THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 26, Issue of November 15, pp. 13984-13988, 1985
Printed in U.S.A.

Primary Structure of the Low-molecular-weight Carbohydrate Chains of *Helix pomatia* α-Hemocyanin

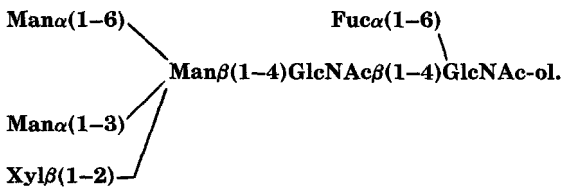
XYLOSE AS A CONSTITUENT OF *N*-LINKED OLIGOSACCHARIDES IN AN ANIMAL GLYCOPROTEIN*

(Received for publication, April 1, 1985)

J. Albert van Kuik, Herman van Halbeek, Johannis P. Kamerling, and Johannes F. G. Vliegenthart

From the Department of Bio-Organic Chemistry, State University of Utrecht, Croesestraat 79, NL-3522 AD Utrecht, The Netherlands

α-Hemocyanin of *Helix pomatia* is a copper-containing glycoprotein which serves as an oxygen carrier in the hemolymph. Its carbohydrate moiety has as constituents fucose, xylose, 3-*O*-methylgalactose, mannose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine residues. Alkaline borohydride did not split off any carbohydrate material, suggesting the absence of *O*-glycosidic chains. The *N*-glycosidic carbohydrate chains of this glycoprotein were liberated by hydrazinolysis of a Pronase digest then fractionated as alditols on Bio-Gel P-4. The fractions containing the low-molecular-weight glycans were investigated by 500-MHz ^{1}H NMR spectroscopy in conjunction with sugar and methylation analysis. The largest, and most abundant, compound was established to be:



Another compound was characterized as the afuco analogue of this structure. *H. pomatia* α-hemocyanin is the first example of an animal glycoprotein having xylose as a constituent of *N*-glycosidic carbohydrate chains.

Hemocyanins are copper-containing glycoproteins which serve as oxygen carriers in some Arthropoda and Mollusca (1). Concerning land and freshwater gastropods, *Helix pomatia* and *Lymnaea stagnalis* are so far the only examples of animal species having glycoproteins with partially *O*-methylated carbohydrate chains (2). The origin of the *O*-methylated sugars is not dietary, as it has been found that *L. stagnalis* can methylate the carbohydrate moiety of its hemocyanin (3). *O*-Methylated sugars have been found earlier as constituents of polysaccharides (4–6) and glycolipids (7). Recently, it has been reported that 3-*O*-methylation of mannose occurs in the processing of high mannose type oligosaccharides in the fungus *Mucor rouxii* (8).

The α-hemocyanin isolated from the hemolymph of *H. pomatia* has a molecular mass of 9×10^6 Da. Its carbohydrate content is 9% (w/w) (9). Sugar analysis using GLC-MS[1] of this material revealed the presence of Fuc, Xyl, 3-*O*-methyl-Gal, Man, Gal, GalNAc, and GlcNAc residues (2, 10). In the present study the isolation of the carbohydrate chains of *H. pomatia* α-hemocyanin will be reported. The chains appeared to be rather heterogeneous. By employment of ^{1}H NMR spectroscopy at 500 MHz, in conjunction with sugar and methylation analysis, the low-molecular-weight structures could be elucidated.

EXPERIMENTAL PROCEDURES

General Data on α-Hemocyanin of H. pomatia—α-Hemocyanin was a gift from Dr. R. Torensma, State University of Groningen, The Netherlands. For storage it was dialyzed at room temperature against 10 mM potassium acetate (pH 5.7) and then lyophilized in the presence of a 2.75 excess of sucrose over glycoprotein (w/w). The lyophilized protein was stored at −20 °C (11).

To check the purity of the glycoprotein material, a small portion of sucrose-stabilized α-hemocyanin was dialyzed for 48 h at room temperature against 1 mM phosphate buffer, pH 7.0, to remove sucrose, and subsequently subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel according to Ref. 12. The following molecular-weight markers were used: myosin (200 kDa), β-galactosidase (166 kDa), phosphorylase *b* (93 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa) (Bio-Rad).

Preparation of Copper-free, Denatured Hemocyanin—To prepare a suitable starting material, the glycoprotein was dialyzed against a number of solutions at 4 °C. The lyophilized glycoprotein/sucrose mixture (2 g corresponding to 533 mg of α-hemocyanin) was taken up in 10 ml of 50 mM Tris/HCl buffer (I = 0.1) pH 7.0, and dialyzed for 48 h against 2 l of the same buffer, with three intermediate changes. Subsequently, copper was removed from the protein by dialysis for 24 h against 2 l of 50 mM Tris/HCl buffer (I = 0.1) pH 8.0, containing 10 mM $CaCl_2$ and 10 mM KCN, with three intermediate changes (13). The resulting colorless, opalescent solution was dialyzed for 24 h against 2 l of 50 mM Tris/HCl buffer (I = 0.1) pH 7.0, with one intermediate change. The apohemocyanin thus obtained was dialyzed for 3 h against 2 l of 100 mM $NaHCO_3$ followed by dialysis for 2.5 h against 2 l of 6 M urea, yielding a clear solution (9). Finally, the solution was dialyzed for 48 h against 2 l of 100 mM ammonium acetate buffer, pH 8.0, with three intermediate changes.

Pronase Digestion—To inactivate possible contaminating glycosidases, Pronase (80 mg) from *Streptomyces griseus* (Boehringer Mannheim, Federal Republic of Germany) was dissolved in 10 ml of 100

* This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), and by the Netherlands Foundation for Cancer Research (KWF, Grant UUKC 83-13). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: GLC-MS, gas-liquid chromatography/mass spectrometry; Fuc, fucose; Xyl, xylose; 3-*O*-methyl-Gal, 3-*O*-methylgalactose; GalNAc, *N*-acetylgalactosamine; NOE, nuclear Overhauser enhancement; DANTE, delays alternated with nutation for tailored excitation; SDS, sodium dodecyl sulfate; l, liter.

mM ammonium acetate buffer, pH 8.0, containing 15 mM $CaCl_2$, and kept for 1 h at 40 °C. Aliquots (2.5 ml) of the Pronase solution (stored at 4 °C until use) were added to the denatured apohemocyanin, dissolved in 20 ml of the same buffer, at 0, 4, 24, and 48 h. The incubation was carried out for 70 h at 40 °C keeping the pH between 7.4 and 8.0. To inactivate proteolytic activity, the solution was kept for 2 min at 100 °C, and, after concentration, passed through a Bio-Gel P-6 column (200–400 mesh, Bio-Rad, 2 × 100 cm) using water as eluent (16 ml/h, 8-ml fractions). The orcinol/H_2SO_4 positive fractions were pooled and lyophilized. The residue was taken up in 20 ml of 100 mM ammonium acetate buffer, pH 8.0, containing 15 mM $CaCl_2$, and subjected to a second Pronase digestion followed by fractionation. The final glycopeptide mixture was lyophilized and dried *in vacuo* over P_2O_5.

Hydrazinolysis Procedure and Fractionation—The thoroughly dried glycopeptide (80 mg) was suspended in 1 ml of anhydrous hydrazine and heated for 8 h at 100 °C. After evaporation of hydrazine, the material was *N*-reacetylated and reduced as described (14), yielding 40 mg of carbohydrate material. For reduction with ^{3}H-labeled $NaBH_4$, 1 mg of the sample was dissolved in 200 μl of 0.08 M NaOH and treated with $NaBH_4$ containing 1.7 mCi NaB^3H_4 in 200 μl of *N,N*-dimethyl formamide (specific activity, 341 mCi/mmol; New England Nuclear). The remaining part was reduced with NaB^2H_4. To facilitate the detection of ^{2}H-labeled oligosaccharide-alditols during the purification and fractionation procedures, 0.30 μCi (8%) of the ^{3}H-labeled oligosaccharide-alditols were added. Paper electrophoresis (Whatman 3MM paper, 4 kV) was carried out using a pyridine/acetic acid/water buffer (3:1:387; v/v), pH 5.4. The oligosaccharide-alditols were recovered from the paper by elution with water.

The neutral oligosaccharide-alditols were fractionated on two connected Bio-Gel P-4 columns (2 × 100 cm each; −400 mesh; Bio-Rad) eluted with water (30 ml/h, 2.5-ml fractions) at 55 °C (15). Oligosaccharide-alditols were monitored by refractive index detection and scintillation counting.

Alkaline Borohydride Treatment—A portion of the Pronase digest (110 μg) was treated with 1 ml of 0.1 M NaOH containing 40 mg of NaB^2H_4. After 20 h at 40 °C the solution was acidified to pH 5.0 with 4 M acetic acid and applied to a column (0.5 × 5 cm) of Dowex 50W-X8, H^+ form (100–200 mesh). The column was washed with 45 ml of water and the eluate was lyophilized. Boric acid was removed by co-evaporation with methanol under reduced pressure (16).

500-MHz ^{1}H NMR Spectroscopy—Oligosaccharide-alditols were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich) with intermediate lyophilization. ^{1}H NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier transform mode at a probe temperature of 27 °C. NOE difference spectroscopy was performed according to Ref. 17 in combination with a DANTE pulse sequence for selective suppression of the HO^2H-line (18). Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation (19). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ = 2.225 ppm) (20).

Carbohydrate Analysis—Samples containing 50 nmol of carbohydrate were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by gas-liquid chromatography of the trimethylsilylated (*N*-reacetylated) methylglycosides on a capillary CPsil5 WCOT fused silica column (0.34 mm × 25 m, Chrompack, Middelburg, The Netherlands) (21).

Methylation Analysis—Methylation analysis of the oligosaccharide-alditol (500 μg) was performed as described by Endo *et al.* (22). The permethylation of the material was carried out with C^2H_3I. Reference partially methylated alditol acetates were used to establish retention times on a capillary CPsil5 WCOT fused silica column (0.34 mm × 25 m, Chrompack, Middelburg, The Netherlands); oven temperature program was 130–220 °C at 2 °C/min. Combined GLC-MS was performed on a Carlo Erba GC/Kratos MS 80/Kratos DS 55 system; electron energy was 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 μA; ion-source temperature, 225 °C; BP1 capillary WCOT fused silica column (0.33 mm × 25 m; Scientific Glass Engineering, Ringwood, Australia); oven temperature program, 130–220 °C at 2 °C/min.

RESULTS

α-Hemocyanin isolated from *H. pomatia* behaved as a single band on SDS-polyacrylamide gel electrophoresis (see Fig. 1). Its sugar analysis is included in Table I. For structural analysis, the *N*-linked carbohydrate chains were released from a Pronase digest of the glycoprotein (sugar analysis, Table I) by hydrazinolysis. After *N*-reacetylation and reduction (sugar analysis, Table I) high-voltage paper electrophoresis showed that the resulting mixture of oligosaccharide-alditols consists of mainly neutral material. Only a small amount (<2%) of acidic material was observed.

The fractionation of the mixture of neutral oligosaccharide-alditols on Bio-Gel P-4 yielded five fractions denoted I–V (Fig. 2). The relative amounts and carbohydrate compositions of the fractions are given in Table I. It is worthy to note that the low-molecular-weight carbohydrate fractions IV and V contain Xyl in addition to the common core residues. In the high-molecular-weight fractions I, II, and III, besides Xyl and the common core residues, also 3-*O*-methyl-Gal, Gal, and GalNAc are found. Alkaline borohydride treatment of the Pronase digest did not give rise to the formation of GalNAc-ol; therefore, it is suggested that the glycoprotein does not

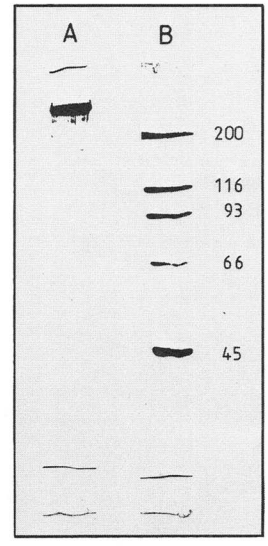


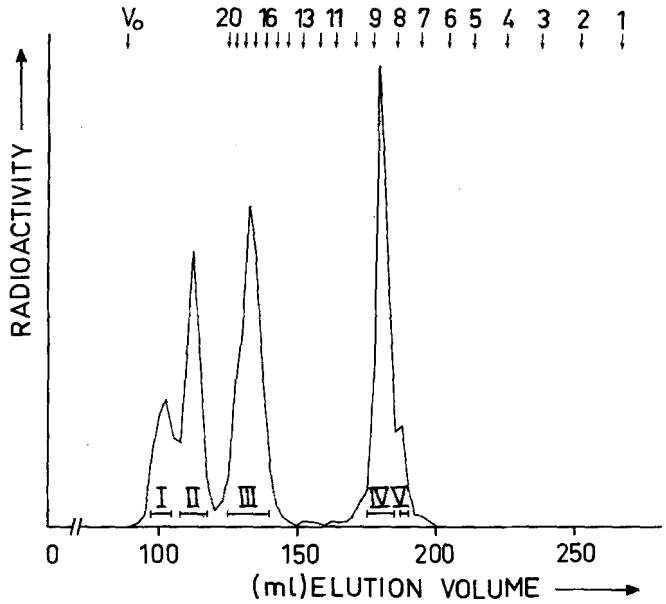
FIG. 1. **SDS-polyacrylamide gel electrophoresis (12) of *H. pomatia* α-hemocyanin after dialysis against 1 mM phosphate buffer, pH 7.0 (*lane A*).** The markers used (*lane B*) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (93 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). The bands were stained with Coomassie Brilliant Blue.

TABLE I

Molar carbohydrate composition of α-hemocyanin from Helix pomatia and related fractions

Monosaccharide	Molar ratio							
	Glycoprotein	Pronase digest	Hydrazinolysate	Bio-Gel P-4 fractions				
				I	II	III	IV	V
Ara[a]		0.52	0.15	0.15	0.03	0.09		
Fuc	0.90	0.54	0.43	0.68	0.59	0.51	0.71	0.27
Xyl	0.94	0.72	0.93	0.69	0.78	0.70	0.75	0.70
3-*O*-Methyl-Gal	2.55	2.28	2.79	6.01	3.46	2.01		
Man[b]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Gal	0.52	0.48	0.36	0.90	0.75	0.15		
Glc	0.01		0.12	0.23	0.05	0.04	0.03	
GalNAc	1.04	0.89	0.77	0.98	0.81	0.87		
GlcNAc	1.52	1.71	1.42	1.58	1.35	1.68	0.63	0.63
GlcNAc-ol			0.63	0.30	1.15	0.81	0.80	0.50
Relative amount (%)				16	21	47	13	2

[a] Ara is a contaminant stemming from Pronase.
[b] Man taken as 3.



contain *O*-glycosidically bound carbohydrate chains. The ratio of GalNAc to Man in the hydrazinolysate is the same as the ratio of GalNAc to Man in the Pronase digest (Table I) which corroborates this presumption.

To elucidate the primary structure of the oligosaccharide-alditols present in fractions IV and V, 500-MHz ^1H NMR spectra of the compounds in ^2H_2O were recorded. The expanded, resolution enhanced, structural-reporter-group regions of the spectrum of fraction IV are presented in Fig. 3. Relevant NMR parameters for both fractions are listed in Table II. For reference purposes, the corresponding data for $Man_3GlcNAcGlcNAc$-ol (compound R) (23) have been included. Close inspection of the ^1H NMR data in Table II

FIG. 2. **Elution profile on Bio-Gel P-4 (−400 mesh) of ^3H-labeled oligosaccharide-alditols derived from *H. pomatia* α-hemocyanin.** The column was eluted with bidestilled water at 55 °C. Fractions of 2.5 ml were collected at a flow rate of 30 ml/h and assayed for ^3H radioactivity. Fractions I–V were pooled. The *arrows* at the *top* indicate the elution positions of glucose oligomers generated by a dextran hydrolysis. The *numbers* at the *top* indicate the glucose units.

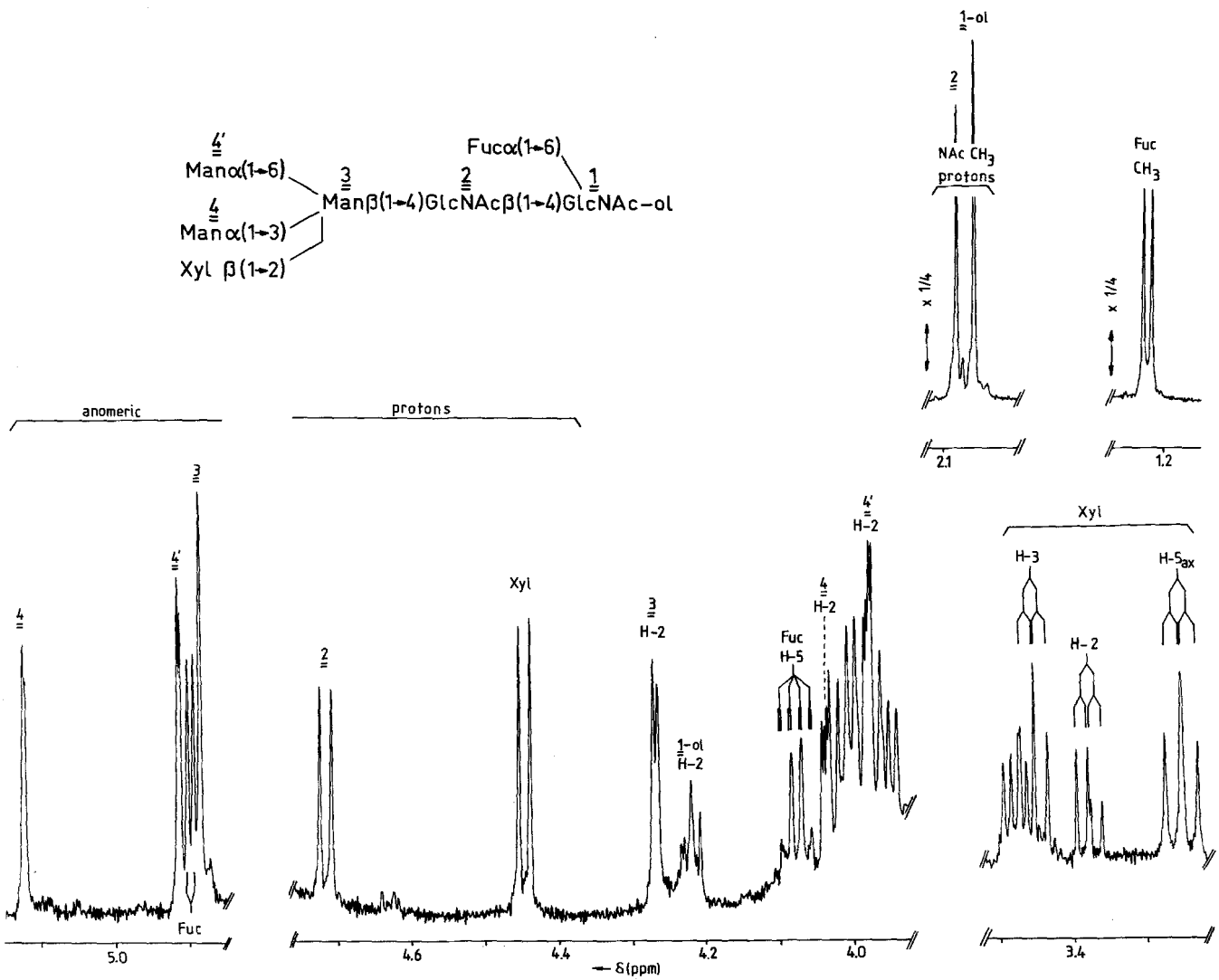
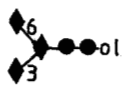
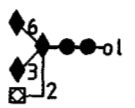
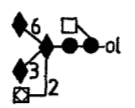


FIG. 3. **Structural-reporter-group regions of the resolution-enhanced 500-MHz ^1H NMR spectrum of oligosaccharide-alditol-1-^2H fraction IV, derived from *H. pomatia* α-hemocyanin recorded in ^2H_2O at 27 °C.** The *numbers* in the spectrum refer to the corresponding residues in the structure. The relative intensity scale of the *N*-acetyl and Fuc CH_3 regions differs from that of the other parts of the spectrum as indicated.

TABLE II

Relevant ^1H NMR parameters of structural reporter groups of constituent monosaccharides for fractions IV and V from the hydrazinolysate of Helix pomatia α-hemocyanin Pronase digest and those for reference compound R

Residue[a]	Reporter group (coupling constant)	Chemical shift (ppm) and coupling constant (Hz) in[b]		
		R	V	IV
				
GlcNAc-ol	H-2	4.244	4.239	4.219
	NAc	2.055	2.057	2.058
GlcNAc 2	H-1 ($J_{1,2}$)	4.637 (8.0)	4.634 (8.0)	4.718 (8.0)
	NAc	2.076	2.073	2.081
Man 3	H-1	4.78	4.883	4.884
	H-2	4.259	4.270	4.270
Man 4	H-1 ($J_{1,2}$)	5.103 (1.9)	5.122 (1.9)	5.124 (1.9)
	H-2	4.067	4.039	4.040
Man 4′	H-1 ($J_{1,2}$)	4.915 (1.8)	4.913 (1.8)	4.914 (1.8)
	H-2	3.974	3.983	3.982
Xyl	H-1 ($J_{1,2}$)		4.449 (7.5)	4.449 (7.7)
	H-2 ($J_{2,3}$)		3.377 (9.2)	3.379 (9.4)
	H-3 ($J_{3,4}$)		3.437 (9.1)	3.453 (9.1)
	H-5_{ax} ($J_{4,5ax}$)		3.250 (10.7)	3.253 (10.5)
	($J_{5ax,5eq}$)		(−11.8)	(−11.6)
Fuc	H-1 ($J_{1,2}$)			4.898 (3.8)
	H-5			4.077
	CH_3 ($J_{5,6}$)			1.225 (6.7)

[a] For numbering of monosaccharide residues and complete structures, see Fig. 4*a*.

[b] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O (27 °C). Compounds are represented by short-hand symbolic notation (20): -●, GlcNAc; -◆, Man; -□, Fuc; -⊠, Xyl.

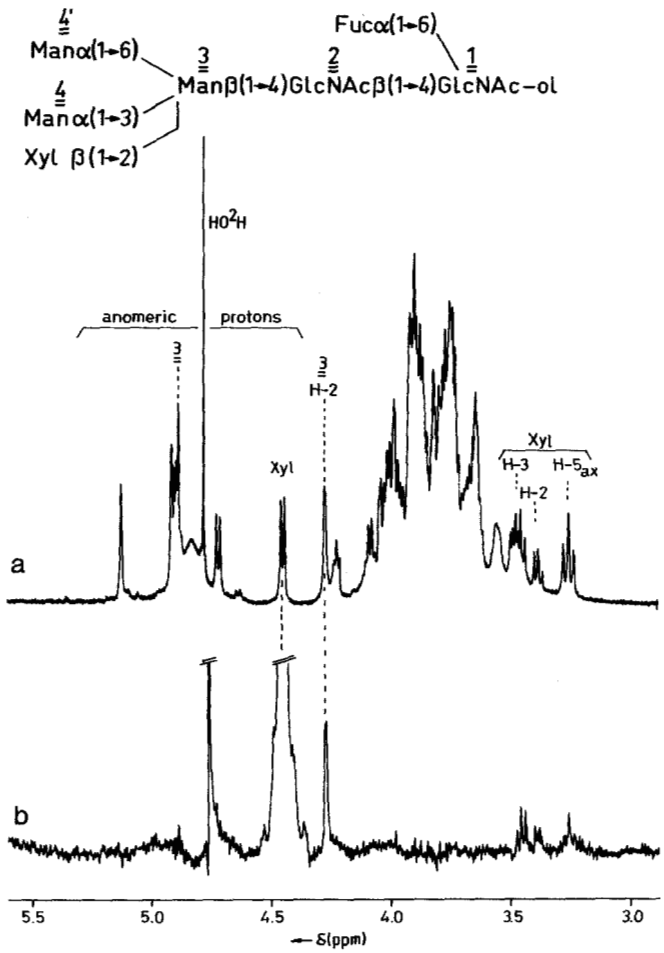


FIG. 4. *a*, Selected region of the 500-MHz ^1H NMR spectrum (^2H_2O at 27 °C) of oligosaccharide-alditol-1-^2H fraction IV, derived from *H. pomatia* α-hemocyanin. The *numbers* in the spectrum refer to the corresponding residues in the structure. *b*, NOE-difference spectrum, with on-resonance irradiation of H-1 of Xyl.

reveals that the compounds in fractions IV and V contain the pentasaccharide-alditol R as fundamental structure. This can be concluded from the chemical shift values of the structural-reporter-group signals known to be typical of the GlcNAcβ(1–4)GlcNAc-ol structural element, together with those of the branching mannotriose core unit. The reporter-group signals for the *N*-acetylchitobiitol unit are the H-2 signal of GlcNAc-ol and the H-1 signal of GlcNAc 2 in combination with two *N*-acetyl signals of equal intensity (Table II) (23, 24). The characteristic patterns of three Man H-1 and three Man H-2 signals (Table II) prove the occurrence of the usual Manα(1–6)[Manα(1–3)]Manβ(1–4) core (20, 23).

The Xyl residue present in fraction IV and V (Table I) is recognized to occur in the pyranose ringform from the ^1H NMR spectra by a set of structural-reporter-group signals, namely, H-1, H-2, H-3, and H-5_{ax}. The chemical shift of the Xyl H-1 doublet (δ = 4.449 ppm), in combination with the coupling constant $J_{1,2}$ (7.7 Hz), are indicative of the β-configuration of its linkage. Assignments of H-2 and H-3 of Xyl are based on sequential, selective ^1H-decoupling experiments on H-1 and H-2, respectively. The signal of H-5_{ax} is readily traced from its triplet-like shape, arising because the absolute value of the geminal coupling constant $^2J_{5ax,5eq}$ (−11.6 Hz) is nearly equal to $J_{4,5ax}$ (10.5 Hz). The characteristic shifts and coupling constants of the Xyl reporter groups (Table II) are in excellent agreement with those reported earlier for Xyl-pβ(1–*O*)Ser (25). When comparing IV or V with R, the apparent extension of the core pentasaccharide with the Xyl residue causes a significant downfield shift of H-1 of Man 3 ($\Delta\delta$ = 0.10 ppm) and also a number of smaller effects on other structural reporters, like H-1 and H-2 of Man 4. It should be noted that, in particular, the positions of H-1 and H-2 of Man 4′ (α(1–6)-linked to Man 3) are hardly affected by attachment of Xyl. These effects suggest that Xyl may be β-linked to C-2 of Man 3. The assignment of the latter glycosidic linkage was further supported by one-dimensional NOE-difference spectroscopy (26, 27). For fraction IV, presaturation of the Xyl H-1 doublet at δ = 4.45 ppm gave rise to a well-pronounced interglycosidic NOE effect on H-2 of Man 3, whereas the effects on other protons (H-1 and H-3) of Man 3 are negligible (see Fig. 4).

The Fuc residue present in fraction IV (Table I) is α(1–6)-linked to GlcNAc-ol as can be deduced from its typical set of structural-reporter-group signals earlier found in an oligosaccharide-alditol obtained from IgM, namely, H-1 (δ = 4.898 ppm; $J_{1,2}$ = 3.8 Hz), H-5 (δ = 4.077 ppm), and CH_3 (δ = 1.225 ppm) (28). The effects of the Fuc in α(1–6)-linkage are expressed by highly characteristic downfield shifts of GlcNAc 2 H-1 ($\Delta\delta$ = 0.08 ppm) and NAc ($\Delta\delta$ = 0.014 ppm). Moreover, H-2 of GlcNAc-ol is shifted slightly upfield in comparison to R ($\Delta\delta$ = −0.02 ppm). Based on the ^1H NMR data, fraction IV contains a single component, the structure of which is proposed to be the following.

TABLE III

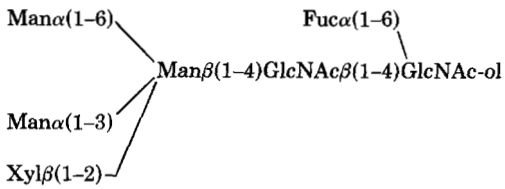
Methylation analysis of the oligosaccharide-alditol in fraction IV of the hydrazinolysate

Partially methylated alditol acetate	Molar ratio	Retention time	
		Fraction IV	Reference
Xylitol			
2,3,4-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	0.2[a]	0.65	0.66
Fucitol			
2,3,4-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	0.3[a]	0.72	0.73
Mannitol			
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)	2.0[b]	1.00[c]	1.00[c]
4-Mono-*O*-methyl-(1,2,3,5,6-penta-*O*-acetyl)	1.3	1.90	1.91
2-*N*-Methylacetamido-2-deoxyglucitol			
1,3,5-Tri-*O*-methyl-(4,6-di-*O*-acetyl)	1.3	1.88	
3,6-Di-*O*-methyl-(1,4,5-tri-*O*-acetyl)	1.2	2.13	2.12

[a] Because of the relatively high volatility of these residues, their values are lower than expected.

[b] 2,3,4,6-Tetra-*O*-methylmannitol taken as 2.0.

[c] The retention time of 2,3,4,6-tetra-*O*-methylmannitol is taken as 1.00 on a capillary CPsil5 WCOT fused silica column (0.34 mm × 25 m, Chrompack; 130–220 °C at 2 °C/min).



This result is confirmed by the methylation analysis data (Table III) showing terminal positions of Fuc, Xyl, and two Man residues together with 2,3,6-trisubstituted Man, 4-monosubstituted GlcNAc, and 4,6-disubstituted GlcNAc-ol.

The ^{1}H NMR data of fraction V (Table II) indicate that the major (80%) constituent of this fraction is the afuco analogue of the aforementioned compound. The minor constituent (20%) corresponds with the structure presented for fraction IV.

DISCUSSION

In this study it has been demonstrated that xylose is covalently bound to an asparagine-linked carbohydrate chain. So far, this type of chain has not been reported to occur in animal glycoproteins. Examples of such glycoproteins from plant material are stem bromelain (29), Tora-bean lectin (30), *Vicia graminea* lectin (31), *Erythrina cristagalli* lectin (32), and lectin from *Griffonia simplicifolia* seeds (33). As far as their primary structures have been reported, these glycoproteins contain Xyl β(1–2)-linked to Man $\underline{3}$ together with Fuc α(1–3)-linked to GlcNAc $\underline{1}$ in the same sugar chain. In contrast, the main component of the low-molecular-weight structures of *H. pomatia* α-hemocyanin contains Xyl β(1–2)-linked to Man $\underline{3}$ in combination with Fuc α(1–6)-linked to GlcNAc $\underline{1}$.

Comparison of the sugar analysis (Table I) of the fractions IV and V with those of fractions I, II, and III strongly suggests that the structures present in the latter fractions contain similar Xyl containing core structures as those in fractions IV and V. To establish the positions of 3-*O*-methyl-Gal, Gal, and GalNAc in the *N*-linked carbohydrate chains, further studies are in progress.

Acknowledgments—We thank Dr. R. Torensma for the kind gift of *H. pomatia* α-hemocyanin, and J. I. M. van Doorn-van Wakeren, Dr. J. H. M. Abbink, and C. Schreuder for their introductory investigations.

REFERENCES

1. Ellerton, H. D., Ellerton, N. F., and Robinson, H. A. (1983) *Prog. Biophys. Mol. Biol.* **41,** 143–248
2. Hall, R. L., Wood, E. J., Kamerling, J. P., Gerwig, G. J., and Vliegenthart, J. F. G. (1977) *Biochem. J.* **165,** 173–176
3. Chaplin, M. F., Corfield, G. C., and Wood, E. J. (1983) *Comp. Biochem. Physiol.* **75B,** 331–334
4. Stephen, A. M. (1983) in *The Polysaccharides* (Aspinall, G. O., ed) Vol. 2, pp. 97–193, Academic Press, London
5. Painter, T. J. (1983) in *The Polysaccharides* (Aspinall, G. O., ed) Vol. 2, pp. 195–285, Academic Press, London
6. Kenne, L., and Lindberg, B. (1983) in *The Polysaccharides* (Aspinall, G. O., ed) Vol. 2, pp. 287–363, Academic Press, London
7. Itasaka, O., and Hori, T. (1979) *J. Biochem. (Tokyo)* **85,** 1469–1481
8. Lederkremer, G. Z., and Parodi, A. J. (1984) *J. Biol. Chem.* **259,** 12514–12518
9. Dijk, J., Brouwer, M., Coert, A., and Gruber, M. (1970) *Biochim. Biophys. Acta* **221,** 467–479
10. Hall, R. L., and Wood, E. J. (1976) *Biochem. Soc. Trans.* **4,** 307–309
11. Konings, W. N., Dijk, J., Wichertjes, T., Beuvery, E. C., and Gruber, M. (1969) *Biochim. Biophys. Acta* **188,** 43–54
12. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680–685
13. Van Driel, R. (1973) *Biochemistry* **12,** 2696–2698
14. Takasaki, S., Mizuochi, T., and Kobata, A. (1982) *Methods Enzymol.* **83,** 263–268
15. Yamashita, K., Mizuochi, T., and Kobata, A. (1982) *Methods Enzymol.* **83,** 105–127
16. Korrel, S. A. M., Clemetson, K. J., Van Halbeek, H., Kamerling, J. P., Sixma, J. J., and Vliegenthart, J. F. G. (1984) *Eur. J. Biochem.* **140,** 571–576
17. Wagner, G., and Wüthrich, K. (1979) *J. Magn. Reson.* **33,** 675–680
18. Haasnoot, C. A. G. (1983) *J. Magn. Reson.* **52,** 153–158
19. Ernst, R. R. (1966) *Adv. Magn. Res.* **2,** 1–135
20. Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* **41,** 209–374
21. Kamerling, J. P., and Vliegenthart, J. F. G. (1982) *Cell Biol. Monogr.* **10,** 95–125
22. Endo, Y., Yamashita, K., Tachibana, Y., Tojo, S., and Kobata, A. (1979) *J. Biochem. (Tokyo)* **85,** 669–679
23. Paz-Parente, J., Strecker, G., Leroy, Y., Montreuil, J., Fournet, B., Van Halbeek, H., Dorland, L., and Vliegenthart, J. F. G. (1983) *FEBS Lett.* **152,** 145–152
24. Mutsaers, J. H. G. M., Van Halbeek, H., Kamerling, J. P., and Vliegenthart, J. F. G. (1985) *Eur. J. Biochem.* **147,** 569–574
25. Van Halbeek, H., Dorland, L., Veldink, G. A., Vliegenthart, J. F. G., Garegg, P. J., Norberg, T., and Lindberg, B. (1982) *Eur. J. Biochem.* **127,** 1–6
26. Brisson, J.-R., and Carver, J. P. (1983) *J. Biol. Chem.* **258,** 1431–1434
27. Prestegard, J. H., Koerner, Jr., T. A. W., Demou, P. C., and Yu, R. K. (1982) *J. Am. Chem. Soc.* **104,** 4993–4995
28. Cahour, A., Debeire, P., Hartmann, L., Montreuil, J., Van Halbeek, H., and Vliegenthart, J. F. G. (1984) *FEBS Lett.* **170,** 343–349
29. Ishihara, H., Takahashi, N., Oguri, S., and Tejima, S. (1979) *J. Biol. Chem.* **254,** 10715–10719
30. Ohtani, K., and Misaki, A. (1980) *Carbohydr. Res.* **87,** 275–285
31. Prigent, M. J., Montreuil, J., and Strecker, G. (1984) *Carbohydr. Res.* **131,** 83–92
32. Iglesias, J. L., Lis, H., and Sharon, N. (1982) *Eur. J. Biochem.* **123,** 247–252
33. Shibata, S., Goldstein, I. J., and Baker, D. A. (1982) *J. Biol. Chem.* **257,** 9324–9329