary complex chains. At the same time, the presence of only two galactose residues showed that one of the terminal nonreducing 2-acetamido-2-deoxyglucose residues is not substituted. Therefore, two most probable variants of oligosaccharide structure (**14** and **15**) could be taken into consideration. The presence of a nonsubstituted, terminal 2-acetamido-2-deoxyglucosyl group was confirmed by treatment of the oligosaccharides with *N*-acetyl-β-D-glucosaminidase, followed by isolation and analysis of oligomeric products (**5a, 6a, 7a,** and **8a**), showing the loss of one of the four

TABLE III

MOLAR RATIO OF MONOSACCHARIDES IN OLIGOSACCHARIDES AND THEIR FRAGMENTS[a]

Oligosaccharide or fragment	*GlcN*	*Man*	*Gal*	*Fuc*	*GlcNol*
5	4.0	3	2.1	[b]	0.8
5a	2.6	3	1.8	0	[c]
5b	2.7	2	[b]	0	0
5c	1.2	2	0	0	[c]
5d	3.2	2	1.8	[b]	[c]
5e	2.3	1	1.6	0	[c]
5f	1.9	1	0.2	[b]	[c]
7	4.1	3	2.3	2.2	0.7
7a	2.7	3	1.9	2.0	[c]
8	3.7	3	2.1	1.8	0.7
8a	2.6	3	1.7	1.7	[c]
6	3.6	3	2.1	1.0	0.6
6a	2.4	3	1.8	0.7	[c]
6b	2.8	2	0	0	0.4

[a]The fragments were isolated after *N*-acetyl-β-D-glucosaminidase treatment (**a**), Smith degradation (**b**), two successive Smith degradations (**c**), and acetolysis (**d, e,** and **f**). See Fig. 9 and Schemes 1 and 2. [b]Traces. [c]Not determined.

5 or **6** →β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-R

4
↑
1
β-D-Glc*p*NAc

5b R = 2-acetamido-2-deoxypentitol

6b R = D-GlcNAcol

→α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-2-acetamido-2-deoxypentitol

5c

Scheme 1

2-acetamido-2-deoxyglucose residues for each oligosaccharide (Table III).

Smith degradation of **5** and **6** (Scheme 1) resulted in the formation of fragments (**5b** and **6b**, Fig. 9b) of similar size, which contained 2-acetamido-2-deoxyglucose and mannose in the ratio of 3:2 (Table III). It is of interest also that

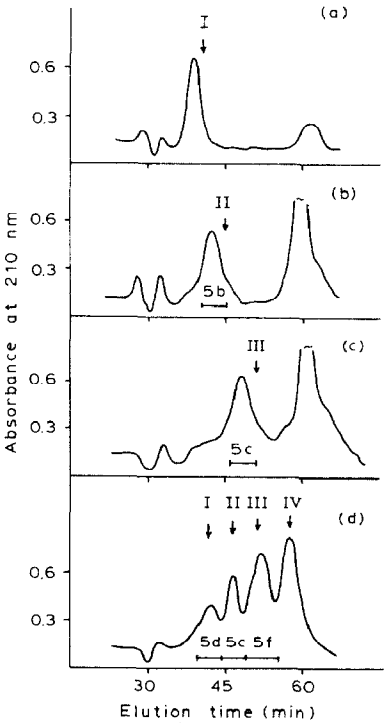
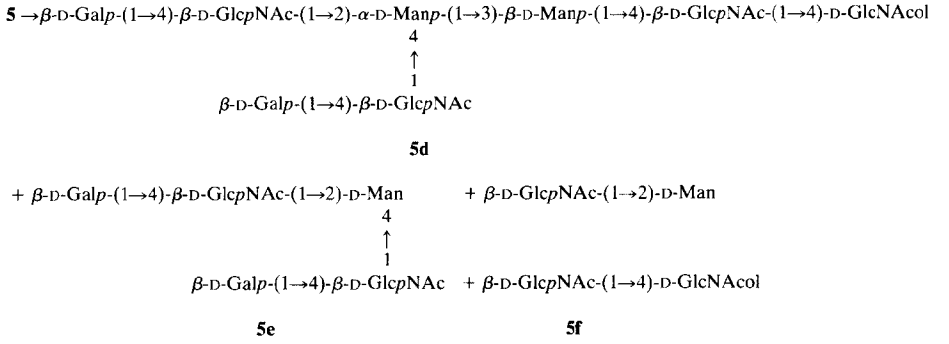


Fig. 9. TSK-40 gel chromatography of: (a) oligosaccharide **5**, and (b) its fragments after a single Smith degradation, (c) two successive Smith degradations, and (d) acetolysis. The arrow show the elution time of reference compounds: I, undecasaccharide $Man_9GlcNAc_1GlcNAcol$; II, hexasaccharide GlcNAc→Gal→GlcNAc→(Fuc→Gal→)GalNAcol; III, trisaccharide GlcNAc→Gal→GalNAcol; and IV, GlcNAc.

the 2-acetamido-2-deoxyglucitol residue of **6** was resistant to the oxidation whereas that of **5** was oxidized into a 2-acetamido-2-deoxypentitol identified (after hydrolysis) by amino acid analysis. Hence, the 2-acetamido-2-deoxyglucitol residue of **6** was substituted at O-6 by a fucosyl group which prevented its oxidation. The structure of the fragment **5b** was confirmed by repeated Smith degradation which resulted in the formation of fragment **5c** (Fig. 9c) that contained one 2-acetamido-2-deoxyglucose and two mannose residues (Table III). Thus, only structure **14** is in agreement with the results of Smith degradation because **15** would have given a trisaccharide Man→GlcNAc→R after the first periodate oxidation. The data also showed that the α-D-Man-(1→3) branch has two 2-acetamido-2-deoxyglucose residues which protect this mannose residue from oxidation.

Additional proof in favor of **14** was obtained by acetolysis of **5**. Although the conditions recommended for acetolysis[22] resulted in the nonselective cleavage of the α-D-Man-(1→3) and -(1→6) linkages, the composition of the fragments isolated (**5d–5f**; Fig. 9d, Table III) was in agreement with the fragmentation presented in Scheme 2. From these data, the structures of the main complex oligosaccharides **5–8** were established[27].



Scheme 2

Thus, the structures of the main oligomannoside and complex chains of hemagglutinin of influenza virus A/Leningrad/385/80 (H3N2) were elucidated showing that the great heterogeneity of carbohydrate fragments is inherent to both heavy and light subunits of HA. The structures of the main chains and their relative amounts are strikingly different from those found for X-31 hemagglutinin[18], confirming the view that variation in glycosylation of HA depends on virus strain[5,28] as well as the host-cell[10,29]. More experimental data on the carbohydrate chain structures of HA from various virus strains and corresponding immunochemical studies are needed for a better understanding of this phenomenon.

EXPERIMENTAL

Materials. — Influenza virus A/Leningrad/385/80 (H3N2), grown in chick embryos, was purified by ultracentrifugation (Beckman L5-65) in sucrose density gradient (10–40%). Bromelain (Merck) was purified by gel chromatography on Sephadex G-75. *N*-Acetyl-β-D-glucosaminidase from bovine kidney was purchased from Sigma Chemical Co. The isolation of HA, HA1, and HA2 was controlled by SDS–PAGE in 7% gel and staining with Coomassie Blue.

General methods. — ^{1}H-N.m.r. spectra were recorded for solutions in ^2H_2O at 80° with a WM-250 Brucker spectrometer and an accumulation of 200–400. The chemical shifts are reported relative to the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. L.c. was performed with an Altex instrument, model 332, with spectrophotometer Knauer and columns (4.6 × 250 mm) of Lichrosorb RP-8, RP-18, and Alltech-NH_2 (Alltech). For microgel chromatography of the oligosaccharides, the column was a Teflon capillar tube (1.6 × 1500 mm) packed with TSK-40 gel (superfine, Toyo Soda) and temperature-controlled at 50°.

Monosaccharide composition was determined, after hydrolysis of the oligosaccharides with 3M trifluoroacetic acid (6 h, 100°), with a liquid chromatograph Biotronik LC 2000 and a DA-X8 column (70°, NaOH–H_3BO_3 (0.4M) buffer, pH 8) and detection with the Cu-bicinchoninate reagent[13]. 2-Amino-2-deoxy-glucose and -glucitol, and amino acids were determined, after hydrolysis of the oligosaccharides with 4M HCl (6 h, 100°), with an amino acid analyzer Biotronik LC-4020 and an Aminex A-5 column (0.35M sodium citrate buffer, pH 5.3).

Isolation of HA. — To a suspension of virions (10 mL, protein ~5 mg/mL) in buffer (0.1M NaCl, 10mM Tris, mM EDTA, 0.02% NaN_3; pH 7.2), were added bromelain (30 mg) and 2-mercaptoethanol (15 mg). The mixture was incubated for 16 h at 37° and centrifuged (150,000*g*, 1.5 h). The supernatant was applied onto a column (0.9 × 118 cm) of Sepharose CL-6B which was eluted with 0.01M NaCl. The absorbance at 280 nm was measured and the fractions showing no protease activity (drop-on-the-film test) were collected as shown in Fig. 1 and lyophilized to give a sample of HA containing usually 10–12 mg of protein; it showed in SDS–PAGE one band (M_r ~85 000) before, and two bands (M_r ~67 000 and 25 000) after, reduction with 2-mercaptoethanol.

Isolation of HA1 and HA2. — The sample of HA prepared as just described was dissolved in a small volume of 0.1M NaCl–2% sodium dodecyl sulfate–0.2% dithiothreitol heated for 3 min at 100°, and loaded onto a column (0.9 × 82 cm) of Ultrogel AcA-34, equilibrated with 0.01M NaCl–0.2% sodium dodecyl sulfate–0.01% dithiothreitol. Fractions were analyzed by SDS–PAGE for the presence of HA1 or HA2, pooled as shown in Fig. 2, and lyophilized. Generally, ~4 mg of HA1 and 2 mg of HA2 (as a protein) were obtained.

Isolation of oligosaccharide chains. — To a solution of glycoprotein (HA1 or HA2, 20 mg of protein) in water (4 mL) were added M LiOH (0.8 mL) and 2-

methyl-2-propanol (11.2 mL), the mixture was cooled in an ice bath, and $LiBH_4$ (0.4 g) was added portionswise. The mixture was heated for 6–8 h at 50° and cooled, and the residual $LiBH_4$ was decomposed with conc. acetic acid. The mixture was concentrated *in vacuo,* and methanol (~5 mL) and several drops of acetic acid were repeatedly added and evaporated. The solution of the residue in water (10 mL) was extracted with butanol (3 × 10 mL) to remove the detergent. The aqueous solution was concentrated and de-ionized on a Sephadex G-15 column with 0.1M acetic acid as an eluent. Orcinol–H_2SO_4-positive fractions were collected and evaporated. The residue was dissolved in 0.05M NaOH (0.5 mL), and then $NaBH_4$ (~15 mg) was added, and the mixture was stored for 16 h at room temperature. The mixture was acidified with acetic acid, and methanol was added and distilled several times from the residue, which was then taken up in water and loaded onto a column (0.5 × 7 cm) of Bio-Rad AG 50 (H^+). The neutral oligosaccharide fraction was eluted with water (20 mL), and the basic fraction with 0.7M NH_4OH (20 mL). Both fractions were analyzed for amino acids, amino sugars, and neutral sugars.

Fractionation of oligosaccharides. — The mixture of oligosaccharides (2–3 mg) was separated on a column (0.9 × 110 cm) of Bio-Gel P-6 (−400 mesh), eluted with 0.1M acetic acid (2 mL/h). The fractions were analyzed with the orcinol–H_2SO_4 reagent and combined as shown in Fig. 3. The monosaccharide composition of fractions isolated from HA is presented in Table I.

Oligosaccharide Fraction I was chromatographed on two successively connected columns of Lichrosorb RP-18 with bidistilled water as an eluent (0.8 mL/h) and u.v.-detection at 210 nm. Components **A–E** (Fig. 4) were isolated from both HA1 and HA2 after several passages through the columns. The separation of oligosaccharide Fraction II was carried out on Lichrosorb RP-8 column with bidistilled water and an elution rate gradient of 0.5 to 2 mL/h in 15 min. Components **F–L** (Fig. 4) were isolated from both HA1 and HA2.

Components **A–L** were further fractionated (separately for HA1 and HA2) on two successively connected Alltech-NH_2 columns with 75% aqueous methanol as an eluent (0.8 mL/min) and u.v.-detection at 210 nm. Thus, 21 oligosaccharides (Figs. 5 and 6) were isolated in preparative form from HA1 and HA2, and the monosaccharide composition was determined (Table II).

Selective fragmentation of oligosaccharides. — Mild acid hydrolysis. An oligosaccharide (5 nmol) in 0.05M trifluoroacetic acid (50 μL) was heated in a sealed tube for 1 or 4 h at 100°, and dried *in vacuo* in the presence of P_2O_5 and NaOH. The residue was dissolved in 0.05M Na_2CO_3 (20 μL) and methanol (10 μL), acetic anhydride (7 μL) was added, and the mixture was kept for 16 h at room temperature. The solution was evaporated after addition of methanol, dried *in vacuo,* and the products were analyzed on a Lichrosorb RP-8 column (Fig. 8).

Enzymic hydrolysis. — (*a*) To a solution of an oligosaccharide (5 nmol) in 0.05M sodium citrate buffer (pH 4.5, 20 μL) was added a suspension of *N*-acetyl-β-D-glucosaminidase (20 U/mL, 1 μL). The mixture was incubated for 20 h at 37°

and, without any treatment, loaded onto a Lichrosorb RP-8 column eluted with water. The only component eluted appeared at the same time as the starting oligosaccharide; it was dried, dissolved in 75% aqueous methanol, and chromatographied on an Alltech-NH_2 column with 75% aqueous methanol; again a single component having a retention time similar to that of the starting oligosaccharide was obtained. This product was hydrolyzed and its monosaccharide composition was determined (fragments **5a, 6a, 7a,** and **8a**, Table III).

(*b*) Oligosaccharide **8** (10 nmol) was treated with *N*-acetyl-β-D-glucosaminidase as just described, and the products were separated on a TSK-40 column. The monosaccharide fraction was hydrolyzed and analyzed for sugar: 2-amino-2-deoxyglucose was the principal component of the monosaccharide mixture.

Smith degradation of oligosaccharides. — A solution of an oligosaccharide (**5** or **6**, 50 nmol) in 0.05M $NaIO_4$ (50 μL) was stored in darkness for 1 day at room temperature, and then $NaBH_4$ (2–3 mg) was added portionwise. After 3–4 h at room temperature, the excess of $NaBH_4$ was destroyed with acetic acid, and the mixture was de-ionized by passage through a column (1–2 mL) of Bio-Rad AG 1-X8 (AcO^-) and AG 50 × 4 (H^+) ion-exchange resins. The aqueous eluate was evaporated three times with addition of methanol, and the residue was dissolved in 0.2M trifluoroacetic acid (0.1 mL). The solution was kept for 16 h at 20° and evaporated, and the residue was fractionated on a TSK-40 column in water (Fig. 9). Fractions **5b** and **6b** were analyzed for monosaccharides (Table III). A second Smith degradation of fragment **5b** was carried out in the same manner.

Acetolysis of oligosaccharide **5**. — The oligosaccharide (20 nmol), carefully dried *in vacuo*, was dissolved in 1:1 acetic anhydride–pyridine (40 μL), and the solution was kept for 16 h at room temperature and evaporated with additions of toluene and methanol. The residue was dissolved in 10:10:1 acetic acid–acetic anhydride–H_2SO_4 (50 μL), and heated for 6 h at 30° (ref. 22). The mixture was passed through a column (~1 mL) of AG 1-X8 (AcO^-) anion-exchange resin, and the products were eluted with 70% aqueous methanol. To a dried sample were added methanol (100 μL) and triethylamine (10 μL), and the solution was kept for 16 h at 5° and then evaporated. The residue was fractionated on a TSK-40 column in water, fractions were collected as shown in Fig. 9, and their monosaccharide composition was determined (Table III).

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