*Glycoconjugate J (1987) 4: 17-32*

# Carbohydrates of Influenza Virus. Structure of the Oligosaccharides Linked to Asparagines 406 and 478 in the Hemagglutinin of Fowl Plague Virus, Strain Dutch

RUDOLF GEYER[1], SILVIA DIABATÉ[1], HILDEGARD GEYER[1], HANS-DIETER KLENK[2], HEINER NIEMANN[2] and STEPHAN STIRM[1]*

[1]*Biochemisches Institut am Klinikum der Universität, Friedrichstrasse 24, D-6300 Giessen, Federal Republic of Germany*
[2]*Institut für Medizinische Virologie am Klinikum der Universität, Frankfurter Strasse 107, D-6300 Giessen, Federal Republic of Germany*

Received December 21, 1986.

*Key words: influenza virus, hemagglutinin, glycoprotein glycans, oligosaccharide structure*

**Fowl plague virus, strain Dutch, was metabolically labeled with D-[2-$^3$H]mannose, or with D-[6-$^3$H]glucosamine, and the small subunit ($HA_2$; 0.8 mg in total) of the viral hemagglutinin was isolated by preparative sodium dodecylsulfate-polyacrylamide gel electrophoresis. After proteolytic digestion, the radioactive oligosaccharides were sequentially liberated from the glycopeptides by treatment with different endo-β-*N*-acetylglucosaminidases and with peptide:*N*-glycosidase or, finally, by hydrazinolysis. In this manner, four groups of glycans could be obtained by consecutive gel filtrations and were subfractionated by HPLC. The structures of the individual oligosaccharides were analyzed by micromethylation, by acetolysis or by digestion with exoglycosidases. The major species amongst the high mannose glycans at Asn-406 of the viral glycopolypeptide were found to be Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc and Manα1-3(Manα1-2Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, while the complex glycans at Asn-478 are predominantly GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (lacking, in part, one of the outer *N*-acetylglucosamine residues) and GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4-GlcNAc.**

**Abbreviations:** BSA, bovine serum albumin; endo D (F,H), endo-β-*N*-acetyl-D-glucosaminidase D (F,H); HA, hemagglutinin ($HA_1$, large subunit of HA; $HA_2$, small subunit); FPV, fowl plague virus; PNGase F, peptide:*N*-glycosidase F; SDS, sodium dodecylsulfate.

*Author for correspondence

The hemagglutinin (HA) and the neuraminidase spikes protruding from the envelope of influenza viruses belong to the best characterized membrane glycoproteins. Thus, the amino acid sequences of many HA antigenic variants have been determined [1, 2], and their conformation has been elucidated by X-ray crystallography [3, 4]. Until recently, however, only over-all structural data were available on the *N*-glycosidically linked carbohydrates in these viral components [5, 6], *i.e.* no detailed studies of the oligosaccharides substituting single Asn-X-Ser/Thr sites had been carried out.

Therefore, we are analyzing [7, 8] the primary structure of the carbohydrates at individual glycosylated asparagines in the hemagglutinin of fowl plaque virus (FPV), which is an avian influenze virus. In FPV strain Dutch, four of these glycosylated asparagines (Asn-12, -28, -123, and -231) occur in the large subunit of the molecule ($HA_1$, about 320 amino acids) and two (Asn-406 and -478) in the small subunit ($HA_2$, 221 amino acids) [9]. Each hemagglutinin spike is a trimer comprising three $HA_1$-$HA_2$ protomers, the three Asn-406 residues of which are part of long $\alpha$-helices in the stem of this structure, while the three asparagines-478 reside in coils between shorter $\alpha$-helical segments at the base of the spike [3, 8, 10]. As shown earlier [7, 8, 11], Asn-406 in the $HA_2$ of FPV strains Rostock and Dutch (the amino acid sequences of which differ by a few single exchanges only [9, 12, 13]) is mainly substituted by glycoprotein *N*-glycans of the oligomannosidic type, whereas Asn-478 carries *N*-acetyllactosaminic oligosaccharides (cf. [14-16]). We have now separated the microheterogeneous $HA_2$ carbohydrates obtained from FPV strain Dutch and have analyzed the single glycan species.

## Materials and Methods

### *Virus*

As described earlier in detail [6], the Dutch strain of fowl plague virus (A/FPV/Dutch/27[H7N7]) was propagated on primary chick fibroblasts; it was metabolically radiolabeled with D-[2-$^3$H]mannose, or with 2-aminodeoxy-D-[6-$^3$H]glucose, and it was harvested and purified by centrifugation techniques.

### *Viral Glycoprotein*

The experimental protocol of Niemann *et al.* [6] was also followed for the disintegration of the virus particles, by heating in the presence of sodium dodecylsulfate (SDS) and 2-mercaptoethanol; and for the isolation of the hemagglutinin subunits by preparative SDS-polyacrylamide gel electrophoresis. Approximately 0.8 mg of FPV $HA_2$ ($4 \times 10^5$ and $6.3 \times 10^5$ cpm of [$^3$H]Man or of [$^3$H]GlcN, respectively) were used for the present study; this material was obtained from the virus harvest of 800 cell culture petri dishes (14.5 cm diameter).

### *Enzymatic Digestions*

FPV $HA_2$ was degraded with pronase from *Streptomyces griseus* (Boehringer, Mannheim, W. Germany) under the conditions described earlier [17]. Also for the digestion of the resulting glycopeptides with endo-$\beta$-*N*-acetyl-D-glucosaminidase D from

*Diplococcus pneumoniae* (endo D) [18] and with endo H from *S. griseus* [18] (both Boehringer), previoisly published experimental protocols [17, 19] were followed. For degradation with the mixture of endo-β-*N*-acetyl-D-glucosaminidase F/peptide:*N*-glycosidase F (endo F/PNGase F) from *Flavobacterium meningosepticum* [20, 21], the endo D- and endo H-resistant glycopeptides ($10^5$ to $5 \times 10^5$ cpm) were dissolved in 200 µl of 100 mM sodium phosphate buffer pH 6.1, containing 50 mM EDTA. They were incubated at 37°C for 8, 16 and 8 h after sequential addition of three portions of approximately 0.1 nkat endo F/PNGase F. The reaction mixture was directly subjected to gel filtration. The endo F/PNGase F preparation used in this study was a kind gift of Dr. John H. Elder, Scripps Clinic, La Jolla, CA, USA.

For experimental details on the degradation of the oligosaccharide alditols with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase from jack bean [22] (all Sigma, Deisenhofen, W. Germany) see also Geyer *et al.* [23].

### *Hydrazinolysis*

The endo F/PNGase F-resistant $HA_2$ glycopeptides were treated with anhydrous hydrazine as described by Takasaki *et al.* [24], except that sodium borohydride was used for reduction, and that the reaction mixture, after removal of the boric acid, was subjected to gel filtration.

### *Chromatography*

Except for the HPLC of complex oligosaccharide alditols, which was carried out by a modification of the method of Bergh *et al.* [25] (cf. legend to Fig. 2), the materials and methods employed for chromatography were the same as in earlier communications. This holds for the reduction of oligosaccharides with $NaBH_4$ [17, 26], for the equipment, column fillings and oligosaccharide alditol standards employed for gel filtration [17, 26], HPLC (of high F-mannose alditols) [19] and deionization [17, 26], as well as for the radioactivity monitoring of the fractions obtained [19].

### *Methylation Analysis*

Samples were methylated [27], hydrolyzed, reduced with $NaBH_4$, and peracetylated. They were analyzed by capillary GLC-MS of the partially methylated alditol acetates [28]. The instrumentation and the microtechniques described earlier [29, 30] were used, except that the Silar 9CP column was replaced by a fused-silica, bonded-phase Durabond DB-210 column (0.25 mm inner diameter; 60 m length; 0.25 µm thickness of the Durabond film; Ict, W. Germany).

### *Acetolysis*

For the acetolysis [31, 32] of the oligomannosidic glycans as well as for the de-*O*-acetylation, reduction (with $NaBH_4$) and chromatographic identification of the products, the experimental protocol of Diabaté *et al.* [19] was followed.

**Table 1.** Distribution of radiolabel into oligosaccharide fractions obtained from the small subunit ($HA_2$) of the hemagglutinin in fowl plague virus (FPV), strain Dutch.

| Oligosaccharide fraction | Virus metabolically labelled with | |
|---|---|---|
| | D-[2-$^3$H]Man | D-[6-$^3$H]GlcN |
| Released by endo D (cf. Fig. 1B) | 9% | 25% |
| Released by endo H from endo D-resistant glycopeptides (cf. Fig. 1C) | 70% | |
| Released by endo F/PNGase F from endo D, H-resistant glycopeptides (cf. Fig. 2B) | 13.5%[a] | 43.5%[b] |
| Released by hydrazinolysis from endo D, H- and endo F/PNGase F-resistant glycopeptides | 7.5% | 25.5% |
| Residual material of high molecular weight[c] | | 6%[c] |

[a] Comprising 4% of *N*-acetylglucosaminyl peptides.
[b] Comprising 7.5% of *N*-acetylglucosaminyl peptides.
[c] *I.e.* radioactivity eluting between 20 and 30 ml in Bio-Gel P-4 chromatography even after hydrazinolysis (cf. Fig. 2A and 2B). This fraction was not further investigated.

## Results

For the glycosylation analysis of *less than 1 mg of glycoprotein* (see the Materials and Methods section), microtechniques had to be used. Therefore, specifically radiolabeled glycoprotein glycans were analyzed throughout, *i.e.* the small subunit ($HA_2$) of the hemagglutinin was isolated from fowl plague virus (FPV; strain Dutch) which had been labeled metabolically with D-[2-$^3$H]Man, or with D-[6-$^3$H]GlcN [6].

### *Liberation and Fractionation of the Glycans in FPV $HA_2$*

After digestion with pronase, the glycans were sequentially liberated from the $HA_2$ glycopeptides by digestion with endo D [18], with endo H [18], with endo F/PNGase F [20, 21], and finally by hydrazinolysis [24]. The oligosaccharides produced were recovered by gel filtration after each step. Following reduction with $NaBH_4$, each of the four groups of oligosaccharide alditols thus obtained were subfractionated by HPLC. The results are summarized in Table 1 and in Fig. 1 and 2, designating the glycan fractions $D_3$, $D_4$ etc., $H_3$, $H_4$ etc., $F_1$, $F_2$ etc., or $Z_1$ and $Z_2$ in accordance with their liberation by endo D, endo H, endo F/PNGase F, or by hydrazinolysis, respectively. Only fraction $F_5$ (see Fig. 2C) was further subfractionated by another Bio-Gel P-4 chromatography, yielding fractions $F_{5.1}$ and $F_{5.2}$ (not shown).

### *Analytical Results*

The FPV $HA_2$ oligosaccharide alditol fractions were desalted and were subjected to micromethylation analysis [30]. The results are presented in Table 2. Methylation of the

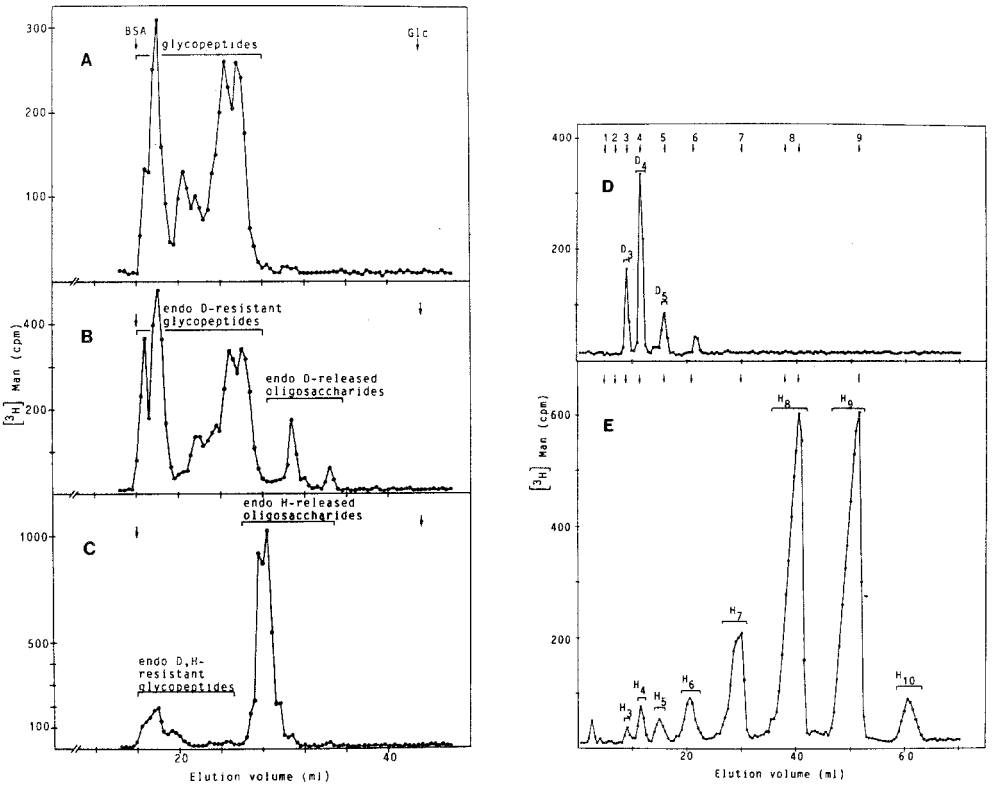



**Figure 1.** Isolation of endo H-sensitive glycans from the small subunit ($HA_2$) of the hemagglutinin of fowl plague virus (FPV) strain Dutch - metabolically radiolabeled with [$^3$H]mannose.
A, FPV $HA_2$ ($4 \times 10^5$ cpm) was exhaustively digested with pronase and the reaction products were chromatographed on a column (200 × 0.6 cm) of Bio-Gel P-4 (-400 mesh) at hydrostatic pressure. 0.02% aqueous $NaN_3$ (1.23 ml/h) served as an eluant and fractions of 0.4 ml were collected and were monitored for radioactivity.
B, As A, after further digestion of the FPV $HA_2$ glycopeptides with endo D.
C, As A, after further digestion of the endo D-resistant glycopeptides (bracket in B) with endo H.
D, The endo D-released oligosaccharides (bracket in B) were reduced with $NaBH_4$. They were subfractionated by HPLC through a column (0.4 × 25 cm) of LiChrosorb Diol (5 $\mu$; Merck, Darmstadt, W. Germany), employing acetonitrile/water, 73/27 by vol, 0.5 ml/min, as an eluant, and collecting fractions of 0.5 ml.
E, The endo H-released oligosaccharides (bracket in C) were analogously reduced and subfractionated. BSA and Glc with arrows; elution volumes of bovine serum albumin and of glucose, respectively. 1, 2 etc. with arrows: elution volumes of oligomannosidic standard alditols ManGlcNAcOH, $Man_2$GlcNAcOH, etc. (two isomers of $Man_8$GlcNAcOH). $D_3$,$D_4$ etc. and $H_3$, $H_4$ etc., oligosaccharide alditol fractions obtained after digestion with endo D and with endo H, respectively (subscripts correlating with designation of co-eluting standards).

hatched peak eluting at 35 ml in Fig. 2B predominantly yielded terminal *N*-acetylglucosamine (not shown in Table 2). Clearly, this fraction consists mainly of [$^3$H]GlcN-labeled *N*-acetylglucosaminyl peptides formed by the action of endo F.

For isomer identification, the oligomannosidic alditol fractions of the D and H series were partially degraded by mild acetolysis [19, 32], and the fragments obtained were

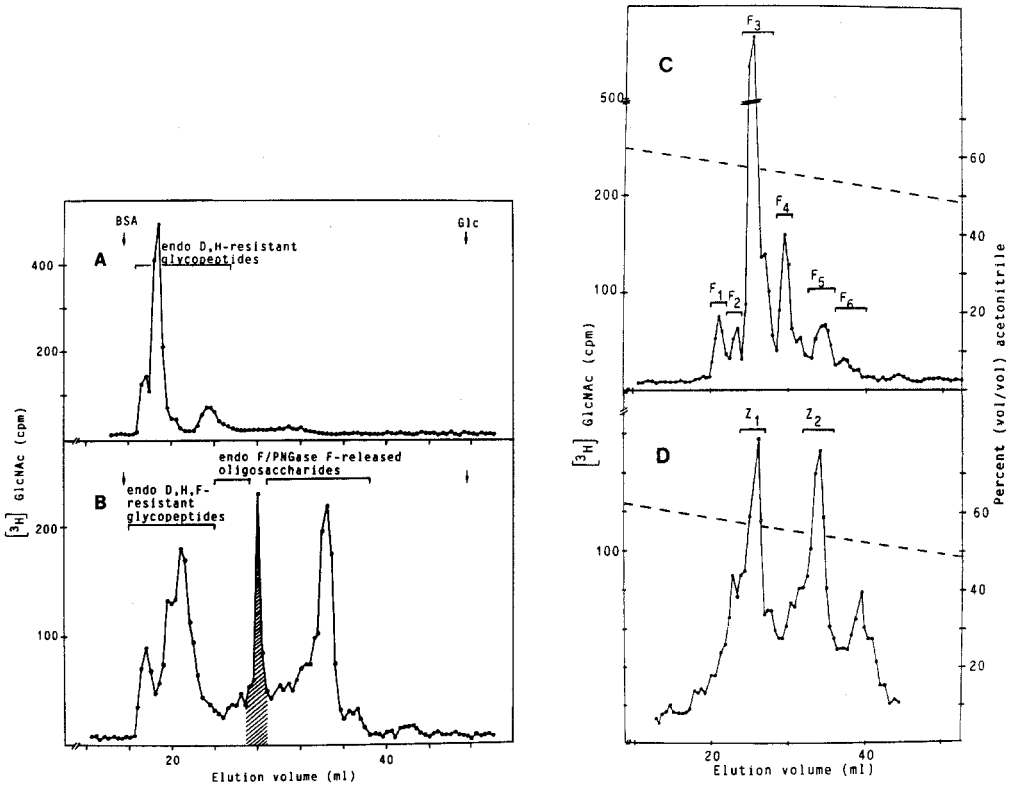



**Figure 2.** Isolation of endo F/PNGase F-released and hydrazinolytic glycans from FPV $HA_2$ - metabolically radiolabeled with 2-aminodeoxy-[$^3$H]glucose.
A, FPV $HA_2$ (6.3 × $10^5$ cpm) was successively digested with pronase, with endo D and with endo H. The resistant glycopeptides were isolated as in Fig. 1 (not shown) and were rechromatographed through Bio-Gel P-4.
B, The same as above after further digestion of the endo D, H-resistant glycopeptides with endo F/PNGase F.
C, The endo F/PNGase-released oligosaccharides (pooled as shown by two brackets in B, *i.e.* omitting the hatched peak of *N*-acetylglucosaminyl peptides) were reduced with $NaBH_4$ and were subfractionated by HPLC.
D, The oligosaccharides finally released from endo D, H, F/PNGase F-resistant glycopeptides (bracket in B) by hydrazinolysis, were passed through Bio-Gel P-4 (not shown) and the major fraction, cochromatographing with the glycans released by endo F/PNGase F, was reduced and was subfractionated by HPLC.
Standards and conditions as in Fig. 1, except that a column of LiChrosorb $NH_2$ (5 $\mu$; Merck) and a linear gradient of acetonitrile (from 65% to 40%, by vol, in 90 min) in a 15 mM potassium phosphate buffer of pH 5.3 (1 ml/min) were used for HPLC, collecting fractions of 0.4 ml. $F_1$, $F_2$, etc. and $Z_1$, $Z_2$, oligosaccharide alditol fractions obtained after treatment with endo F/PNGase F, or with anhydrous hydrazine, respectively.

analyzed by gel filtration and by HPLC after de-*O*-acetylation and reduction with $NaBH_4$ (see the example in Fig. 3 and the summary in Table 3).

By sequential treatment with the respective exoglycosidases [22], oligosaccharide alditol fractions $F_1$, $F_3$ and $F_4$ (labeled with [$^3$H]GlcN) could be degraded to radioactive fragments co-eluting with *N*-acetylglucosamine and ManGlcNAcOH in gel filtration. In this manner, one $\beta$-*N*-acetylhexosamine and two $\alpha$-mannose residues were removed

**Table 2.** Methylation analysis of oligosaccharide alditol fractions (see Fig. 1 and 2) isolated from the small subunit ($HA_2$) of the hemagglutinin in fowl plague virus (FPV), strain Dutch. Results are expressed in peak ratios of the partially methylated alditol acetates obtained [30]. 2,3,4,6-ManOH, 2,3,4,6-tetra-*O*-methyl-D-mannitol etc.; 1,3,5,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methyl) acetamido-1,3,5,6-tetra-*O*-methyl-D-glucitol, etc.

| Peracetate of | Oligosaccharide alditol fractions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $H_3$[a,b] | $H_4$[a,c] | $H_5$[b,d] | $H_6$[b] | $H_7$[b] | $H_8$[b] | $H_9$[b] | $H_{10}$[e] |
| 2,3,4,6-ManOH | 1.7 | 1.15 | 1.4 | 1.3 | 2.4 | 2.8 | 2.85 | 1.6 |
| 3,4,6-ManOH | 0.3 | 1.0 | 1.35 | 1.8 | 2.55 | 3.25 | 4.65 | 4.6 |
| 2,4,6-ManOH | 0.15 | — | 0.5 | 0.45 | — | — | — | 0.85 |
| 2,3,4-ManOH | — | — | — | 0.15 | — | — | — | — |
| 2,4-ManOH[f] | 0.85 | 1.05 | 1.75 | 2.15 | 2.05 | 1.95 | 1.5 | 2.1 |
| 2-ManOH[f] | — | — | — | 0.15 | — | — | — | — |
| 2,3,4,6-GalOH | — | — | 0.25 | 0.2 | — | — | — | — |
| 2,3,4,6-GlcOH | — | — | — | — | — | — | — | 0.85 |
| 1,3,5,6-GlcN(Me)AcOH[g] | 0.6 | 0.55 | 0.4 | 0.3 | 0.9 | 0.7 | 0.9 | 0.1 |
| 3,4,6-GlcN(Me)AcOH | — | 1.15 | 1.6 | 0.55 | — | — | — | — |

| Peracetate of | Oligosaccharide alditol fractions | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $F_1$[c] | $F_1$[c,h] | $F_2$[c] | $F_3$[c] | $F_4$[c] | $F_4$[c,i] | $F_{5.1}$[j] | $F_{5.2}$[j] | $F_6$[j] | $Z_1$[c] | $Z_2$[j] | $Z_2$[c,i] |
| 2,3,4-FucOH | — | — | — | — | — | — | 0.45 | 0.55 | 0.55 | — | 0.45 | 0.7 |
| 2,3,4,6-ManOH | 1.05 | 0.25 | — | — | — | — | — | — | — | — | — | 2.1 |
| 3,4,6-ManOH | 1.0 | 1.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.65 | 1.7 | 1.65 | 2.0 | 1.8 | 1.0 |
| 2,4,6-ManOH | — | 0.35 | — | — | — | — | — | — | — | — | — | — |
| 2,3,4-ManOH | — | 0.15 | — | — | — | 0.7 | — | — | — | — | — | 1.4 |
| 3,4-ManOH | — | — | — | — | — | — | 0.35 | 0.3 | 0.35 | — | 0.2 | — |
| 2,4-ManOH[f] | 0.95 | 0.2 | 0.9 | 1.05 | 1.0 | — | 0.5 | 0.85 | 0.4 | 0.85 | 0.8 | 0.45 |
| 2-ManOH[f] | — | — | — | — | — | — | 0.5 | — | 0.55 | — | 0.25 | — |
| 2,3,4,6-GalOH | — | — | — | — | 0.9 | 1.0 | 0.25 | 1.3 | 0.65 | — | 0.25 | 0.45 |
| 2,4,6-GalOH | — | — | — | — | — | — | — | — | 0.15 | — | — | — |
| 2,3,4-GalOH | — | — | — | — | — | — | — | 0.2 | — | — | — | — |
| 1,3,5,6-GlcN(Me)AcOH[g] | 1.1 | 1.1 | — | 1.25 | 1.05 | 0.5 | 0.1 | 1.1 | 0.4 | 0.1 | — | trace |
| 3,4,6-GlcN(Me)AcOH | 1.25 | 1.15 | 2.7 | 2.75 | 1.35 | — | 2.85 | 2.3 | 2.5 | 2.0 | 3.25 | 0.4 |
| 1,3,5-GlcN(Me)AcOH | — | — | — | — | — | — | 0.65 | 0.2 | 0.6 | — | 0.2 | 0.65 |
| 3,6-GlcN(Me)AcOH | — | — | 1.05 | — | 1.15 | 0.95 | 1.05 | 1.05 | 1.65 | 1.0 | 1.05 | 2.2 |
| 6-GlcN(Me)AcOH | — | — | — | — | — | — | — | 0.7 | 0.1 | — | — | — |

[a] Similar results were obtained with fractions $D_3$ and $D_4$.
[b] Based on sum of mannose residues = 3 in $H_3$, = 5 in $H_5$ etc.
[c] Based on 3,4,6-ManOH peracetate = 1 or 2.
[d] Amounts of fractions $D_5$ were insufficient for methylation analysis.
[e] Based on sum of mannose and glucose residues = 10.
[f] Corrected for losses during hydrolysis [33].
[g] Including small amounts of 3,5,6-GlcN(Me)AcOH peracetate. Due to de-*O*-methylation during hydrolysis and side reactions during hydrazinolysis, low values are often obtained.
[h] *I.e.* after digestion with α-mannosidase.
[i] *I.e.* after digestion with β-*N*-acetylhexosaminidase and with α-mannosidase.
[j] Based on sum of 3,4,6- and 3,4-ManOH peracetates = 2.

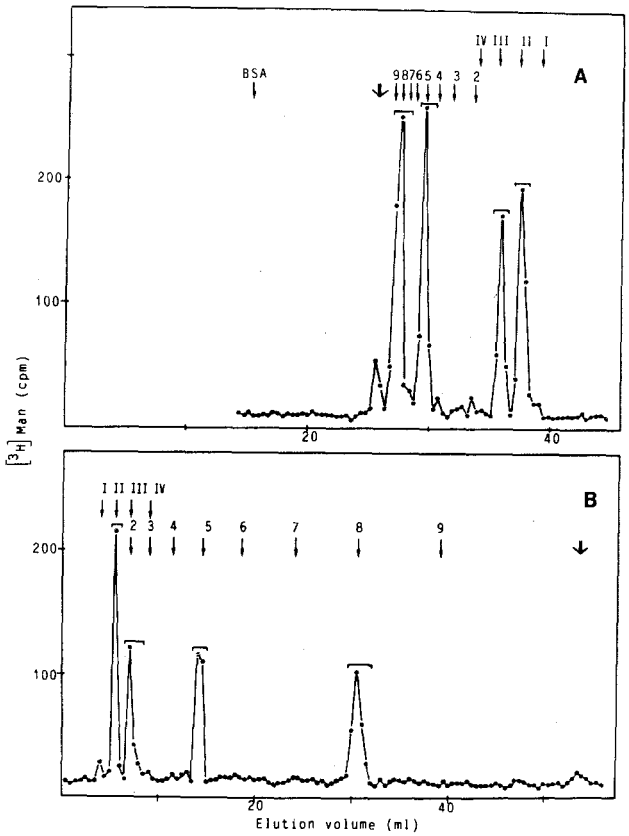



**Figure 3.** Acetolysis of oligosaccharide alditol fraction $H_{10}$ from FPV $HA_2$. Fraction $H_{10}$ (see Fig. 1E; $6 \times 10^3$ cpm) was acetylated and treated with glacial acetic acid/acetic anhydride/conc. sulfuric acid, 11/10/1 by vol, at 30°C for 7 h. The reaction products were de-*O*-acetylated and reduced with $NaBH_4$.
A, Bio-Gel P-4 chromatogram of $H_{10}$ acetolysis fragments.
B, HPLC of the same fragments. Bold arrows indicate elution volume of intact $H_{10}$. Brackets show fractions as pooled for radioactivity monitoring (see Table 3). Conditions of chromatography and calibration of column with BSA and with oligomannosidic standards as in Fig. 1, except that a LiChrosorb Diol column of 15 × 0.4 cm and acetonitrile/water, 75/25 by vol, were used for HPLC. Roman numerals with arrows, elution volumes of $GlcO^3H$ and $(Glc)_{1\text{-}3}GlcO^3H$ of the isomaltose series.

from $F_1$; two β-*N*-acetylhexosamine and two α-mannose residues from $F_3$; and one β-galactose, two β-*N*-acetylhexosamine and two α-mannose residues from $F_4$ (see Fig. 4 for an example).

For an assignment of the differing antennae, asymmetrically substituted glycans were digested with α-mannosidase ($F_1$) or with β-*N*-acetylhexosaminidase and with α-mannosidase ($F_4$ and $Z_2$), and the truncated products were subjected to methylation analysis. The results are included in Table 2.

**Table 3.** Acetolysis of oligomannosidic oligosaccharide alditol fractions (see Fig. 1) isolated from the small subunit ($HA_2$) of the hemagglutinin in fowl plague virus (FPV), strain Dutch. The results are expressed in per cent [$^3$H]Man radioactivity found in the fragments produced. The latter were analyzed by gel filtration and by HPLC after reduction with $NaBH_4$ (see Fig. 3).

| Acetolysis fragments (reduced)[a] | Oligosaccharide alditol fraction | | | | | | |
|---|---|---|---|---|---|---|---|
| | $D_3$ | $H_3$ | $D_5$ | $H_7$ | $H_8$ | $H_9$ | $H_{10}$[b] |
| ManOH | 31.2 | 31 | 20.1 | 5.4 | 4.4 | 6.0 | 2.1 |
| ManManOH | | | 17.5 | 42.4 | 39.0 | 29.0 | 25.6 |
| $(Man)_2$ManOH | | | 4.6 | | 3.2 | 14.4 | 17.5 |
| $(Man)_2$GlcNAcOH | 68.8 | 69 | 20.9 | | | | |
| $(Man)_3$GlcNAcOH | + | + | 6.6 | 16.5 | | 2.5 | |
| $(Man)_4$GlcNAcOH | | | 30.2 | 5.9 | 16.6 | 18.8 | |
| $(Man)_5$GlcNAcOH | | | + | 26.3 | 4.0 | 3.7 | |
| $(Man)_6$GlcNAcOH | | | | 3.5 | 32.8 | | |
| $(Man)_7$GlcNAcOH | | | | + | | 25.6 | |
| $(Man)_8$GlcNAcOH | | | | | + | | |
| $(Man)_9$GlcNAcOH | | | | | | + | |
| Glc$(Man)_4$GlcNAcOH[c] | | | | | | | 25.8 |
| Glc$(Man)_7$GlcNAcOH[c] | | | | | | | 29.0 |
| Glc$(Man)_9$GlcNAcOH | | | | | | | + |

[a] ManOH, mannitol; GlcNAcOH, *N*-acetylglucosaminitol.
[b] Mean values from the chromatograms shown in Fig. 3.
[c] Not separated from $(Man)_5$GlcNAcOH or from $(Man)_8$GlcNAcOH, respectively. Composition deduced from methylation results of $H_{10}$ (See Table 2).

## Discussion

The structures of the oligomannosidic alditols identified in the glycan fractions obtained from FPV $HA_2$ are shown in Table 4. They were deduced from the analytical results as follows.

Fraction $H_9$ clearly originates form the common biosynthetic intermediate $Man_9GlcNAc_2$-polypeptide [14-16]: it co-elutes with the respective $Man_9$GlcNAcOH isomer standard both on HPLC and gel filtration; it contains approximately three terminal, four 2-substituted and two 3,6-disubstituted mannose residues (Table 2) and it yields the expected major acetolysis fragments ManManOH, $Man_2$ManOH, $Man_4$GlcNAcOH, and $Man_7$GlcNAcOH (see Table 3 and arrows in Table 4). By an analogous line of reasoning, the major species in the second main oligomannosidic alditol fraction, $H_8$, is concluded to lack Man-**$D_2$**. This follows especially from the acetolysis results: only small amounts of $Man_2$ManOH, and $Man_6$GlcNAcOH instead of $Man_7$GlcNAcOH were obtained.

Fraction $H_{10}$ contains about one residue each of terminal glucose and of 3-substituted mannose, as well as approximately two residues only of terminal mannose and four of

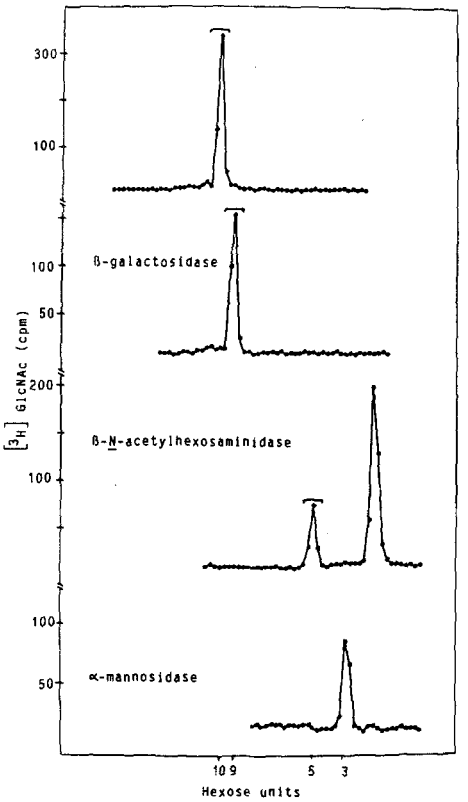



**Figure 4.** Sequential digestion of FPV $HA_2$ oligosaccharide alditol fraction $F_4$ (see Fig. 2C) with exoglycosidases. After passage through a column of Bio-Gel P-4 (top chromatogram), fraction $F_4$ ($7 \times 10^3$ cpm of [$^3$H]GlcNAc) was successively digested with the enzymes indicated. After each step, the products were rechromatographed and the oligomeric species (brackets) were degraded further. Conditions of chromatography as in Fig. 1. Calibration in "hexose units" according to Yamashita *et al.* [34] (1 hexose = 1 unit, 1 *N*-acetylhexosamine = 2 units).

2-substituted mannose. Upon acetolysis, it yields, compared to $H_9$, $Hex_5GlcNAcOH$ instead of $Hex_4GlcNAcOH$, and $Hex_8GlcNAcOH$ instead of $Hex_7GlcNAcOH$. $H_{10}$ is therefore proposed to originate from the trimming precursor of $Man_9GlcNAc_2$-polypeptide [16]. Similarly, *i.e.* especially from the predominance of acetolysis fragments $Man_3GlcNAcOH$ and $Man_5GlcNAcOH$, it may be concluded that the major constituent in fraction $H_7$ is the $Man_7GlcNAcOH$ isomer without Man-**D**$_2$ and Man-**D**$_1$.

As evident from their methylation analyses, fractions $H_6$ and $H_5$ do not consist of high mannose glycans only. These fractions could not be subfractionated to sufficiently pure species and were not further investigated. The amounts available of fraction $D_5$ did not allow methylation analysis; the general trimming pathway of oligomannosidic oligosaccharides [16], the substrate specificity of endo D [18, 36], and the finding that fraction $D_5$ yielded ManOH, ManManOH, $Man_2GlcNAcOH$ and $Man_4GlcNAcOH$ as major

**Table 4.** Structures proposed for the oligomannosidic oligosaccharide alditols in the glycan fractions obtained from the small subunit ($HA_2$) of the hemagglutinin in fowl plague virus (FPV), strain Dutch. The mol % of each glycan in total $HA_2$ is also shown, estimated from the distribution of [$^3$H]Man and of [$^3$H]GlcN radioactivity in the different structures.

| Oligosaccharide alditol fractions[a] | Structure[b,c] | Mol % |
|---|---|---|
| $H_{10}$ | Manα1-2Manα1<br>6<br>Manα1<br>3 \|<br>Manα1-2Manα1 6<br>Manβ1-4GlcNAcOH<br>3<br>Glc1-3Manα1-2Manα1-2Manα1 | 2.3 |
| $H_9$[b] | **$D_3$ B**<br>Manα1-2Manα1<br>6 **4'**<br>Manα1<br>3 \|<br>Manα1-2Manα1 6<br>**$D_2$ A** Manβ1-4GlcNAcOH<br>3 **2**<br>Manα1-2Manα1-2Manα1 **3**<br>**$D_1$ C 4** | 15.3 |
| $H_8$ | Manα1-2Manα1<br>6<br>Manα1<br>3 \|<br>Manα1 6<br>Manβ1-4GlcNAcOH<br>3<br>Manα1-2 Manα1-2Manα1 | 19.1 |
| $H_7$ | Manα1-2Manα1<br>6<br>Manα1<br>3 \|<br>Manα1 6<br>Manβ1-4GlcNAcOH<br>3<br>Manα1-2Manα1 | 7.7 |
| $D_5$ | Manα1<br>6<br>Manα1<br>3 \|<br>Manα1 6<br>Manβ1-4GlcNAcOH<br>3<br>Manα1 | 1.0<br>Total 45.4 |

[a] See Fig. 1D and 1E.

[b] In the structure of $H_9$, the sites of preferential cleavage by acetolysis (arrows), as well as the designation of the sugar residues in oligomanosidic *N*-glycans [35] are also shown.

[c] GlcNAcOH, *N*-acetylglucosaminitol.

**Table 5.** Structures proposed for the (incomplete) *N*-acetyllactosaminic oligosaccharide alditols in the glycan fractions obtained from the small subunit ($HA_2$) of the hemagglutinin in fowl plague virus (FPV), strain Dutch. The mol % of each glycan in total $HA_2$ is also shown.

| Oligosaccharide alditol fraction[a] | Structure[b] | Mol % |
|---|---|---|
| $H_3$, $D_3$ | Manα1<br>6<br>Manβ1-4GlcNAcOH<br>3<br>Manα1 | 2.7 |
| $F_1$, $D_4$ ($H_4$) | Manα1<br>6<br>Manβ1-4GlcNAcOH<br>3<br>GlcNAcβ1-2Manα1<br><br>GlcNAcβ1-2Manα1<br>6<br>Manβ1-4GlcNAcOH<br>3<br>Manα1 | 11.3 |
| $F_2$, $F_3$, $Z_1$ | GlcNAcβ1-2Manα1<br>6<br>Manβ1-4GlcNAc(β1-4GlcNAc)OH<br>3<br>GlcNAcβ1-2Manα1 | 20.9 |
| $F_4$ | **6′ 5′ 4′**<br>Galβ1-4GlcNAcβ1-2Manα1<br>6<br>Manβ1-4GlcNAcOH<br>3 ↑ **2**<br>GlcNAcβ1-2Manα1 **3**<br>**5** **4** | 3.3 |
| $F_{5.1}$, $Z_2$ | **7′**<br>(GlcNAcβ1)$_{0.2\text{-}0.35}$<br>6<br>(Galβ1-4)$_{0.25}$ { Manα1<br>2<br>GlcNAcβ1 \|<br>(Fucα1)$_{0.5}$<br>6 6<br>(GlcNAcβ1-4)$_{0.24\text{-}0.5}$Manβ1-4GlcNAcβ1-4GlcNAcOH<br>**9** 3 **1**<br>GlcNAcβ1-2Manα1 | 5.3 |
| | | Total: 43.5 |

[a] See Fig. 2C and 2D.

[b] The designation of the central and of some additional sugar residues [15] is shown with the structure of $F_4$ and of $F_{5.1}Z_2$, respectively. The glycan produced by the action of PNGase F or by hydrazinolysis include GlcNAc-**1**, those produced by endo F do not.

acetolysis fragments, however, let us assume that it consists of the $Man_5GlcNAcOH$ isomer shown in Table 4. Fractions $H_3$ and $D_3$, finally, are concluded to contain Man$\alpha$1-3(Man$\alpha$1-6)Man$\beta$1-4GlcNAcOH as a main constituent. This follows from their major acetolysis fragments and from the methylation results obtained. This structure cannot be explained by the major trimming pathway of oligomannosidic glycans and is included in Table 5 (see below). Equally unexplained is the liberation of this oligosaccharide by endo H, which is in contradiction to the substrate specificity of this enzyme as reported by Kobata [18].

The structures of the (incomplete) *N*-acetyllactosaminic glycans identified amongst the oligosaccharide alditols from FPV $HA_2$ are summarized in Table 5. The methylation analyses of fractions $F_3$, $F_2$ and $Z_1$ all are consistent with a simple bi-antennary glycan lacking galactoses. In all three cases, about two terminal *N*-acetylglucosamines, two 2-substituted mannoses and one 3,6-disubstituted mannose residue were found. In the case of $F_3$, the major component, this assumption was confirmed by sequential exoglycosidase digestion. $F_3$ and $F_2$ then appear to result from the action of endo F followed by incomplete reduction with $NaBH_4$ (4-substituted GlcNAcOH-**2** or GlcNAc-**2** found, respectively), while $Z_1$ may be due to the incomplete action of endo F on bi-antennary glycopeptides, and/or to the PNGase F resistance of glycosyl asparagines with free amino or carboxyl groups [37]. $Z_1$ was not found to contain fucose or 4,6-disubstituted *N*-acetylglucosaminitol, which is in agreement with the result that no appreciable amounts of fucosylated GlcNAc-peptides could be identified amongst the endo F/PNGase F products (see Fig. 2B and text on methylation results).

Fraction $F_4$ represents a monogalactosylated $F_3$ as evident from the methylation results (one equivalent each of terminal galactose and of terminal and 4-substituted *N*-acetylglucosamine) and from its successive digestion with exoglycosidases (Fig. 4). Since, after its sequential treatment with $\beta$-*N*-acetylhexosaminidase and with $\alpha$-mannosidase, only position 3 of Man-**3** became accessible to methylation (see Table 2; replacement of 3,6-di- by 6-monosubstituted mannose), $F_4$ is concluded to carry Gal-**6'**.

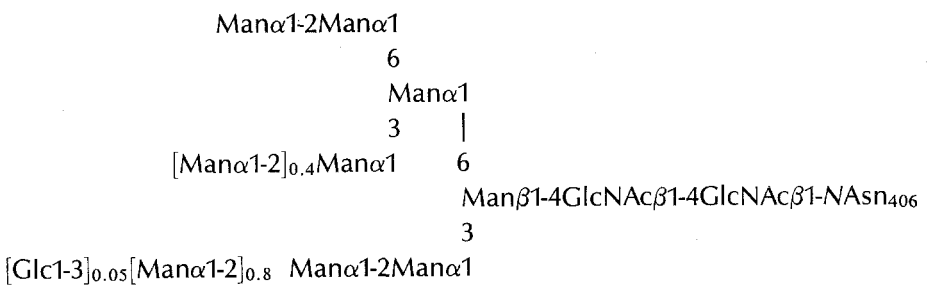
Fractions $F_1$, $H_4$ and $D_4$ yielded the methylation results of an $F_3$ lacking one outer *N*-acetylglucosamine (one equivalent each of terminal mannose, of 2-substituted mannose and of terminal *N*-acetylglucosamine) and this conclusion is corroborated by the exoglycosidase digestion of $F_1$. Since, after exposure of this fraction to $\alpha$-mannosidase alone, positions 3 and 6 of Man-**3** could be methylated in a ratio of 3:7, $F_1$ is concluded to consist of a mixture of oligosaccharides carrying GlcNAc-**5** or **5'**. Because of the substrate specificity of endo D, the latter species must also constitute fraction $D_4$, while the structure of $H_4$ remains somewhat uncertain, since according to Kobata [18], neither isomer should be liberated by endo H. Our results on the GlcNAc-peptides produced also argue against an appreciable fucosylation of GlcNAc-**1** in the original glycosyl asparagines corresponding to $F_4$, $F_1$, $D_4$ and $H_4$.

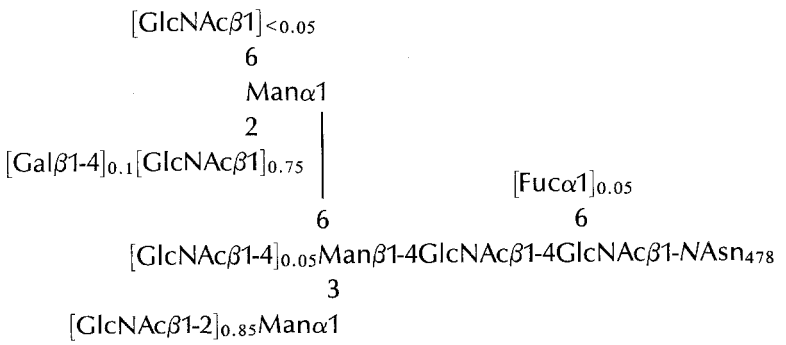
Fractions $F_{5.1}$ and $Z_2$ clearly comprise mixtures of oligosaccharide alditols with the following two additional features: (i) They are intersected or (and) tri-antennary as judged from the occurrence of 3,4,6-trisubstituted and of 2,6-disubstituted mannose, respectively; (ii) they contain about 0.5 equivalents of Fuc$\alpha$1-6 residues at GlcNAc-**1** (see values for terminal fucose and for 4,6-disubstituted *N*-acetylglucosaminitol). The presence of GlcNAc-**1** in $F_{5.1}$ may be ascribed to the resistance of multi-antennary species towards endo F, resulting in predominant cleavage of the corresponding glycosyl peptides by

PNGase F (if their peptide portions were large enough) [37]. As in $F_4$, the 0.25 equivalents of galactose in $Z_2$ also appear to be linked preferentially to GlcNAc-**5'** or **-7'**, since by digestion of this fraction with β-*N*-acetylglucosaminidase and with α-mannosidase, only 6-substituted mannose is formed. These conclusions are summarised in Table 5.

Finally, minority fractions $F_{5.2}$ and $F_6$ characteristically contain additional substituted galactose and 3,4-disubstituted *N*-acetylglucosamine residues. Our results do not allow an identification of the extra substitutents. Since influenza viruses contain a neuraminidase, it cannot be sialic acids; a fucosylation of the antennal *N*-acetylglucosamine residues, however, appears possible.

We have shown earlier [7, 8, 11] that the oligosaccharides substituting Asn-406 and Asn-478 in the small subunits of FPV hemagglutinins mainly belong to the high mannose or to the complex type, respectively. We therefore conclude that the major glycans at these two asparagines in the $HA_2$ from strain Dutch are the following (cf. Tables 4 and 5):




Clearly, the biosynthetic processing [16, 38] of the oligosaccharides at Asn-406 stopped (in the rough endoplasmic reticulum or in the Golgi apparatus of the chick fibroblasts) during the action of trimming α-mannosidases I or, to a small extent, already during that of glucosidase II. The glycans at Asn-478, on the other hand, were processed further, *viz.* mainly until elongation by the Golgi *N*-acetylglucosaminyltransferases I and II, with a few β-Gal-**6'** residues added by the corresponding galactosyltransferase.

A comparison of these data with those previously obtained for the Rostock strain of FPV [8] shows especially that the substituents at Asn-406 in the hemagglutinin of this other strain comprise much less $Man_8GlcNAc_2$, and those at Asn-478 carry distinctly more outer $\beta$-galactose units. This is an interesting finding, because it shows that not all the glycosylation sites in the hemagglutinin of FPV Dutch are processed further than the corresponding ones in the HA of FPV Rostock. It appears that the glycosylation differences between these two similar viral components are, at least in part, due to the few amino acid exchanges [9, 12, 13] and not, as discussed earlier on the basis of carbohydrate analyses of the two $HA_1$ subunits alone [6, 8], only a consequence of the higher replication rate of the Rostock strain.


## Acknowledgements

We thank M. Rosing (growth of virus), W. Mink (methylations) and S. Kühnhardt (GLC-MS) for excellent technical assistance. This project was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47, Teilprojekte A6 and B3; Habilitationsstipendium for H.N.) and by Fonds der Chemischen Industrie (341).


## References


1 Webster RG, Laver WG, Air GM (1983) in Genetics of Influenza Viruses, eds. Palese P, Kingsbury DW, Springer-Verlag, Wien, p 127-68.
2 Ward CW (1981) Curr Top Microbiol Immunol 94:1-74.
3 Wilson IA, Skehel JJ, Wiley DC (1981) Nature 289:366-73.
4 Wiley DC, Wilson IA, Skehel JJ (1981) Nature 289:373-78.
5 Matsumoto A, Yoshima H, Kobata A (1983) Biochemistry 22:188-96.
6 Niemann H, Dabrowski J, Dabrowski U, Geyer R, Keil W, Klenk H-D, Stirm S (1985) Eur J Biochem 146:523-32.
7 Keil W, Niemann H, Schwarz RT, Klenk H-D (1984) Virology 133:77-91.
8 Keil W, Geyer R, Dabrowski J, Dabrowski U, Niemann H, Stirm S, Klenk H-D (1985) EMBO J 4:2711-20.
9 Pritzer E (1985) Diplom thesis, University of Giessen, W. Germany.
10 Wilson IA, Ladner RC, Skehel JJ, Wiley DC (1983) Biochem Soc Trans 11:145-47.
11 Keil W (1982) Dr. rer. nat. thesis, University of Giessen, W. Germany.
12 Porter AG, Barber C, Carey NH, Hallewell RA, Threlfall G, Emtage JS (1979) Nature 282:471-77.
13 Garten W, Kuroda K, Schuy W, Naruse H, Scholtissek C, Klenk H-D (1985) Vaccine 3:227-29.
14 Montreuil J (1982) Comp Biochem Physiol [B] (Part II) 19:1-188.
15 Vliegenthart JFG, Dorland L, van Halbeek H (1983) Adv Carbohydr Chem Biochem 41:209-374.
16 Kornfeld R, Kornfeld S (1985) Annu Rev Biochem 54:631-64.
17 Diabaté S, Geyer R, Stirm S (1983) Arch Virol 76:321-34.
18 Kobata A (1979) Anal Biochem 100:1-14.
19 Diabaté S, Geyer R, Stirm S (1984) Eur J Biochem 139:329-36.

20 Elder JH, Alexander S (1982) Proc Natl Acad Sci USA 79:4540-44.
21 Plummer TH, Elder JH, Alexander S, Phelan AW, Tarentino AL (1984) J Biol Chem 259:10700-4.
22 Li Y-T, Li S-C (1972) Methods Enzymol 28:702-13.
23 Geyer R, Geyer H, Stirm S, Hunsmann G, Schneider J, Dabrowski U, Dabrowski J (1984) Biochemistry 23:5628-37.
24 Takasaki S, Mizuochi T, Kobata A (1982) Methods Enzymol 83:263-68.
25 Bergh MLE, Koppen P, van den Eijnden DH (1981) Carbohydr Res 94:225-29.
26 Geyer R, Geyer H, Hunsmann G, Schneider J, Stirm S (1982) Biochim Biophys Acta 717:491-501.
27 Hakomori S (1964) J Biochem (Tokyo) 55:205-7.
28 Björndal H, Hellerqvist CG, Lindberg B, Svensson S (1970) Angew Chem 82:643-52.
29 Geyer R, Geyer H, Kühnhardt S, Mink W, Stirm S (1982) Anal Biochem 121:263-74.
30 Geyer R, Geyer H, Kühnhardt S, Mink W, Stirm S (1983) Anal Biochem 133:197-207.
31 Kocourek J, Ballou CE (1969) J Bacteriol 100:1175-81.
32 Vijay IK, Perdew GH (1982) FEBS Lett 139:321-24.
33 Conchie J, Hay AJ, Lomax JA (1982) Carbohydr Res 103:129-32.
34 Yamashita K, Mizuochi T, Kobata A (1982) Methods Enzymol 83:105-26.
35 van Halbeek H, Dorland L, Veldink GA, Vliegenthart JFG, Strecker G, Michalski J-C, Montreuil J, Hull WE (1980) FEBS Lett 121:71-77.
36 Mizuochi T, Amano J, Kobata A (1984) J Biochem (Tokyo) 95:1209-13.
37 Tarentino AL, Gomez CM, Plummer TH (1985) Biochemistry 24:4665-71.
38 Schachter H (1986) Biochem Cell Biol 64:163-81.