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Carbohydrates of influenza virus. Structural elucidation of the individual glycans of the FPV hemagglutinin by two-dimensional ^{1}H n.m.r. and methylation analysis

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The structures of the oligosaccharides of the hemagglutinin of fowl plague virus [influenza A/FPV/Rostock/34 (H7N1)] have been elucidated by one- and two-dimensional ^{1}H n.m.r. spectroscopy at 500 MHz and by microscale methylation analysis. N-Glycosidic oligosaccharides of the oligomannosidic (OM) and of the N-acetyllactosaminic type have been found, the latter type comprising biantennary structures, without (A) or with (E) bisecting N-acetylglucosamine, and triantennary (C) structures. Analysis of the tryptic and thermolytic glycopeptides of the hemagglutinin allowed the allocation of these oligosaccharides to the individual glycosylation sites. Each attachment site contained a unique set of oligosaccharides. Asn12 contains predominantly structures C and E which are highly fucosylated. Asn28 contains OM and A structures that lack fucose and sulfate. Asn123 shows A that has incomplete antennae but is highly fucosylated and sulfated. Asn149 has fucosylated A and E. Asn231 shows fucosylated A and E with incomplete antennae. Asn406 has OM oligosaccharides. Asn478 has A and E with little fucose. Localization of the oligosaccharides on the three-dimensional structure of the hemagglutinin revealed that the oligomannosidic glycans are attached to glycosylation sites at which the enzymes responsible for carbohydrate processing do not have proper access. These observations demonstrate that an important structural determinant for the oligosaccharide side chains is the structure of the glycoprotein itself. In addition, evidence was obtained that the rate of glycoprotein synthesis also has an influence on carbohydrate structure.

Key words: hemagglutinin/influenza virus/oligosaccharides/n.m.r.

Introduction

The hemagglutinin of influenza virus, the major viral antigen (Webster *et al.*, 1983) and the initiator of infection at the cellular level (Klenk and Rott, 1980) is of considerable biological interest, and is amongst the best characterized membrane glycoproteins. The amino acid sequence of many hemagglutinin subtypes has been elucidated (Ward, 1981), and its conformation has been studied by X-ray crystallography (Wilson *et al.*, 1981). The hemagglutinin is synthesized as a precursor HA which is cleaved by post-translational limited proteolysis into the amino-terminal fragment HA_1 and the carboxy-terminal fragment HA_2. The hemagglutinin spike is a trimer containing three $HA_{1,2}$ subunits. Each subunit comprises two structurally distinct regions, a rod-like structure composed predominantly of HA_2 and a globular domain formed by HA_1 sitting on top of the stalk (Wilson *et al.*, 1981).

The carbohydrates of the hemagglutinin are attached by N-glycosidic linkages to the protein (Keil *et al.*, 1979) and their biosynthesis follows the general pathways established for this class of glycans (Hubbard and Ivatt, 1981). Chromatographic analysis of metabolically labeled glycopeptides (Schwarz *et al.*, 1977; Collins and Knight, 1978; Nakamura and Compans, 1978; Nakamura *et al.*, 1980) and chemical analysis of constituent sugars (Ward *et al.*, 1981) indicated the presence of N-acetyllactosamine (complex, type I), oligomannosidic (mannose-rich, type II), and hybrid side chains. Recently, the more sophisticated techniques of sequential exoglycosidase digestion, methylation analysis and ^{1}H n.m.r. spectroscopy have been applied to the hemagglutinin carbohydrates (Matsumoto *et al.*, 1983; Niemann *et al.*, 1985). However, the information provided by both of these studies was still limited in that structural analyses were performed on oligosaccharides derived from total glycoproteins rather than from individual glycosylation sites.

We have analyzed the hemagglutinin of the Rostock strain of fowl plague virus [A/FPV/Rostock/34 (H7N1)], which, as indicated by nucleotide sequence analysis (Porter *et al.*, 1979), has seven potential carbohydrate attachment sites. Previously we had obtained evidence by lectin affinity studies that each glycosylation site has a typical set of heterogenous oligosaccharides (Keil *et al.*, 1984). We have now analyzed the hemagglutinin carbohydrates employing ^{1}H n.m.r. spectroscopy, and we have carried out methylation analyses of the glycans derived from the individual glycosylation sites. Using this procedure the structure of the oligosaccharides at each attachment site was established.

Results

^{1}H n.m.r. analysis of the oligosaccharides of the FPV-Rostock hemagglutinin

Mixtures of several 'complex type' oligosaccharides derived from glycoproteins can successfully be analyzed with the aid of two-dimensional proton-proton shift correlated n.m.r. spectroscopy (Geyer *et al.*, 1984; Niemann *et al.*, 1985; see also Battacharyya *et al.*, 1984). We show now that this technique enables one to analyse even more heterogeneous mixtures containing both the complex type and the high-mannose oligosaccharides.

The basic, one-dimensional spectrum of the HA_1 mixture of oligosaccharides has been measured at 300 and 318 K to uncover the signals overlapped by the HDO peak. In spite of a good instrumental resolution at 500 MHz, a number of diagnostic peaks in the H-1 and H-2 region, as well as all of the H-3 peaks, remained unresolved (Figure 1). Nevertheless, we were able to analyse this complex mixture by taking advantage of the increased dispersion of the chemical shift information in the two-dimensional ^{1}H/^{1}H correlated spectrum. The identification of the structures present in the mixture is based on extensive reference data referring to individual compounds that have been collected chiefly by the groups of Vliegenthart (Vliegenthart *et al.*, 1983)

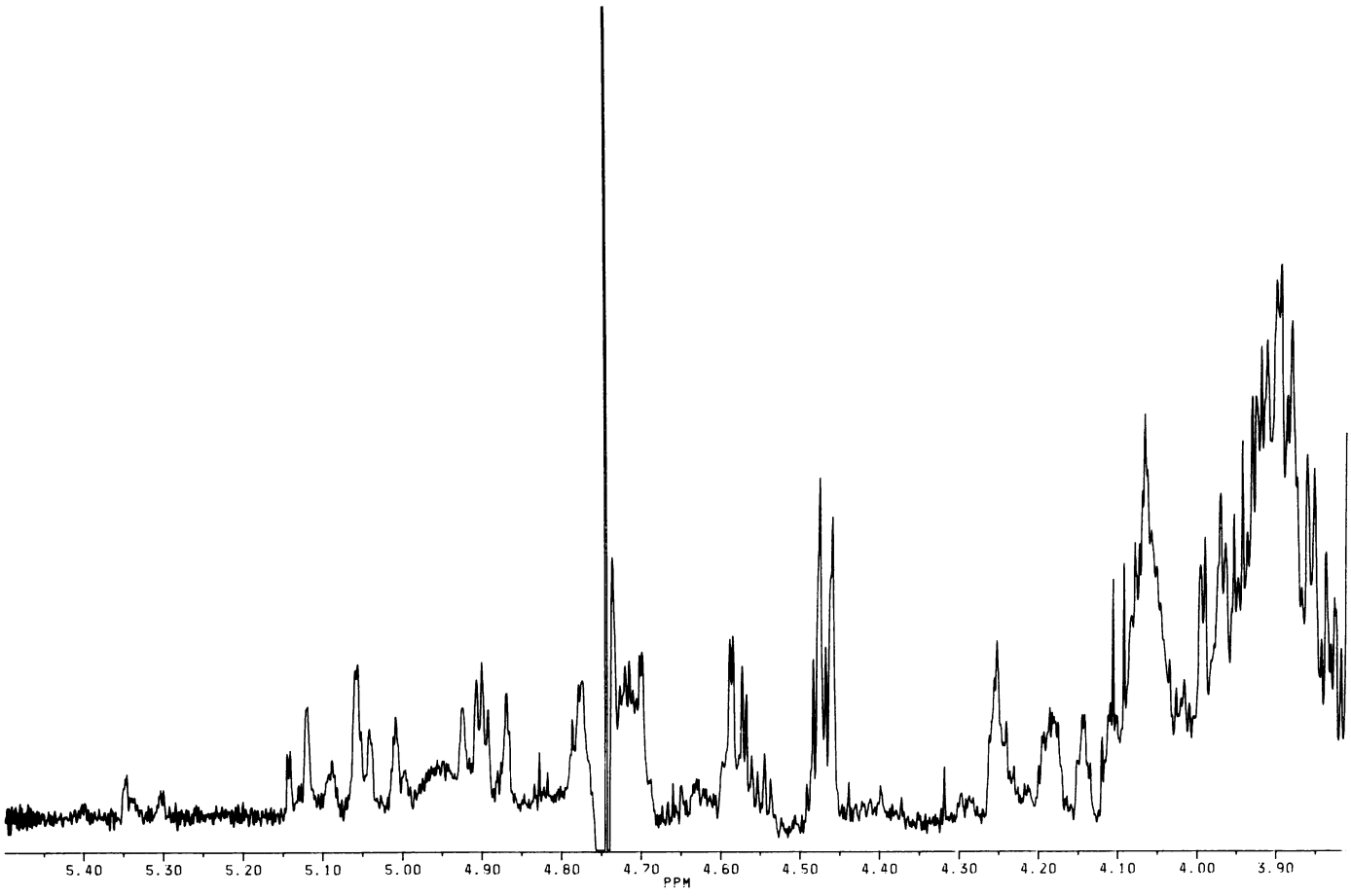

Fig. 1. Resolution enhanced 500 MHz ^{1}H n.m.r. spectrum of subunit HA_1 (mixture of glycans A and E and of oligomannosidic compounds) measured in D_2O at 300 K. HDO peak suppressed by pre-saturation.

and Carver (Carver and Grey, 1981; Carver *et al.*, 1981). The data published by Cohen and Ballou (1980), Ceccarini *et al.* (1983,1984), Bock *et al.* (1982), Homans *et al.* (1984) and Townsend *et al.* (1982), as well as our material concerning the analyses of related mixtures (Geyer *et al.*, 1984; Niemann *et al.*, 1985; Battacharyya *et al.*, 1984), have also been utilized. For defining sugar residues, we adopted Vliegenthart's notation (Vliegenthart *et al.*, 1984), except that we used small letters (a – d) to label the mannose arm residues in high-mannose oligosaccharides, in order to avoid confusion with the capitals referring to oligosaccharides of complex type (Figure 3).

As far as an individual compound is concerned, one is able in most cases to recognize immediately its structure by screening the reference data for a combination of Man H-1 and H-2 resonances that fit its experimental spectrum. Recently, a computer program was developed to facilitate this procedure (Hounsell *et al.*, 1984). A number of GlcNAc, Gal, Fuc and NeuAc signals are also of diagnostic value. Although the analysis of mixtures rests on a similar fitting, a close inspection of Figures 3 and 4 shows that in a signal-rich spectrum there are many ways to combine H-1 and H-2 signals into formally possible but incorrect pairs. It is the connectivity information that reveals the true pairs of coupled protons. In the proton-proton shift correlated spectrum of the HA_1 fraction (Figure 2), the connectivities between scalar-coupled vicinal protons can be read in a routine manner (Geyer *et al.*, 1984; Niemann *et al.*, 1985; Battacharya *et al.*, 1984; Bock *et al.*, 1982; Homans *et al.*, 1984). The ω_1 and ω_2 coordinates of an off-diagonal contour correspond to the chemical shifts of the given two coupled protons. Since the same information follows from the ω'_1, ω'_2 coordinates of another contour, located symmetrically across the diagonal ($\omega'_1 = \omega_2$, $\omega'_2 = \omega_1$), only one of such twin contours has been labelled in Figure 2 for each pair. The values thus obtained are gathered in Table I. Along with the Man H-1 and H-2 chemical shifts, a number of Man H-3, as well as GlcNAc H-1 and H-2, and Gal H-1 - H-4, have been assigned in this way. On the other hand, the contours that would correspond to the H-1/H-2 connectivity for β-Man-3, are absent from this spectrum. The failure to detect them can be explained by the ineffectiveness of the magnetization transfer mediated by the small coupling of ~ 1 Hz. Although the same connectivity has been found for a sample of 20 mM concentration (Homans *et al.*, 1984), it was not observed for solutions whose concentrations, as in the present work, were smaller by order of magnitude (Geyer *et al.*, 1984; Niemann *et al.*, 1985; Battacharyya *et al.*, 1984). However, two pairs of contours: 4.25/3.77 and 4.18/3.87 p.p.m., though showing no connectivity toward the H-1 region, correspond exactly to what is expected for the H-2/H-3 connectivity of β-Man-3 of compounds A and E, respectively (Niemann *et al.*, 1985; Homans *et al.*, 1984). Obviously, magnetization transfer mediated by $^3J_{2,3} \approx 3.5$ Hz was sufficiently effective. By analogy, and in agreement with reported values for H-2 (Vliegenthart *et al.*, 1983; Carver *et al.*, 1981), we tentatively ascribe another pair, showing no coupling with H-1 and located at 4.23/3.74 p.p.m., to H-2 and H-3 of β-Man-3

Table I. Proton chemical shifts (p.p.m.[a] at 300 K in ^2H_2O) for complex oligosaccharide components A and E[b] of hemagglutinin subunits HA_1 and HA_2

Residue[b]	Protons	Component A[b] 6-5-4 / 3-2-1 / 6′-5′-4′ F		Component E[b] 6-5-4 / 9-3-2-1 / 6′-5′-4′ F	
		HA_1	HA_2	HA_1	HA_2
Man-3	H-1	4.775[c]			
	H-2	4.25		4.173	
	H-3	3.77		3.865	
Man-4-	H-1	5.124	5.124	5.062	5.060
	H-2	4.186		4.252	
	H-3	3.895		3.900	
Man-4′	H-1	4.927	4.927	5.008	5.008
	H-2	4.113		4.136	
	H-3	3.87		3.82	
GlcNAc-5,-5′[d]	H-1	4.581	4.584	4.581	4.584
	H-2	3.736		3.736	
GlcNAc-9	H-1	–	–	4.469	4.470
	H-2	–	–	3.687	
Gal-6,-6′	H-1	4.471	4.470	4.471	4.470
	H-2	3.538		3.538	
	H-3	3.66		3.66	
	H-4	3.92		3.92	
Fuc	H-1	4.900	4.894	4.900	4.894
	H-2	3.805		3.805	

[a]Relative to acetone set equal to 2.225 p.p.m.
[b]The shorthand notation corresponds to the numbering given in Figure 3 but F denotes Fuc (1-- 6).
[c]Measured at 318 K.
[d]Values for terminal GlcNAc-5,-5′ residues in incomplete structures: 4.55 p.p.m. (H-1) and 3.72 p.p.m. (H-2).

of oligomannosidic structures.

For a full analysis of a mixture of oligosaccharides, the connectivity information, obtained from a two-dimensional spectrum or by other means, should be combined with integration of the one-dimensional spectrum (Geyer *et al.*, 1984; Niemann *et al.*, 1985; Battacharyya *et al.*, 1984). Concerning the oligosaccharides of complex type, the presence of compounds A and E is satisfactorily documented (Figure 2 and Table I). As for component C, inspection of Figure 3 shows that its H-1 and H-2 signals of Man-3 and -4 are expected to coincide with those of compound A. At the same time, a potentially diagnostic correlation between H-1 and H-2 of Man-4′ (4.87/4.09 p.p.m.), which proved that compound C occurs in the Dutch strain of FPV (Niemann *et al.*, 1985), is absent from the two-dimensional spectrum (Figure 2), whereas a similar correlation for the OM components (4.87/4.14 p.p.m.) is distinctly seen in this spectrum. Although there is a weak signal at 4.55 p.p.m. (Figures 1 and 2), that could be attributed to GlcNAc-7′, i.e., to compound C (Niemann *et al.*, 1985), its integral intensity would require that there be more than 25% of C in the mixture, this being clearly at variance with the results of elementary calculations based on the integrals of the H-1 signals of Man-3 and Man-4 of A, and of Man-4′ of OM, potentially coinciding with the corresponding signals of C (Figure 3). Thus, although some 2,6-disubstituted mannose, corresponding to structure C, has been found by methylation analysis (Table V), there is no n.m.r. evidence of this component.

Returning to the resonance at 4.55 p.p.m., we ascribe it to terminal GlcNAc residues, i.e., to incomplete structures of complex type, in agreement with the results of methylation analysis (Table V) and data of Vliegenthart *et al.* (1985). The H-2 resonance occurs at 3.70 p.p.m. (Figure 2). The alternative assignment of these resonances to GlcNAc-5,-5′ residues of component E, which would be in agreement with the assignment by Homans *et al.* (1984), must be ruled out because it would require that HA_1 contains only 12% of E, whereas a content of 37% follows from the integrals in the region of mannose H-1 resonances.

The total content of oligomannosidic structures can be calculated from the integral of the peak at 4.870 p.p.m. (H-1 of Man-4′ of OM) and the share of A and E can be derived from the integrals of the signals at 5.124 p.p.m. (H-1 of Man-4 of A) and 5.008 p.p.m. (H-1 of Man-4′ of E), respectively. Accordingly, the mole percentage of the components of HA_1 is estimated to amount to 38% A, 37% E and 25% OM. Although, a number of signals could theoretically fit 'hybrid' structures as well, it is concluded that the latter are absent, since the integral of the GlcNAc-5,5′ resonances at 4.55 and 4.58 p.p.m. is exactly twice the integral sum of the Man-4- and Man-4′ signals for A and E just discussed.

A subdivision of the group of high-mannose oligosaccharides is possible to some extent (Table II). Although both the H-1 and H-2 resonances of the terminal mannose residues d_1, d_2 and d_3 may be overlapped, the amount of these residues can be determined from the integrated intensity of the separate H-1 peaks of the subterminal mannose residues c_i, a_i and b_i, respectively. It is not clear whether a_i is present at all, since the relevant weak signal at 5.38 p.p.m. does not correlate with the Man H-2 region, whereas its correlation with a resonance at 3.48 p.p.m. shows that it is due, at least partly, to an impurity. Thus, there is little, if any, d_2 present. The molar fraction of the terminal mannose residue d_1 is 0.4 according to the integral of the c_i signal at 5.307 p.p.m., the integral of the Man-4′ signal of OM at 4.870 p.p.m. being set equal to 1.0. Similarly, a mole fraction of 0.7 for d_3 has been determined from the integral of the b_i peak at 5.141 p.p.m. The share of $c_i + c_t$, which must be equal, by definition, to OM 4_i, amounts to 0.8, based on the integral of the H-1 peak of the latter at 5.347 p.p.m. Since 0.4 was found above for c_i, 0.4 also follows for c_t. It is clear that several incomplete oligomannosidic species are feasible, but because the chemical shifts referring to sugar residues in a given arm are practically unaffected by the structure of the remaining arms, it is hardly possible to define these species.

The HA_2 fraction was available in a small quantity and was only examined by one-dimensional n.m.r. spectroscopy. A, E and OM oligosaccharides were found. The oligomannosidic part was rather homogeneous. Since the integrals of the OM H-1 signals of 4_i (5.334 p.p.m.), a_i (5.045 p.p.m.), b_i (5.140 p.p.m.) and c_i (5.307 p.p.m.) were approximately equal (Table II) and no signals of terminal residues of incomplete structures were visible (data not shown), it can be concluded that the structure depicted in Figure 4, containing the full complement of nine mannose residues, dominates here.

Comparison of the HA_1 oligosaccharides of Dutch and Rostock strains of FPV

In a previous study employing ^1H n.m.r. spectroscopy and methylation analysis, oligosaccharides A, C and E were also found in the HA_1 subunit of the hemagglutinin of the Dutch strain of FPV. The general similarity in the HA_1 oligosaccharides of the Rostock and Dutch strains is underscored by the comparative methylation analysis shown in Table III. The failure to detect 3,6-ManOH indicates that both strains contain neither compound

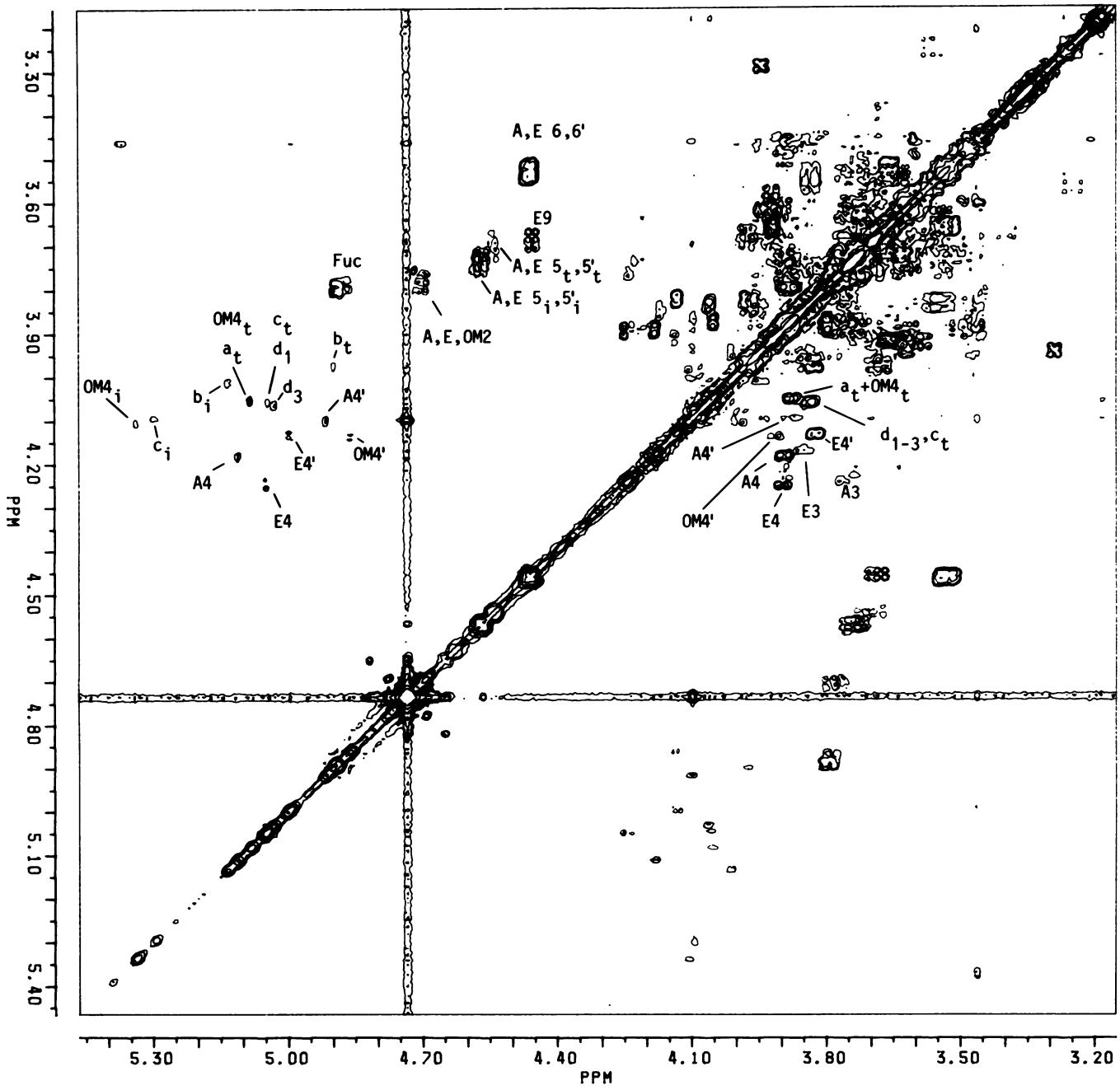

Fig. 2. The 3.15 – 3.45 region of the two-dimensional scalar shift-correlated (COSY) spectrum of subunit HA_1 (mixture of glycans A and E and of oligomannosidic compounds) measured in D_2O at 300 K. Labelling of the correlation contours corresponds to the formulae in Figures 3 and 4, but OM additionally means 'oligomannoside'. In order to avoid crowding, only one of each pair of correlation contours located symmetrically across the diagonal has been labelled. The H-1/H-2 connectivities are labeled in the upper triangle, and the labels for the H-2/H-3 connectivities are placed in the lower one. Correspondingly, the δ_2 coordinate (horizontal scale) of a given contour in the upper triangle denotes the H-1 chemical shift and its δ_1 co-ordinate (vertical scale) stands for the H-2 chemical shift; in the lower triangle, δ_2 = δ(H-3) and δ_1 = δ(H-2).

B nor compound D (Figure 3). It is interesting to note, however, that the Rostock strain exhibits an increase in 2,3,4,6-ManOH paralleled by a decrease in 2,3,4,6-GalOH, suggesting that there is a relatively high proportion of incomplete oligosaccharides terminating with mannose residues 4 or 4′ (Figure 3).

Isolation and identification of the tryptic and thermolytic glycopeptides of HA_1 of FPV Rostock

According to the amino acid sequence of the FPV hemagglutinin (Porter *et al.*, 1979), each glycosylation site should be located on a distinct tryptic peptide (Figure 5). Purified HA_1 was therefore digested with trypsin and the cleavage products were pre-fractionated by gel filtration on Biogel P6 (400 mesh) using 0.1 M pyridine acetate buffer, pH 5.0 as eluant. For analytical separations the column was loaded with digests obtained from ~0.8 mg HA_1. The elution pattern comprised five glycopeptide peaks designated I, II, III, IV and V (Figure 6). Preparative runs with 10-fold the amount of material applied to the column were carried out to obtain sufficient material for further analysis of the glycopeptides. Under these conditions, only fractions IV and V were obtained as separate peaks, whereas fractions I, II and III eluted as one peak. To achieve separation of the latter frac-

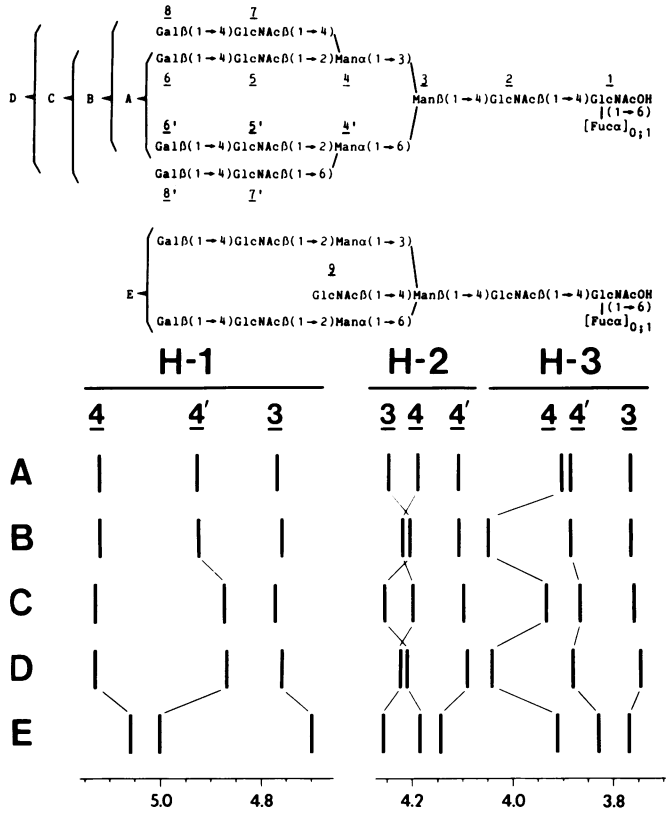

Fig. 3. The general formulae of the oligosaccharide alditols A – E and the expected chemical shifts for their H-1, H-2, and H-3 resonances of mannose residues 3, 4 and 4′, as compiled from the literature (Vliegenthart *et al.*, 1983; Carver *et al.*, 1981; Carver and Grey, 1981) and from our results (Geyer *et al.*, 1984; Niemann *et al.*, 1985; Battacharyya *et al.*, 1984).

tions the following strategy was adopted. Fraction III could be isolated by gel chromatography on Biogel P4 with distilled water as eluant (Figure 7, upper panel). Peak I/II was subjected to h.p.l.c. on LiChrosorb RP-18 which allowed only partial separation of both fractions (Figure 7, middle panel). This material was, therefore, further digested with thermolysin. H.p.l.c. now yielded two clearly separated fractions designated I_{Th} and II_{Th} (Figure 7, lower panel). Fractions IV and V obtained after gel filtration (Figure 6) were also subjected to h.p.l.c., each one yielding one major peak (Figure 8). The small amount of material eluting between fractions 30 and 40 could be converted into the major fraction by re-digestion with trypsin.

The identity of the isolated glycopeptides was established by determining amino acid sequences employing Edman degradation and by metabolic labelling with radioactive glucosamine and specific amino acids. The radioactive amino acids selected for this purpose were cysteine, histidine and tryptophan, which are present in different combinations in each glycopeptide (Figure 5) and therefore provide a suitable system for differential labeling (Figures 6 and 7, middle panel). The data obtained by both procedures are summarized in Table IV. They demonstrate that glycopeptides I and I_{Th} contain glycosylation site Asn231, II and II_{Th} Asn123, III Asn 12, IV Asn28 and V Asn149.

The tryptic glycopeptides of HA_2 containing attachment sites Asn406 and Asn478, respectively, were separated by chromatography on Biogel P6 as described previously (Keil *et al.*, 1984). They were further purified by h.p.l.c. on LiChrosorb RP-18.

Allocation of oligosaccharides to individual glycosylation sites

To analyze the oligosaccharide structures attached to individual

Table II. Proton chemical shifts (p.p.m.[a] at 300 K in ^2H_2O) for oligomannoside components of hemagglutinin subunits HA_1 and HA_2

Residue[b,c]	Protons	d_1-c-4, 3-b, d_2-a, 4, d_3-b HA_1	HA_2
3	H-1	4.775[d]	
	H-2[e]	4.23	
	H-3[e]	3.74	
4_i	H-1	5.347	5.334
	H-2	4.116	
$4'_i$	H-1	4.870	
	H-2	4.141	
	H-3	3.92	
a_i	H-1	5.405[e]	5.392
a_t	H-1	5.093	
	H-2	4.058	
	H-3	3.88	
b_i	H-1	5.141	5.140
	H-2	4.017	
b_t	H-1	4.908	
	H-2	3.979	
c_i	H-1	5.307	5.307
	H-2	4.11	
c_t	H-1	5.058	
	H-2	4.060	
d_1	H-1	5.058	5.060
	H-2	4.060	
d_2			5.060
d_3	H-1	5.044	5.045
	H-2	4.070	

[a]Relative to acetone set equal to 2225 p.p.m.
[b]The shorthand notation corresponds to the numbring given in Figure 4.
[c]Subscript 'i' refers to internal, i.e., glycosylated sugar residues and 't' denotes terminal residues in incomplete structures.
[d]Measured at 318 K.
[e]Tentative assignment.

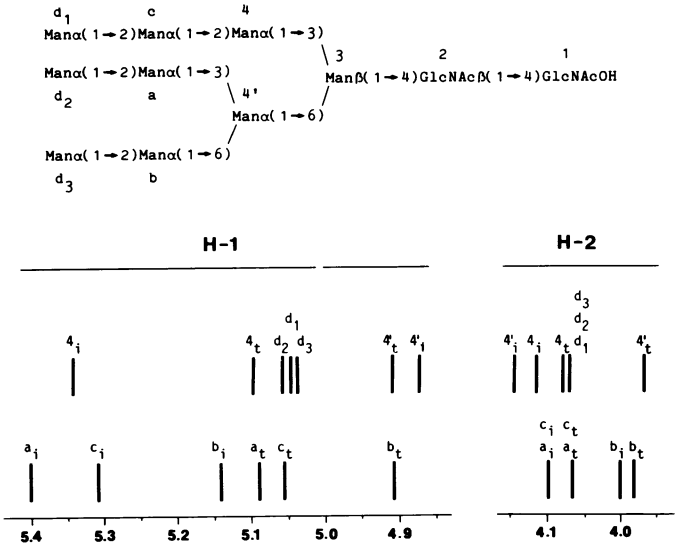

Fig. 4. The formulae of the nonamannosidic oligosaccharide and the expected Man H-1 and H-2 chemical shifts for the complete structure (d_1, d_2, d_3 are present, and a, b and c are in the internal position, as labeled by the subscript i) and for different incomplete structures (terminal units labeled by t). The relevant data have been compiled from the literature (Vliegenthart *et al.*, 1985; Carver *et al.*, 1981; Carver and Grey, 1981; Cohen and Ballou, 1980; Ceccarini *et al.*, 1983,1984).

Table III. Methylation analysis of the complex oligosaccharides of the HA_1 subunits Dutch and Rostock[a] strains of FPV

Peracetate of[b]	FPV/Dutch[c]	FPV/Rostock
2,3,4-FucOH	0.25	0.2
2,3,4,6-ManOH	0.2[d]	0.5[d]
3,4,6-ManOH	1.65[d]	1.4[d]
2,4,6-ManOH	trace	–
3,4-ManOH	0.15[d]	0.1[d]
2,4-ManOH[e]	0.7	0.5
2-ManOH[e]	0.35	0.3
2,3,4,6-GalOH	1.7	0.9
1,3,5,6-GlcN(Me)AcOH[f]	trace	0.1
3,4,6-GlcN(Me)AcOH	0.7	0.9
1,3,5-GlcN(Me)AcOH	0.3	0.2
3,6-GlcN(Me)AcOH	3.4	2.1

[a]HA_1 was exhaustively digested with Pronase, and the major fraction obtained after chromatography on Biogel P-4 comprising 90% of the total complex oligosaccharides (Niemann *et al.*, 1985) was analyzed. Glycopeptides were permethylated and hydrolyzed, and the methylated sugar components were reduced and per-O-acetylated. The partially methylated alditol acetates were analyzed by capillary gas chromatography-mass fragmentography.
[b]2,3,4-FucOH, 2,3,4-tri-O-methylfucitol; 1,3,5,6-GlcN(Me)AcOH, 2-deoxy-2-(N-methyl)acetamido-1,3,5,6-tetra-O-methylglucitol, etc.
[c]FPV/Dutch data from Niemann *et al.* (1985).
[d]Peak ratios based on sum of 2,3,4,6-ManOH, 3,4,6-ManOH, and 3,4-ManOH = 2.0.
[e]Corrected for losses during hydrolysis (for ref. see Niemann *et al.*, 1985).
[f]During alkaline hydrolysis, the polypeptide proximal GlcNAc residue is partially degraded and epimerized, especially when it is not fucosylated (for ref. see Niemann *et al.*, 1985). Therefore, the value for 1,3,5,6-GlcN(Me)AcOH is always too low.

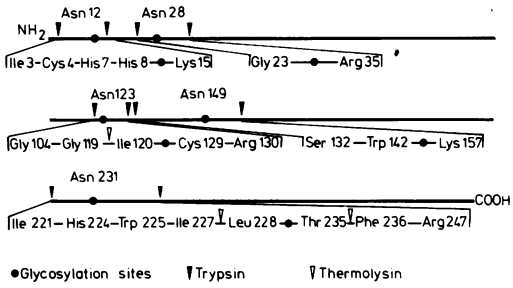

Fig. 5. Map of tryptic and thermolytic glycopeptides of HA_1 of the Rostock strain of FPV. The amino acid sequence is derived from the work of Porter *et al.* (1979). Asparagine residues representing potential glycosylation sites and amino acids at adjacent cleavage sites for trypsin and thermolysin are shown. The positions of cysteine, histidine and tryptophan residues which were radiolabeled to identify the fragments are also indicated.

glysocylation sites, permethylation studies have been carried out on glycopeptides I_{Th}, II_{Th}, III, IV and V and on the tryptic glycopeptides derived from HA_2 containing attachment sites Asn406 and Asn478 (Table V). The table also includes a fraction of the glycopeptide containing Asn28 that was sensitive to endoglucosaminidase H. Oligosaccharides sensitive to this enzyme were also separated from the glycopeptide containing Asn123, but they were obtained in amounts too small to allow permethylation analysis. The glycopeptides containing the individual glycosylation sites were also analyzed for their affinity to concanavalin A and *Lens culinaris* agglutinin (Table VI). By affinity chromatography on concanavalin A, structures of the oligomannosidic type (strong binding) can be discriminated from complex oligosaccharides of type A (weak binding) and types C and E (no binding) (Baenziger and Fiete, 1979; Narasimhan *et al.*, 1979). Binding to *L.*

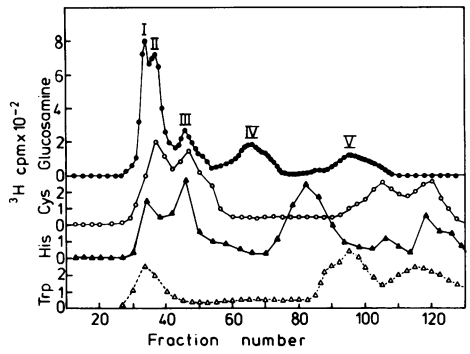

Fig. 6. Identification of the tryptic glycopeptides of HA_1 by differential labeling with radioactive glucosamine and amino acids. Tryptic digests of HA_1 labeled with either [^{3}H]glucosamine, [^{3}H]histidine, [^{3}H]tryptophan or [^{35}S]cysteine were subjected to chromatography on Biogel P6 (400 mesh). The column (30 × 2 cm) was eluted with pyridine-acetate buffer (0.1 M, pH 5.0). 0.7 ml fractions were collected.

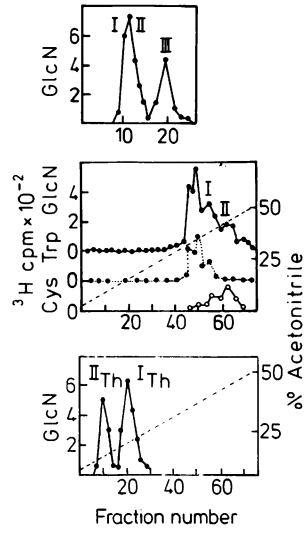

Fig. 7. Purification of glycopeptides I, II and III. **Upper panel:** the combined fractions I, II and III obtained from a [^{3}H] glucosamine-labeled tryptic digest prefractionated on the Biogel P6 column (Figure 6) were subjected to chromatography on a Biogel P4 column (25 × 1 cm). The eluent was distilled water, 1 ml fractions were collected. **Middle panel:** analysis of fractions I/II of the P4 column **(upper panel)** labeled with either [^{3}H]glucosamine, [^{3}H]tryptophan or [^{3}H]cysteine by h.p.l.c. on LiChrosorb RP18 using a 0.1 M ammonium acetate-acetonitrile gradient as eluent. **Lower panel:** the glycopeptides present in peak I/II obtained from the Biogel P4 column **(upper panel)** were digested for 5 h with thermolysin and subjected to h.p.l.c. on LiChrosorb RP18.

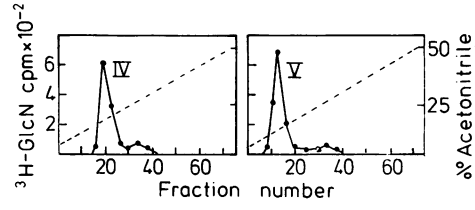

Fig. 8. Purification of glycopeptides IV and V. Fractions IV **(left panel)** and V **(right panel)** obtained from a [^{3}H]glucosamine-labeled tryptic digest pre-fractionated on the Biogel P6 column (Figure 6) were subjected to h.p.l.c.

culinaris agglutinin requires fucosylation of oligosaccharides A, C and E (Kornfeld *et al.*, 1981).

The permethylation and lectin affinity studies allowed the assignment of specific patterns of complex and oligomannosidic oligosaccharides to each attachment site (Table VII). In addition to the basic structures, other features could be evaluated for each set of oligosaccharides. These include the completeness of glycosylation as indicated by the amount of terminal mannose (2,3,4,6-ManOH) and N-acetylglucosamine (3,4,6-GlcN(Me)AcOH) residues, fucosylation as deduced from methylation analysis and affinity to *L. culinaris* agglutinin, and sulfation as indicated by the proportion of charged oligosaccharides (Keil *et al.*, 1984). According to these criteria, Asn12 contains predominantly the complex structures C, and E which are highly fucosylated. The prevalent complex oligosaccharide at Asn28 is of type A. It lacks fucose and sulfate. The endoglucosaminidase H-sensitive fraction obtained from this attachment site contains oligomannosidic oligosaccharides with 6 – 7 mannose residues. Partial trimming of these oligomannosidic structures is not only indicated by the methylation data, but also by the n.m.r. spectra described above. Asn123 contains predominantly A oligosaccharides which are highly incomplete, but readily sulfated and fucosylated. Asn149 has fucosylated A and E oligosaccharides. These oligosaccharides prevail also at Asn231, but here they show a fair degree of incompleteness and heavy sulfation and fucosylation. Asn478 is substituted predominantly with the biantennary oligosaccharide A and, to a smaller extent, with the intersected structure E. Asn406 contains oligomannosidic oligosaccharides. There may be a small amount of other structures in this position which, however, have not been identified yet. Figure 9 shows the h.p.l.c pattern of the oligosaccharides liberated by endoglucosaminidase H from Asn406. The predominant oligosaccharides contains nine mannose residues. Smaller amounts of oligosaccharides with six, seven and eight mannose residues are also present.

Table IV. Identification of the tryptic glycopeptides of HA_1

Fraction	N-terminal amino acid sequence	Differential labeling with			Glycopeptide
		Cys	His	Trp[a]	
I	Ile-Asp-Phe	–	+	+	Ile221-Asn123-Arg247
I_{Th}	Leu-Asp-Pro	–	–	–	Leu228-Asn231-Thr235
II[b]	Gly	+	–	–	Gly104-Asn123-Arg130
II_{Th}	not determined	+	–	–	Ile120-Asn123-Arg130
III	Ile	+	+	–	Ile3-Asn12-Lys15
IV	Gly-Val-Glu-Val	–	–	–	Gly23-Asn28-Arg35
V	not determined	–	–	+	Ser132-Asn149-Lys157

[a]Data on amino acid labeling of the tryptic glycopeptides are derived from the experiments shown in Figure 6 and Figure 7 (middle panel). Data on amino acid labeling of thermolytic glycopeptides A_{Th} and B_{Th} are not shown.
[b]This glycopeptide contains two additional potential tryptic cleavage sites that appear not to be cleaved by the enzyme. The resistance of the peptide bond at Lys110 can be explained by the negative charge at Glu111. The peptide bond at Arg121 presumably remains uncleaved because of steric hindrance by the adjacent oligosaccharide attached to Asn123.

Table V. Methylation analysis of glycopeptides of FPV/Rostock hemagglutinin[a]

Peracetate of[b]	Peak ratios obtained from oligosaccharides linked to							
	Asn12	Asn28 (endo H^-)	Asn28[c] (endo H^+)	Asn123	Asn149	Asn231	Asn406[c]	Asn478
2,3,4-FucOH	0.5	–	–	0.2	0.6	0.25	–	0.2
2,3,4,6-ManOH	0.15[d]	0.2[d]	3.0[e]	0.65[d]	0.25[d]	0.45[d]	3.0[e]	0.25[d]
3,4,6-ManOH	1.55[d]	1.6[d]	1.3	1.25[d]	1.75[d]	1.40[d]	3.6	1.7[d]
2,4,6-ManOH	–	–	0.1	–	–	–	–	–
2,3,4-ManOH	0.1	–	0.2	–	–	–	–	–
3,4-ManOH	0.3[d]	0.2[d]	–	0.1[d]	–	0.15[d]	–	0.05[d]
2,4-ManOH[f]	0.6	0.55	1.3	0.6	0.5	0.4	1.3	0.55
2-ManOH[f]	0.5	0.2	–	0.25	0.6	0.5	–	0.15
2,3,4,6-GalOH	1.55	1.6	0.1	0.9	1.4	1.0	–	1.0
3,4,6-GlcN(Me)AcOH	0.5	0.75	0.1	0.4	0.9	1.05	–	0.25
3,6-GlcN(Me)AcOH	2.35	4.0	1.2	1.4	2.3	2.5	1.0	3.5
3-GlcN(Me)AcOH	0.25	–	–	0.2	0.4	0.25	–	0.1

[a]Individual glycopeptides were permethylated and hydrolyzed, and the methylated sugar components were reduced and per-O-acetylated. The partially methylated alditol acetates were analyzed by capillary gas chromatography-mass fragmentography.
[b]2,3,4-FucOH, 2,3,4-tri-O-methylfucitol; 3,4,5-GlcN(Me)AcOH, 2-deoxy-2-(N-methyl)acetamido-3,4,5-tri-O-methylglucitol, etc.
[c]Only oligosaccharides released by endoglycosidase H without subsequent reduction were analyzed.
[d]Peak ratios based on sum of 2,3,4,6-ManOH, 3,4,6-ManOH, and 3,4-ManOH = 2.0.
[e]Peak ratios based on 2,3,4,6-ManOH = 3.0.
[f]Corrected for losses during hydrolysis (for ref. see Niemann *et al.*, 1985).

Table VI. Lectin affinity of glycopeptides of the hemagglutinin of FPV/Rostock

Lectin	Lectin-bound fraction (%) obtained from glycopeptide containing						
	Asn12	Asn28	Asn123	Asn149	Asn231	Asn406	Asn478
Lens culinaris	90	3	65	85	80	5	50
Con A (weak binding)[a]	30	35	45	40	60	–	75
Con A (strong binding)[b]	–	40	15	–	–	85	–

[a]Elution with 10 mM α-methyl-mannoside.
[b]Elution with 500 mM α-methyl-mannoside.

Table VII. Oligosaccharide structures attached to individual glycosylation sites

Attachment sites	Basic structures				Other features of structures A, C and E		
	A[a]	C[b]	E[c]	OM[d]	Incomplete antennae[e]	Fucosylation[f]	Sulfatation[g]
Asn12	25%	30%	45%	–	15%	90%	25%
Asn28	32%	12%	16%	40%	20%[h]	–	–
Asn123	45%	10%	30%	15%[i]	65%[j]	65%	70%
Asn149	45%	–	55%	–	25%	85%	20%
Asn231	30%	15%	55%	–	45%	80%	80%
Asn406	–	–	–	85%[k]	–	–	–
Asn478	75%	5%	20%	–	25%	50%	20%

[a]Data derived from methylation analyses (Table V). Ratios: 100% minus ratio C minus ratio E minus ratio OM.
[b]Data derived from methylation analyses (Table V). Ratios are based on the proportion of 3,4-ManOH at mannose residue 4′ (half sum of 2,3,4,6-, 3,4,6- and 3,4-ManOH).
[c]Data derived from methylation analyses (Table V). Ratios based on the proportion of 2-ManOH at mannose residue 3 (sum of 2- and 2,4-ManOH).
[d]Percentage derived from concanavalin A binding studies (Table VI).
[e]Based on values for terminal mannose (2,3,4,6-ManOH) in Table V.
[f]Values are derived from binding studies to *Lens culinaris* agglutinin (Table VI).
[g]Values are derived from electrophoretic mobilities of glycopeptides (Keil *et al.*, 1984).
[h]Value applies only to the complex oligosaccharides of this glycosylation site.
[i]Oligomannosidic structure in this fraction have not been substantiated by methylation analysis.
[j]Value has not been corrected for presumably 15% OM oligosaccharides present in this fraction.
[k]This glycosylation site contains, in addition to the OM type, a small fraction (~15%) of unidentified oligosaccharides.

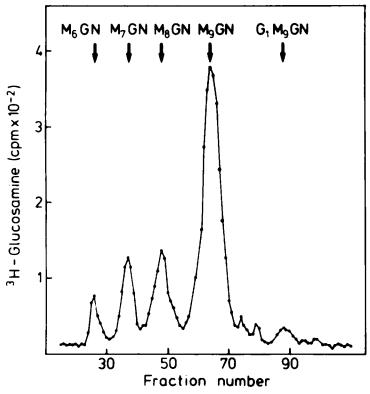

Fig. 9. H.p.l.c. of oligomannosidic glycans released from Asn406 by treatment with glucosaminidase H. Radiolabeled [^{3}H]GlcN-oligosaccharide alditols were separated on LiChrosorb Diol as described in Materials and methods. Arrows indicate elution volumes of $(Glc)_{0-1}(Man)_{6-9}GlcNAcOH$ standards.

Discussion

We have elucidated here the structure of the oligosaccharides attached to the individual glysocylation sites of the hemagglutinin of FPV (Rostock strain) employing primarily ^{1}H n.m.r. spectroscopy and methylation analysis. Only N-glycosidic oligosaccharides have been detected. The glycans of the N-acetyllactosamine type comprised biantennary oligosaccharides without (A) or with (E) bisecting N-acetylglucosamine and, to a lesser extent, triantennary oligosaccharides (C). These structures showed variations in the completeness of the antennae, in the amount of fucose attached to N-acetylglucosamine residue 1, and in the extent of sulfation. There are also oligomannosidic (OM) glycans on both subunits of the FPV hemagglutinin. Previously, we have identified this oligosaccharide type only on HA_2 (Schwarz *et al.*, 1977; Klenk *et al.*, 1981). On HA_1 it has probably escaped detection, because here it is only a minor component of the total carbohydrate. It should be pointed out that none of these carbohydrate structures is unique to the hemagglutinin of influenza virus. They are also present in a large variety of other membrane and secretory glycoproteins (Montreuil, 1982). This observation supports the concept that it is not so much the specific structure of the oligosaccharides that contributes to the antigenic properties of the hemagglutinin, but their position on the polypeptide. Evidence has indeed been obtained that immune recognition of an antigenic site of the hemagglutinin is modulated by the presence or absence of an oligosaccharide (Skehel *et al.*, 1984). It is, therefore, tempting to speculate that the acquisition of oligosaccharides antigenically indifferent to the host is one of the mechanisms underlying antigenic drift and, thus, helps the virus

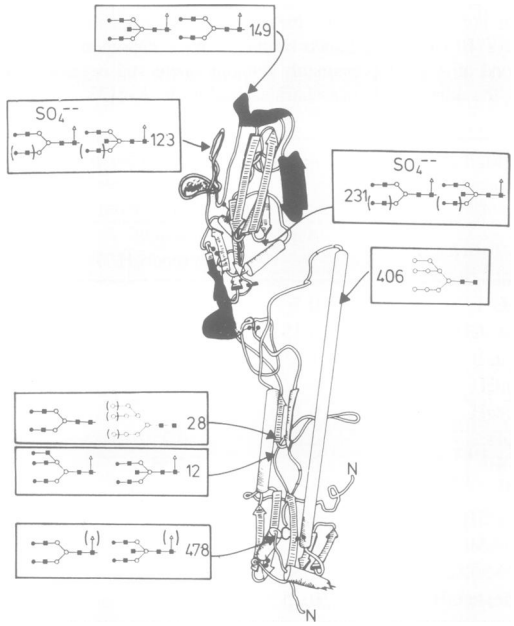

Fig. 10. The positions of the individual glycans on the three-dimensional structure of the hemagglutinin. The model of Wilson *et al.* (1981) is shown. The shaded areas indicate antigenic sites. The glycosylation sites are marked by the sequence numbers of the asparagine residues. Only the oligosaccharides prevalent at each attachment site are shown. ■ = N-Acetylglucosamine, ○ = mannose; ● = galactose; △ = fucose.

to escape the immune defense of the host organism.

The data presented here demonstrate that all potential carbohydrate attachment sites of the hemagglutinin of the Rostock strain of FPV are glycosylated. Our previous failure to detect carbohydrate at Asn231 (Keil *et al.*, 1984) appears to be because the cyanogen bromide fragment containing this attachment site co-migrated with other glycopeptides, mainly with the one containing Asn28. The cyanogen bromide cleavage also provides an explanation for the observation in that study that, compared with the present data, most carbohydrate side chains had a lower affinity to *L. culinaris* agglutinin. It appears that the formic acid treatment employed in that procedure had removed a significant fraction of the fucose residues, thus reducing the binding of the oligosaccharides to this lectin.

The carbohydrate side chains attached to the individual glycosylation sites of the FPV hemagglutinin display a high degree of structural specificity, despite some microheterogenicity in each position. Similar results have been obtained with human IgD (Mellis and Baenziger, 1983), the murine major histocompatibility antigen (Swiedler *et al.*, 1983), Sindbis virus glycoproteins (Hsieh *et al.*, 1983) and the glycoproteins of Friend murine leukemia virus (Schlüter *et al.*, 1984,1985). The FPV hemagglutinin, however, is the only glycoprotein where the individual carbohydrate side chains can be localized on the three-dimensional structure of the molecule. This is shown in Figure 10. We have previously pointed out that the oligomannosidic side chain at Asn406 of the FPV hemagglutinin and the oligomannosidic glycans of the H3 hemagglutinin are located in niches at interfaces between different domains of the glycoprotein that may prevent proper access of the enzymes responsible for trimming and re-elongation of the oligosaccharides (Keil *et al.*, 1984). The presence of oligomannosidic structures as Asn28 of the FPV hemagglutinin can also be explained by steric hindrance. It appears that, in this case, the adjacent oligosaccharides at Asn12 interferes with the processing enzymes. These observations demonstrate that an important structural determinant for the oligosaccharide side chains is the structure of the glycoprotein itself. Since each glycosylation site has its own unique environment on the hemagglutinin, none of the carbohydrate side chains should be exactly like the other, as has indeed been observed in the present study. This concept may explain also, at least in part, the variations in the oligosaccharide pattern of the FPV and the H3 hemagglutinins (Matsumoto *et al.*, 1983) which carry most of their carbohydrate side chains at different positions.

Our data also provide, on the other hand, evidence for additional factors that may have an influence on the structure of the carbohydrate side chains. Comparison of the Rostock and Dutch strains of FPV reveals, despite similar glycan patterns in both hemagglutinins, a high proportion of incomplete oligosaccharides with the Rostock strain. Both proteins have closely related amino acid sequences and their carbohydrates attached in identical positions, except for one glycosylation site that is missing in the hemagglutinin of the Dutch strain (H.-D. Klenk, R.T. Schwarz, E. Pritzer and W. Garten, unpublished results). The Rostock strain, however, has a higher replication rate in chick embryo (CE) cells and grows to titers about four times higher than the Dutch strain. It is therefore unlikely that the protein structure of the hemagglutinin is responsible for the variations in the completenes of the oligosaccharides. It appears rather that, in the case of the Rostock strain, glycosylation cannot fully cope with the rapid transit of the hemagglutinin through the Golgi apparatus. Thus, there is evidence that the rate of hemagglutinin biosynthesis is also a determinant of the structure of the carbohydrate side chains.

Materials and methods

Virus

Unless stated otherwise the Rostock strain of FPV [A/FPV/Rostock/34 (H7N1)] has been used. Seed stocks were grown in the allantoic cavity of 11-day-old embryonated eggs and were stored as allantoic fluids at −80°C. Virus used for carbohydrate analyses was grown in primary cultures of CE cells and purified from the culture medium by centrifugation on sucrose density gradients as described previously (Schwarz *et al.*, 1977).

Metabolic labeling of the virus

When radioactively labeled virus was needed, isotopes were added to the culture medium at the following concentrations: [6-^{3}H]glucosamine 2 μCi/ml, [5-^{3}H]tryptophan 2 μCi/ml, [2,5-^{3}H]histidine 2 μCi/ml, [^{35}S]cysteine 5 μCi/ml, and [^{35}S]sulfate 100 μCi/ml. The composition of the medium used in the labeling experiments has been described previously (Keil *et al.*, 1984).

Isolation of the hemagglutinin subunits HA_1 and HA_2

HA_1 and HA_2 were isolated from purified virus by preparative polyacrylamide gel electrophoresis in SDS essentially as described previously (Nagai and Klenk, 1977). In a typical experiment, virus samples obtained from 160 Petri dishes (14 cm diameter) were separated on 10 column gels (120 × 7 mm) to yield ~1.6 mg HA_1 and 0.8 mg HA_2. Carbohydrate analyses were carried out on pooled glycoprotein samples containing ~20 mg HA_1 and 10 mg HA_2 that were obtained from a total of ~2000 Petri dishes.

Digestion with trypsin and thermolysin

Pooled glycoprotein samples were dissolved in 4−5 ml 0.25 M Tris-HCl buffer, pH 7.8, containing 0.5% SDS and incubated with 1.0 trypsin for 5 h at 37°C. In a similar fashion, tryptic glycopeptides of HA_1 were further degraded with thermolysin. The enzymes were pre-digested for 1 h at 37°C to eliminate contaminating glycosidases.

Fractionation of glycopeptides

Glycopeptides obtained after trypsin digestion were separated on Biogel P6 (400 mesh) columns using 0.1 M pyridine acetate buffer, pH 5.0, or distilled water as eluants (Keil *et al.*, 1984). Fractions of 0.74−1.0 ml were collected. The final step in the purification of tryptic and thermolytic glycopeptides involved h.p.l.c. which was performed on a Lichrosorb RP18 column using a 0−50% gradient of acetonitrile in 0.1 M ammonium acetate as eluant (Schlüter *et al.*, 1984). The flow-rates were 1.1 ml/min. 0.7 ml fractions were collected.

Analysis of amino-terminal sequences

Amino termini of peptides were analyzed by the microsequencing method developed by Chang *et al.* (1978) using the colored Edman reagent 4-N,N-dimethylaminoazobenzene-4′-isothiocyanate (DABITC). The colored 4-N,N-dimethylaminoazo-benzene-4′-thio-hydantoine amino acids (DABTH-amino acids) were identified by two-dimensional chromatography on polyacrylamide gels (Schuy *et al.*, 1984).

Liberation and fractionation of HA_1 and HA_2 glycans for n.m.r. and methylation analysis

The procedure has been described in detail elsewhere (Niemann *et al.*, 1985). Briefly, after Pronase digestion of HA_1 and HA_2, oligosaccharides were liberated as alditols by hydrolysis in 2 N aqueous NaOH containing 2 M $NaBH_4$ (4 h at 100°C). After re-N-acetylation and de-O-acetylation the oligosaccharide alditols were purified by filtration through Biogel P-4. 10 mg of each HA_1 and HA_2 yielded ~1.0 and 0.5 mg oligosaccharides, respectively, as determined by gas chromatographic analysis.

Digestion with endo-β-N-acetylglucosaminidase H

Digestion of hemagglutinin subunits and of glycopeptids was carried out as described previously (Keil *et al.*, 1984).

^1H n.m.r. spectroscopy

Prior to measurements HA_1 and HA_2 fractions were exchanged with D_2O with intermediate lyophilisation and then dissolved in 0.3 ml of D_2O containing a trace of acetone, which was used as internal reference (δ2.225). The spectra were obtained at 500 MHz on a Brucker WM-500 spectrometer. A two-dimensional scalar shift-correlated spectrum (COSY, 90°-t-90° pulse sequence) of fraction HA_1 was measured at 300 K. A total of 256 free induction decays was aquired, each with 176 transients. One zero-filling in the F_1 dimension and a magnitude calculation produced a 512 × 512 data matrix with digital resolution of 2.7 Hz/point.

H.p.l.c. separation of oligomannosidic oligosaccharide alditols

A Waters (Milford, MA) model 6000 A pump and an U6K injection system were

used. The radiolabeled oligosaccharide alditols were chromatographed at 7 – 10 MPa through a column (0.4 × 15 cm) of LiChrosorb Diol (5 μm; Merck, Darmstadt, FRG) using acetonitrile/water (75/25, v/v; 0.5 ml/min) as an eluant. Fractions of 0.5 ml were collected and monitored for radioactivity.

Lectin affinity chromatography

Affinity chromatography of glycopeptides was carried out on concanavalin A and *L. culinaris* agglutinin bound to Sepharose 4B essentially as described previously (Keil *et al.*, 1984).

Methylation analysis

Isolated glycopeptides or oligosaccharides were permethylated according to Hakomori (1964), and the partially methylated alditol acetates obtained after hydrolysis were analyzed by capillary gas chromatography-mass fragmentography at a microscale as described in detail elsewhere (Geyer *et al.*, 1982,1983).

Chemicals and isotopes

Biogel P6 (400 mesh) was from Bio-Rad (München, FRG). Endo-β-acetylglucosaminidase H was from Miles (Frankfurt, FRG). *L. culinaris* agglutinin Sepharose 4B and Concanavalin A-Sepharose 4B were from Deutsche Pharmacia (Freiburg, FRG). Thermolysin and TPCK-trypsin were from Serva (Heidelbrg, FRG) LiChrosorb RP18 was from Merck, (Darmstadt, FRG). All radioactive compounds were from Amersham Buchler (Braunschweig, FRG). These included D-[6-^{3}H]glucosamine-HCl (10 Ci/mmol), [^{35}S]sulfate (25 Ci/mg), L-[^{35}S]cysteine (28 mCi/mmol), L-[5-^{3}H]tryptophan (30 Ci/mmol) and L-[2,5-^{3}H]histidine (50 mCi/mmol).

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