should be possible also to use these reactions for coupling of polysaccharide antigens to polysaccharide supports.

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Linkage and Sequence Analysis of Mannose-Rich Glycoprotein Core Oligosaccharides by Proton Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The anomeric proton (H-1) chemical shifts of D-mannopyranosides in aqueous solution are affected both by the aglycon and by substitution on the ring [Lee, Y. C., & Ballou, C. E. (1965) *Biochemistry 4*, 257]. We have examined the ^{1}H NMR spectra for a variety of linear and branched mannooligosaccharides and have assigned the H-1 resonances to the component sugars. The chemical shifts, which range from δ 4.76 to 5.36, provide information regarding the linkages, sequences, and anomeric configurations of mannose residues in an oligomer. Thus, ^{1}H NMR spectroscopy can complement enzymatic hydrolysis, methylation analysis, and acetolysis for the structural characterization of oligosaccharides. Furthermore, small structural differences between otherwise identical oligosaccharides are often accompanied by long-range chemical shift changes for the anomeric protons. Because sugars three or more residues away from the structural alteration can be affected, the changes must reflect conformational differences. We have placed emphasis on the mannose-rich oligosaccharides from glycoproteins, particularly those produced by endo-β-*N*-acetylglucosaminidase digestion. Two mannose-rich glycopeptides were isolated from a monoclonal human IgM and their positions of origin on the polypeptide chain were determined. The oligosaccharides were released with endo-β-*N*-acetylglucosaminidase and fractionated into several size classes. Our structural studies show that each glycopeptide possessed a unique set of oligosaccharides, in agreement with a recent report [Chapman, A. & Kornfeld, R. (1979) *J. Biol. Chem. 254*, 816]. The NMR spectra were particularly valuable in detecting and quantitating isomeric fragments not observed previously, and our results suggest a modification of the scheme presented by Chapman and Kornfeld for the processing of mannose-rich IgM oligosaccharides.

Despite the diversity of glycoproteins, their carbohydrate components have common structural features. For the asparagine-linked carbohydrates, two major classes are known. These have been termed *complex* and *high mannose* (or *simple*) to reflect their sugar compositions (Kornfeld & Kornfeld, 1976). The complex oligosaccharides typically contain fucose, galactose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine attached to asparagine by way of a pentasaccharide core of mannose and di-*N*-acetylchitobiose, $Man_3GlcNAc_2$.[1] Mannose-rich (high-mannose) oligosaccharides lack these other sugars but contain additional mannose that is linked to protein through the $Man_3GlcNAc_2$ core.

Two facts make the mannose-rich structures of particular interest. First, these oligosaccharides appear to have been conserved through evolution, as evidenced by the resemblance

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[1] Abbreviations used: GlcNAc or GNAc, *N*-acetylglucosamine; GlcNAc-H_2 or GNAc-H_2, *N*-acetylglucosaminitol; reduced oligosaccharides may be indicated with the prefix "r" (rGP-563-I is reduced oligosaccharide GP-563-I); T_1, spin–lattice relaxation time.

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of yeast mannoprotein core oligosaccharides (Nakajima & Ballou, 1974) to carbohydrate fragments derived from human γM immunoglobulin (Chapman & Kornfeld, 1979a,b). Similar mannooligosaccharides also occur in glycoproteins of other fungi such as α-amylase from *Aspergillus oryzae* (Yamaguchi et al., 1971), in insect vitogellin (Kunkel et al., 1978), in hen ovalbumin (Tai et al., 1975), and in calf thyroglobulin (Ito et al., 1977). Second, the biosynthesis of both types of asparagine-linked oligosaccharides begins with the transfer of a glucosylated mannose-rich fragment from dolichol pyrophosphate to the protein (Turco et al., 1977; Spiro et al., 1978; Li et al., 1978). The glucose units and some of the mannoses are removed to leave a mannooligosaccharide core that is processed to give either complex or mannose-rich structures (Robbins et al., 1977; Tabas et al., 1978, Hunt et al., 1978; Staneloni & Leloir, 1978; Chen & Lennarz, 1978; Kornfeld et al., 1978). The modification of core mannooligosaccharides to yield complex forms has a counterpart in *Saccharomyces cerevisiae* wherein a long mannose outer chain is constructed on the core (Ballou, 1976). Like IgM (Shimizu et al., 1971), the yeast glycoprotein invertase contains both simple and modified core oligosaccharides (Lehle et al., 1979).

A detailed knowledge of mannose-rich oligosaccharide structures is a prerequisite to understanding their biosynthesis and processing. Such characterization, moreover, should facilitate an assessment of the significance of the microheterogeneity commonly found in mannose-rich oligosaccharides (Huang et al., 1970; Nakajima & Ballou, 1975; Chapman & Kornfeld, 1979a,b). A complete characterization of mannose-rich oligosaccharides is difficult, and it has been pointed out (Kornfeld & Kornfeld, 1976) that the term "simple" to describe these compounds is a misnomer in that sequence and linkage analysis of complex saccharide chains composed of several different sugars is usually easier. The difficulty lies in determining linkages and anomeric configurations for a homopolymer; by conventional methods, a combination of degradative techniques must be employed (Kornfeld & Kornfeld, 1976).

The anomeric proton (H-1) chemical shifts of hexoses and their glycosides are known to be affected by the anomeric configuration and the nature of reducing end substituents (Lemieux & Lineback, 1963; Van der Veen, 1963; Pasika & Cragg, 1963). For α-D-mannopyranosides in aqueous solution, these shifts are also influenced by substitution around the ring, allowing both linkage information and sequence information to be derived (Lee & Ballou, 1965a). Similar observations have been made for other sugars, and ^{1}H NMR spectroscopy has been applied to complex-type oligosaccharides from glycoproteins (Dorland et al., 1977, 1979; Fournet et al., 1978) and glycolipids (Strecker et al., 1977; Falk et al., 1979a–c).

In the present study, we have examined the ^{1}H NMR spectra for a variety of compounds, which include all linkage combinations commonly found in the mannose-rich oligosaccharides of glycoproteins. The H-1 chemical shifts have been assigned and can be correlated with nearest-neighbor sequences. In addition, we have isolated the two mannose-rich glycopeptides from a human IgM and have determined their positions of origin on the peptide chain. The core oligosaccharides were released from the glycopeptides, and NMR spectra were used for structural assignments. While this work was in progress, Chapman & Kornfeld (1979a,b) published a chemical study of similar oligosaccharides, and our results are consistent with and extend their findings. A preliminary report of some of these results has appeared (Cohen & Ballou, 1979), and some of our assignments have been used in a published study (Lehle et al., 1979).

Experimental Procedures

Materials. Bio-Gel P-2 (−400 mesh), P-4 (−400 mesh), and P-6 (200–400 mesh) and Dowex AG 50-W-X8, AG 50-W-X2, and AG 1-X8 were from Bio-Rad, and Sephadex G-25M was from Pharmacia. Endo-α1→6-D-mannanase was purified from *Bacillus circulans* ATCC 29101 (Nakajima et al., 1976). An endo-β-*N*-acetylglucosaminidase from the same source (Nakajima & Ballou, 1974) was purified by ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration to yield a preparation free of other glycosidases (Cohen, 1980). The specificity of this enzyme is similar to that reported for endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus* (Tarentino & Maley, 1974). Pronase, grade B, was from Calbiochem, and sodium borotritide (200 Ci/mol) was from New England Nuclear.

Analytical Methods. Total hexose was determined by the phenol–sulfuric acid method (Dubois et al., 1956) with D-mannose as the standard. Neutral sugar compositions were determined after hydrolysis in 1.0 N trifluoroacetic acid under N_2 at 120 °C for 1.5 h. The monosaccharides were converted to alditol acetates (Sloneker, 1972), which were analyzed on a Varian Aerograph 1400 gas chromatograph equipped with a 6-ft column packed with 3% ECNSS-M on 100–200 mesh Gas Chrom Q (Applied Science). The column temperature was maintained at 170 °C for 30 min following sample injection, and then it was increased to 190 °C at 1 °C/min. *N*-Acetylneuraminic acid was assayed with periodate–resorcinol (Jourdian et al., 1971).

Glycopeptides and oligosaccharides were methylated as described by Lindberg (1972). Permethylated samples were formolyzed in 90% formic acid at 100 °C for 2 h and then hydrolyzed in 0.3 N HCl at 100 °C for 6 h. The products were reduced and acetylated according to Albersheim et al. (1967). The partially methylated alditol acetates were analyzed on a Varian Aerograph 1400 gas chromatograph equipped with a flame ionization detector and coupled to a Du Pont Model 21-491 mass spectrometer. A glass column (1/8 in. by 4 ft), packed with OV-210 (3% w/w) on Supelcoport, was used at 180 or 210 °C with He as the carrier gas. Peaks were identified by mass spectrometry (Björndal et al., 1967) and by comparison of their retention times with those of standards.

Acetolysis of sodium borotritide reduced oligosaccharides (5×10^4 cpm; 2–10 nmol) was done as described by Tai et al. (1975), except that the acetylation was carried out in 250 μL of acetic anhydride–pyridine (1:1) at 100 °C for 60 h, and the acetolysis was conducted at 40 °C for 10 or 12 h.

Electrophoresis of glycopeptides was done on Whatman GF83 glass-fiber paper. Samples were spotted in the middle of 48-cm strips and electrophoresed in a pyridine–acetic acid–water (1:10:289) buffer system, pH 3.7, at 1000 V for 60 min. Samples were detected either by spraying the strip with 50% sulfuric acid in ethanol and charring it at 120 °C or by spraying with ninhydrin. Whatman No. 1 paper was used for paper chromatography, and radioactivity was detected by cutting the chromatograms into 1 × 2 cm strips that were counted in 0.2 mL of water and 5 mL of Biofluor (New England Nuclear) in a Beckman LS-3150 T liquid scintillation counter. Aqueous samples were counted directly in Biofluor.

Amino acid compositions and glucosamine were determined with a Beckman Model 120C amino acid analyzer. Glycopeptide samples were hydrolyzed in 2 N HCl at 120 °C for 12, 24, and 36 h, at concentrations below 0.3 mg/mL, and under N_2 in sealed tubes to minimize side reactions. Ami-

no-terminal sequencing of glycopeptides was done by the manual Edman degradation/dansylation procedure (Gray, 1967), which was carried through three cycles. Dansyl amino acids were identified by chromatography on Cheng Chin polyamide sheets (Woods & Wang, 1967).

Yeast Strains and Mannans. *S. cerevisiae* X2180 (wild type) and the *mnn2* and *mnn3* mutants (Raschke et al., 1973) were obtained from the laboratory collection, as was the *mnn5* mutant (Cohen et al., 1978). The yeasts were grown aerobically at 30 °C to stationary phase in 1% yeast extract, 2% Bacto-peptone, and 2% D-glucose. Mannans were extracted according to Peat et al. (1961) and precipitated with Fehling's solution (Peat et al., 1961) or by the Cetavlon method (Lloyd, 1970). *Pichia mucosa* YB-1344 extracellular mannan was provided by Dr. M. E. Slodki, Northern Regional Research Laboratory, Peoria, IL. The mannans of *Hansenula polymorpha* (formerly *Hansenula angusta*), *Hansenula wingei* Y-2340 *type* 5, and *Saccharomyces italicus* Y66 were available from previous studies.

Preparation of IgM Glycopeptides. Serum from a patient (Ca) with Waldenström's macroglobulinemia was provided by Dr. H. H. Fudenberg, Medical University of South Carolina, Charleston. IgM was purified from this serum (Hickman et al., 1972), and immunoelectrophoresis and zone electrophoresis showed the protein to be greater than 95% pure. Glycopeptides were produced by two cycles of Pronase digestion and Sephadex G-25 chromatography (Spragg & Clamp, 1969).

Reference Oligosaccharides and Glycopeptides. Mannooligosaccharides are listed in Table I, and glycopeptides and GlcNAc-terminated oligosaccharides are in Table II. Most of the mannooligosaccharides were prepared by acetolysis of yeast mannans (Kocourek & Ballou, 1969). In this way, α1→2-linked mannobiose through mannotetraose were prepared from *H. polymorpha* mannan (Lipke et al., 1974), αMan1→3αMan1→2αMan1→2Man from *S. cerevisiae* X2180 mannan (Lee & Ballou, 1965a), and αMan1→3αMan1→2Man was from the *mnn5* mutant of *S. cerevisiae* (Cohen et al., 1978). The pentasaccharides αMan1→3αMan1→3αMan1→2αMan1→2Man and αMan1→3αMan1→2αMan1→2αMan1→2Man were from *S. italicus* (Ballou et al., 1974) and *H. wingei* mannans (Yen & Ballou, 1974), respectively, the latter preparation being a gift from Carl Hashimoto of this laboratory. α1→3-Linked mannooligosaccharides were obtained by acetolysis of *P. mucosa* mannan (Seymour et al., 1976), whereas the α1→6-linked oligomers were released by endo-α1→6-D-mannanase digestion of *S. cerevisiae mnn2* mannan (Nakajima et al., 1976). The same enzyme was used to prepare the branched tetrasaccharide (**15** in Table I) from *S. cerevisiae mnn3* mannan (Cohen, 1980). A mixture of α- and β1→4-mannobiose was made by acetolysis of ivory nut (*Phytelephas macrocarpa*) mannan and resolved by preparative paper chromatography (Aspinall et al., 1958).

βMan1→4GlcNAc was a gift from Dr. Y. C. Lee, The Johns Hopkins University, Baltimore, MD. The oligosaccharides αMan1→3βMan1→4GlcNAc, αMan1→2αMan1→3βMan1→4GlcNAc, and αMan1→2αMan1→2αMan1→3βMan1→4GlcNAc. were provided by Dr. S. Svensson, University Hospital, Lund, Sweden (Nordén et al., 1973). The branched tetrasaccharide $Man_3GlcNAc$ (**20** in Table II) is from the core of IgM complex oligosaccharides (Hickman et al., 1972). It was prepared by glycosidase digestion of IgM (Ca) complex glycopeptides by Dr. C. Reading (Reading et al., 1978). Hen ovalbumin glycopeptides (Tai et al., 1975; Conchie & Strachan, 1978) were purified by the procedure of Huang et al. (1970), and oligosaccharides were released from them by endo-β-*N*-acetylglucosaminidase digestion. Glycopeptides **23** and **24** (Table II) correspond to AC-E and AC-D in the nomenclature of Huang et al. (1970).

NMR Spectroscopy. ^{1}H NMR studies were made at 180 MHz with a Bruker superconducting magnet and Nicolet 1180 computer operated in the Fourier transform mode with quadrature phase detection. Spectra were obtained using a 90° pulse width, 1500 Hz spectral width, and 3.1-s cycle time, with data being accumulated into 8192 addresses. The probe temperature was 40 ± 0.2 °C. Occasionally, other temperatures from 20.0 to 60.0 °C were used, but these produced no significant deviation of sugar proton chemical shifts from those measured at 40.0 °C. A digital broadening of up to 0.40 Hz, but usually 0.05 or 0.10 Hz, was used to enhance sensitivity. Chemical shifts are expressed relative to sodium 3-(trimethylsilyl)propanesulfonate as the internal standard or are referenced indirectly with an internal acetone standard (δ 2.217 at 40.0 °C). Samples were prepared by passing the aqueous solutions through 1-mL columns of Chelex 100 (Bio-Rad) to remove metal ions, exchanging them 3 times with D_2O by lyophilization, and dissolving the product in 100.0% D_2O (low in paramagnetic ions grade, from Aldrich Chemical). Concentrations ranged from 0.1 to 10 mM, and 5-mm sample tubes were used. Borate complexation studies employed ultrapure sodium tetraborate from Alfa-Ventron.

For some of the core oligosaccharide spectra, the H-1 resonances were integrated by using the 1180 computer. Errors introduced by different spin–lattice (T_1) relaxation times were judged to be negligible because the 3.1-s interval between excitation pulses exceeded 5 times the estimated T_1 values for H-1. Hall & Preston (1976) have determined that the H-1 T_1 relaxation times decrease with increasing oligosaccharide size and were 0.63 s or less for a tetrasaccharide in aqueous solution. We confirmed this for $Man_9GlcNAc$, in which the glycosidic protons had T_1 values between 0.35 and 0.55 s (unpublished experiments).

Results

General Features of Mannooligosaccharide ^{1}H Spectra. Interpretation of ^{1}H NMR spectra of oligosaccharides is simplified because the anomeric protons resonate at lower frequencies than the other ring protons (Van der Veen, 1963). Furthermore, the conformation for D-mannopyranosides can be determined from the H-1 coupling constants, since in either anomeric configuration of the $^4C_1$ chair form splitting by H-2 is less than 3 Hz (H-1/H-2 dihedral angle is ~60°), whereas for the $^1C_4$ chair conformation a larger coupling constant is predicted (Karplus, 1959). As expected, in aqueous solution the $^4C_1$ form of mannopyranose and its glycosides predominates. In accordance with the rules formulated by Lemieux & Stevens (1965), the anomers can be distinguished because equatorial protons such as H-1 of mannose in the α configuration, give signals at lower field than their axial counterparts.

Typical ^{1}H NMR spectra of mannooligosaccharides in D_2O are shown in Figure 1. H-1 proton signals appear downfield of the HDO resonance, between 4.7 and 5.4 ppm, whereas the ring protons form a broad envelope further upfield between 3.5 and 4.0 ppm. Although the complexity of the ring proton region precludes its analysis, C-2 protons often are deshielded and appear between 4.0 and 4.3 ppm, depending upon linkage position. That the signals in this region are from H-2 was confirmed by homonuclear decoupling experiments. Selective irradiation at the H-1 frequencies collapsed all signals between 4.0 and 4.3 ppm from quartets to doublets, and the remaining

Table I: Anomeric Proton Chemical Shifts for Reference Mannooligosaccharides[a]

	sugar residue[b]					δ (ppm)[c]					
compound	E	D	C	B	A	E	D	C	B	A_α	A_β
1					M					5.165	4.879
2					$M^1\rightarrow{}^2M$				5.036	5.363	4.900
3					$M^1\rightarrow{}^3M$				5.133	5.143	4.896
4					$M^1\rightarrow{}^4M$				5.236	5.160	4.874
5					$M^1\rightarrow{}^6M$				4.896	5.159	–[d]
6				$M^1\rightarrow{}^2M^1\rightarrow{}^2M$				5.039	5.280	5.352	4.897
7				$M^1\rightarrow{}^3M^1\rightarrow{}^3M$				5.139	5.120	5.151	4.905
8				$M^1\rightarrow{}^6M^1\rightarrow{}^6M$				4.886	4.907	5.163	–[d]
9				$M^1\rightarrow{}^3M^1\rightarrow{}^2M$				5.138	5.033	5.363	4.912
10			$M^1\rightarrow{}^2M^1\rightarrow{}^2M^1\rightarrow{}^2M$				5.038	5.275	5.275	5.354	4.898
11			$M^1\rightarrow{}^3M^1\rightarrow{}^3M^1\rightarrow{}^3M$				5.133	5.117	5.117	5.145	4.897
12			$M^1\rightarrow{}^3M^1\rightarrow{}^2M^1\rightarrow{}^2M$				5.134	5.026	5.278	5.354	4.900
13	$M^1\rightarrow{}^3M^1\rightarrow{}^2M^1\rightarrow{}^2M^1\rightarrow{}^2M$					5.135	5.027	5.274	5.274	5.350	4.894
14	$M^1\rightarrow{}^3M^1\rightarrow{}^3M^1\rightarrow{}^2M^1\rightarrow{}^2M$					5.129	5.117	5.031	5.278	5.354	4.901
15				$M^1\rightarrow{}^6M^1\rightarrow{}^6M$ $\uparrow{}^2$ M^1				4.925	5.107 5.032	5.158	4.891

[a] The columns headed by capital letters correlate sugar residues with their anomeric proton chemical shifts. [b] All glycosidic linkages have the α configuration; M, mannose. [c] Chemical shifts were determined with a precision of ±0.002 ppm. [d] The reducing end β-anomeric proton resonance was obscured by the signal from the non-reducing terminal mannose.

Table II: Anomeric Proton Chemical Shifts for Reference Core Oligosaccharides and Glycopeptides[a]

	sugar residue[b]					δ (ppm)[c]					
compound	E	D	C	B	A	E	D	C	B_α	B_β	A
16			$M^1_\beta\rightarrow{}^4GNAc$					4.755	5.202	4.706	–
17			$M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow$ M^1					4.773 5.104	5.196	4.715	–
18			$M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow$ M^1 $\uparrow{}^2$ M^1					4.765 5.340 5.042	5.193	4.713	–
19			$M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow$ M^1 $\uparrow{}^2$ M^1 $\uparrow{}^2$ M^1					4.774 5.343 5.290 5.054	5.214	4.715	–
20			$M^1\rightarrow{}^6M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow$ M^1					4.910 5.103	4.772	5.204	4.711	–
21			$M^1\rightarrow{}^6M^1\rightarrow{}^6M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow{}^3$ $\uparrow$ M^1 M^1			4.904		4.868 5.102	4.773 5.102	5.232	4.712	–
22			$M^1\rightarrow{}^6M^1\rightarrow{}^6M^1_{3\beta}\rightarrow{}^4GNAc$ $\uparrow{}^3$ $\uparrow$ M^1 M^1 $\uparrow{}^2$ M^1			4.903		4.868 5.082 5.046	4.766 5.335	5.235	4.710	–
23			$M^1\rightarrow{}^6M^1\rightarrow{}^6M^1_{3\beta}\rightarrow{}^4GNAc^1_\beta\rightarrow{}^4GNAc^1_\beta Asn$ $\uparrow{}^3$ $\uparrow$ M^1 M^1			4.893		4.857 5.089	4.758 5.089	–	4.608	5.07[d]
24			$M^1\rightarrow{}^6M^1\rightarrow{}^6M^1_{3\beta}\rightarrow{}^4GNAc^1_\beta\rightarrow{}^4GNAc^1_\beta Asn$ $\uparrow{}^3$ $\uparrow$ M^1 M^1 $\uparrow{}^2$ M^1			4.902		4.864 5.093 5.045	4.756 5.329	–	4.612	5.07[d]

[a] The columns headed by capital letters correlate sugar residues with their anomeric proton chemical shifts. M, mannose; GNAc, N-acetylglucosamine. [b] Unless otherwise indicated, glycosidic linkages have the α configuration. [c] Chemical shifts were determined with a precision of ±0.003 ppm. [d] This value is only approximate (±0.01 ppm) due to overlap with other signals.

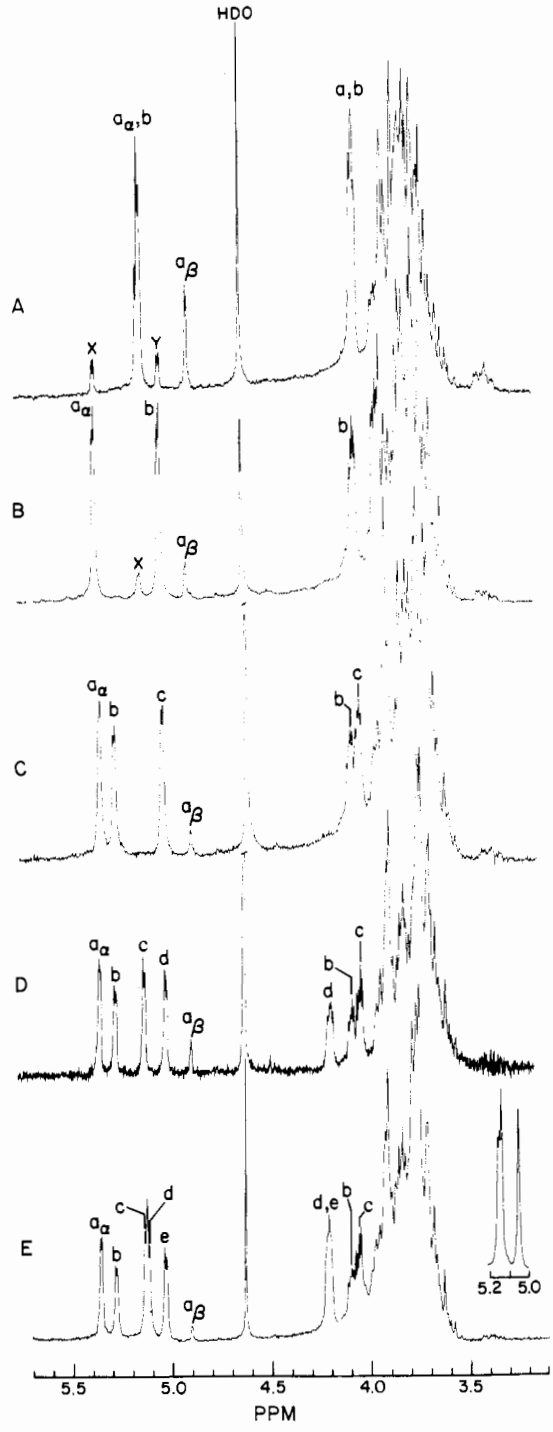

FIGURE 1: Selected NMR spectra of mannooligosaccharide standards. The samples are (A) αMan1→3Man, compound **3**, (B) αMan1→-2Man, compound **2**, (C) αMan1→2αMan1→2Man, compound **6**, (D) αMan1→3αMan1→2αMan1→2Man, compound **12**, and (E) αMan1→3αMan1→3αMan1→2αMan1→2Man, compound **14**. Anomeric proton assignments are listed in Table I. The letters a–e correlate spin-coupled H-1 and H-2 protons, as determined by homonuclear decoupling. In (A) and (B), the small peaks x and y are due to cross contamination of the two samples. The inset in (E) shows the effect on the anomeric proton signals c, d, and e of selectively irradiating the resonance at 4.21 ppm. Spectra were acquired at a probe temperature of 40.0 °C.

major splitting came from H-2/H-3 interactions.

In assigning the H-1 resonances we followed the strategy of Lee & Ballou (1965a). For a series of disaccharides, the C-1 protons of both mannoses can be assigned (a) by identifying the reducing-end H-1 because it is split into two components, α and β, and (b) by comparing the spectra for sodium borohydride reduced compounds in which the reducing-end H-1 signal is moved into the ring proton region of the spectrum. As mannose units are added, new H-1 signals appear. If these are sufficiently resolved from other resonances, assignment is straightforward. For example, comparison of α1→2-linked mannobiose (Figure 1B) with the homologous mannotriose (Figure 1C) suggests that peak b in Figure 1C arises from H-1 of the middle sugar. This was confirmed with the tetrasaccharide in which a peak at 5.28 ppm, the same position as peak b in Figure 1C, is twice the intensity of the other two α-anomeric proton resonances.

Assigning the H-1 resonances for αMan1→3αMan1→-2αMan1→2Man was more difficult, as noted earlier (Lee & Ballou, 1965a). This is because addition of an α1→3-linked mannose to the nonreducing end of α1→2-mannotriose results in two new H-1 lines (peaks c and d in Figure 1D), one of these arising from the change in the H-1 shift of the nonreducing terminal sugar in α1→2-mannotriose upon 3-O-substitution. These signals were resolved by use of borate ion which, on complexing the 2,3-*cis*-hydroxyls of mannose, promotes a downfield shift and broadening of the H-1 resonance of the complexed sugar (Gorin et al., 1968). By this technique, the H-1 chemical shifts have been correlated with the sequence of this and related oligosaccharides (for example, **7**, **9**, **12**, and **13** in Table I) by Gorin & Spencer (1970).

Analysis of the homologous α1→3-linked mannobiose, mannotriose, and mannotetraose indicated a chemical shift for H-1 of the nonreducing terminal sugar to be ~5.135 ppm. This corresponds to peak c in Figure 1D, leaving peak d to account for the penultimate mannose residue. This assignment was confirmed by selective decoupling experiments. The C-2 protons of 3-O-substituted mannopyranosides with a free 2-hydroxyl are found at ~4.2 ppm, significantly further downfield than the C-2 protons of mannose in other linkages (Gray & Ballou, 1971; R. E. Cohen and C. E. Ballou, unpublished data). Thus, coupling between the quartet at 4.207 ppm and the H-1 signal at 5.026 ppm (peaks d in Figure 1D) supports the conclusion that signals c and d in Figure 1D are from the nonreducing terminal and penultimate sugar units, respectively.

The NMR spectrum for αMan1→3αMan1→3αMan1→-2αMan1→2Man is shown in Figure 1E, and the anomeric proton assignments are in Table I (compound **14**). Integration of the lowest frequency C-2 proton resonance (δ 4.213) showed two protons, which is consistent with two 3-O-substituted mannoses in the structure. These C-2 protons were spin coupled to separate C-1 protons, indentified as peaks d and e in Figure 1E. Decoupling by irradiation at the H-2 frequencies allowed the overlapping H-1 peaks c and d to be assigned, since each could be made to collapse independently to a singlet (see the inset of Figure 1E). Note that oligosaccharides **12** and **14** in Table I differ by a single unit, →3αMan1→3. The obvious differences between parts D and E of Figure 1 are in peaks d, which decoupling experiments show can arise from →3αMan1→3. The nonterminal sugars in α1→3-mannotriose and mannotetraose possess this structure and give similar H-1 (δ 5.12) and H-2 (δ 4.22) chemical shifts. Assignments for the remaining linear oligosaccharides in Table I were made with similar arguments.

The H-1 signals for the branched tetrasaccharide **15** were assigned by considering the effect of 2-O-substitution upon the middle residue of the α1→6-mannotriose (compound **8**). We assumed that H-1 for the terminal sugars of this trisaccharide would be relatively unaffected and that the shift

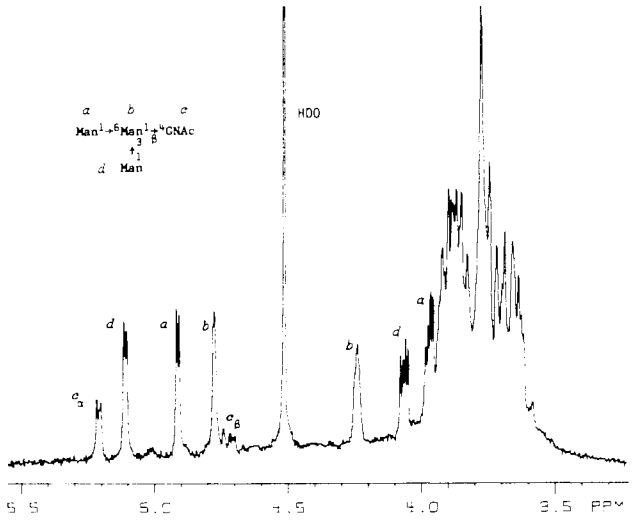

FIGURE 2: NMR spectrum of a branched core tetrasaccharide. The small letters indicate which anomeric resonances correspond to the sugars in the figure and correlate spin-coupled H-1 and H-2 signals; chemical shifts are listed in Table II (compound **20**). In the presence of 0.2 M sodium tetraborate, peaks a and d are broadened while peaks b and c remain unchanged. The sample was maintained at 50.0 °C.

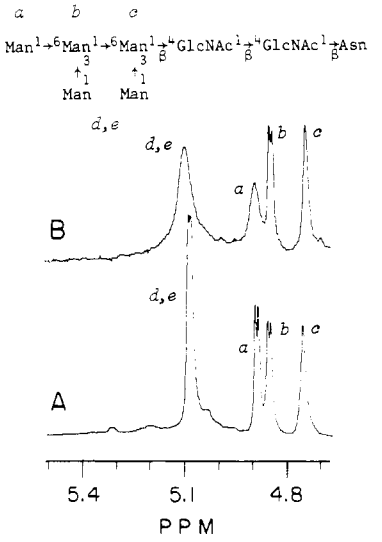

FIGURE 3: Anomeric proton region of the NMR spectra of an ovalbumin glycopeptide. Spectrum A is for the sample alone in D_2O, and spectrum B shows the effect of 0.2 M sodium tetraborate; the sample was maintained at 50.0 °C in both experiments. Assignments are listed in Table II (compound **23**).

for H-1 of the terminal α1→2-mannosyl residue would be close to that of the nonreducing termini of compounds **2**, **6**, and **10** (δ 5.03–5.04). Thus, the resonance at δ 4.925 was assigned to the terminal α1→6-residue, δ 5.158 and 4.891 was assigned to the α and β anomers of the reducing residue, and δ 5.032 was assigned to the terminal α1→2-residue. Assignment of the reducing sugar H-1 signals was confirmed by their decreased heights due to the presence of two anomers. The resonance at δ 5.107 was left to account for H-1 of the branched residue. The chemical shift change (0.20 ppm) of the middle sugar of **8** upon 2-O-substitution is similar to that found by comparing compounds **1**, **2**, **6**, and **10**.

^{1}H NMR Spectra of Reference Core Oligosaccharides and Glycopeptides. All glycoprotein-derived mannose-rich oligosaccharides contain the disaccharide βMan1→4GlcNAc, which is usually branched as in tetrasaccharide **20** (Table II), that serves as the core in most complex oligosaccharides (Kornfeld & Kornfeld, 1976). Figure 2 shows the ^{1}H NMR spectrum of **20**, and Table II lists the H-1 chemical shift assignments for this and related structures. The chemical shift for H-1 of the β-mannopyranosyl unit is furthest upfield of the mannose anomeric protons. The C-1 protons for the reducing *N*-acetylglucosamine are easily identified because the large proportion of β anomer and H-1/H-2 spin coupling ($J_\alpha \approx 2.5$ Hz; $J_\beta \approx 7.5$ Hz) diminish the heights of these signals.

Assignments for typical core hexa- and heptasaccharides and the parent glycopeptides are in Table II (structures **21**, **22**, **23**, and **24**), and Figure 3A shows the H-1 spectrum of glycopeptide **23**. The two H-1 signals of the di-*N*-acetylchitobiosyl unit are at δ 4.612, for the mannose-linked residue, and δ 5.07 as assigned by Dorland et al. (1977). The resonance at δ 5.07 lies under the signal (d,e in Figure 3A) from both terminal α1→3-linked mannoses and probably accounts for the broad base of this peak. Unambiguous assignment of the two α1→6-linked mannose units was made after addition of sodium tetraborate, which complexes with the three terminal mannoses, causing broadening of the affected resonances and a slight downfield shift (Figure 3B). Resonances b and c are unaffected and are due to the two 3,6-di-O-substituted sugars. Peak c, at the higher frequency, is assigned to the β-mannosyl residue, b is assigned to the 3,6-di-O-substituted α-mannosyl unit, and a is assigned to the terminal α1→6-linked mannose.

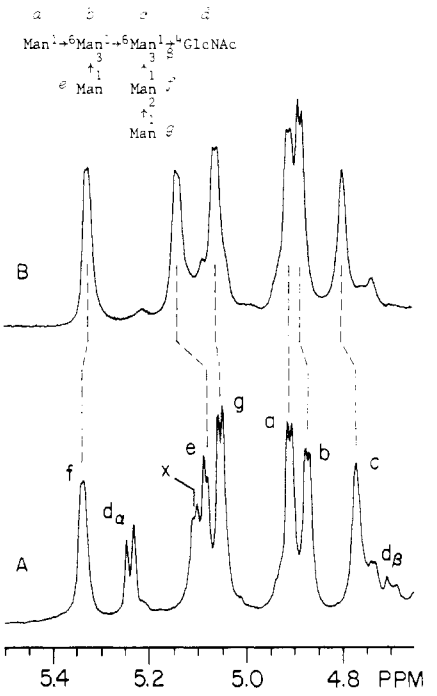

FIGURE 4: Anomeric proton region of the NMR spectrum of an ovalbumin-derived core oligosaccharide. Spectrum A is for the compound represented in the figure, and the small letters correlate the sugar residues with their anomeric proton resonances. Chemical shifts are listed in Table II (compound **22**). The origin of peak x is uncertain and is discussed in the text. Spectrum B is for the oligosaccharide after reduction with sodium borohydride. Both spectra were acquired at a probe temperature of 45.0 °C.

The H-2 resonances that correspond to these signals are at δ 3.970 (a), 4.119 (b), 4.218 (c), and 4.071 (d,e). Endo-β-*N*-acetylglucosaminidase digestion afforded the hexasaccharide **21**. The H-1 spectrum of this compound was similar to that of the parent glycopeptide except for the loss of one GlcNAc signal and a downfield shift of the other as a consequence of its free reducing end. The α1→3- and β-mannosyl H-1 signals also experienced small (0.015 ppm) changes.

Ovalbumin glycopeptide **24** and heptasaccharide **22** gave spectra that were easily interpreted after assignments for the

Table III: Amino Acid and Sugar Compositions of IgM (Ca) Glycopeptides

glycopeptide pool	mol/mol of glycopeptide[a]													
	Man	Gal	Fuc	$GlcNH_2$[b]	Asx	Val	Ser	Thr	His	Glx	Ala	Lys	Pro	Gly
a	1.89	0.87	0.66	2.11	1.00		0.29	0.14		0.36	0.15			
b	2.10	0.59	0.33	1.23	1.00	0.10	0.38	0.12	0.18	0.52	0.19	0.14	0.21	0.20
c	5.98	0.23	0.12	1.74	1.00	0.70	0.42	0.10	0.17					
d	5.82			1.77	1.00				1.70		0.73		0.49	

[a] Values are normalized to Asx. [b] Presumably as GlcNAc in the native glycopeptides.

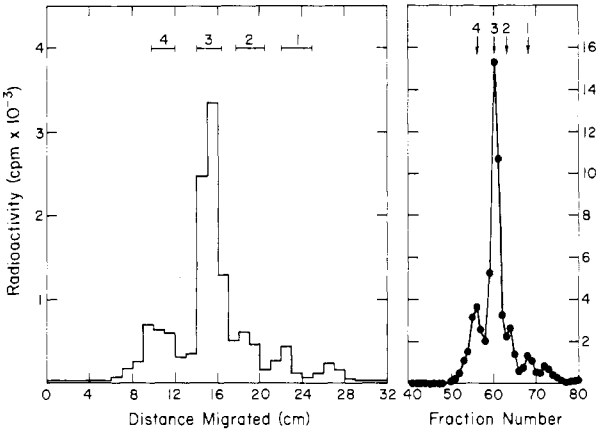

FIGURE 5: Chromatography of the acetolysis products from ^{3}H-reduced ovalbumin oligosaccharide **22**. The material (1 × 10^5 cpm) was acetolyzed for 12 h at 40 °C, and the deacetylated fragments were analyzed. In the left panel, a portion of the products was spotted on Whatman No. 1 paper and irrigated with 1-butanol–pyridine–water (4:3:4) for 18 h. Radioactivity was detected by cutting the chromatogram into 2-cm wide strips and counting 1-cm wide horizontal bands. In the right panel, a portion of the products was applied to a Bio-Gel P-2 (−400 mesh) column (0.4 × 100 cm) which was eluted with 0.01 M acetic acid; 2-drop fractions were collected and counted. The elution positions of standards for paper and gel chromatography are indicated as bars and arrows, respectively. The standards, made by reducing compounds **16**–**19** in Table II, were (1) reduced ManGlcNAc, (2) reduced $Man_2GlcNAc$, (3) reduced $Man_3GlcNAc$, and (4) reduced $Man_4GlcNAc$.

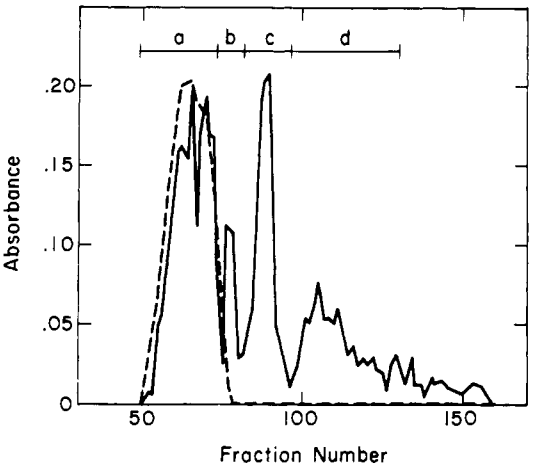

FIGURE 6: Separation of IgM glycopeptides by chromatography on Bio-Gel P-6. IgM (Ca) was digested twice with Pronase, desalted on a column of Sephadex G-25, applied to a Bio-Gel P-6 (200–400 mesh) column (2 × 200 cm), and eluted with water. Fractions of 3 mL were collected, assayed for total hexose (—) and *N*-acetylneuraminic acid (---), and pooled as indicated.

Table IV: Sugar Linkages in IgM (Ca) Mannose-Rich Glycopeptides Determined by Methylation Analysis

glycopeptide	molar ratios of linkage types[a]				
	Man1→	→2Man1→	→3Man1→	→6 →3Man1→	→4GlcNAc1→
GP-402	1.54	0.98	0.12	1.00	+[b]
GP-563	1.41	1.37	0	1.00	+[b]

[a] Ratios are normalized to the 3,6-di-O-substituted mannose. [b] This sugar was present but not quantitated.

smaller homologues were made. The H-1 region of the spectrum for **22** is shown in Figure 4A. This compound may be considered a composite of the fragments αMan1→2αMan1→3βMan1→4GlcNAc (**18** in Table II) and αMan1→6αMan1→6(αMan1→3) (a portion of **21** in Table II), and the signals were assigned accordingly. Some question arose, however, regarding the origin of the low-field shoulder (x) of peak e in the spectrum.[2] The sample did not contain oligosaccharides of different size because it was homogeneous by gel filtration and high-pressure liquid chromatography (not shown). Moreover, only a single component was observed when the ^{3}H-reduced oligosaccharide was subjected to high-voltage paper electrophoresis in a pH 9.5 borate buffer (Tai et al., 1975). We conclude that the sample is a mixture of isomers (see Discussion); that the major component has the structure **22** in Table II was supported by partial acetolysis of the ^{3}H-reduced material. This procedure, which preferentially cleaves the α1→6 linkages, yielded $Man_3GlcNAc\text{-}H_2$ as the predominant radioactive fragment (Figure 5).

Isolation and Characterization of Mannose-Rich IgM Glycopeptides. The glycopeptide mixture from IgM (Ca) was fractionated into four components by gel filtration (Figure 6), and compositions were determined. As seen from Table III and Figure 6, glycopeptide pools a and b contained sugars expected for complex oligosaccharides, whereas pools c and d contained mostly mannose and *N*-acetylglucosamine. The ratio of 2 mol of *N*-acetylglucosamine/mol of asparagine suggested that pools c and d consisted of mannose-rich glycopeptides.

The amino acid compositions of glycopeptide pools c and d were different. Val and Ser were found only in pool c, whereas His, Ala, and Pro were unique to pool d. Upon high-voltage electrophoresis, the two fractions migrated toward the cathode as single spots, with pool d moving 10% faster than pool c. These results implied that pools c and d originated from different places on the IgM polypeptide chain, and subsequent amino acid sequencing confirmed this. The sequence for pool c was Asn-Val-(Ser) and that for pool d was His-Pro-Asn-Ala-(His), the amino acids in parentheses being included solely from the composition data. Except for the second His residue in pool d, these sequences are identical with those reported for the two mannose-rich glycosylation sites in IgM (Ou) (Putnam et al., 1973; Shimizu et al., 1971). Consequently, glycopeptide pool c can be assigned to Asn-563 and d can be assigned to Asn-402 of the IgM μ chain, and the notations

[2] A similar observation has been made independently for this ovalbumin oligosaccharide (P. H. Atkinson, personal communication).

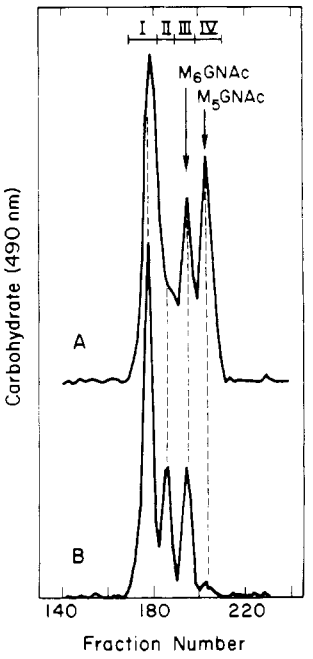

FIGURE 7: Gel filtration chromatography of IgM mannose-rich oligosaccharides on Bio-Gel P-4. The products from endo-β-*N*-acetylglucosaminidase digestion of IgM glycopeptides were applied to a Bio-Gel P-4 (−400 mesh) column (2 × 200 cm). The column was eluted with water, and 2-mL fractions were collected. The profile for the oligosaccharides from GP-402 is in (A) and that for GP-563 is in (B). Fractions were pooled as indicated. M_5GNAc and M_6GNAc identify the elution positions of reference core oligosaccharides (compounds **21** and **22** in Table II).

GP-563 and GP-402 are given to the glycopeptides and to the oligosaccharides derived from them.

Assuming only 2 GlcNAc units/oligosaccharide, the Man to GlcNAc ratios in Table III indicate an average carbohydrate chain size of 8.9 sugars for GP-563 and 8.6 for GP-402. The methylation results in Table IV indicate that all branching in each glycopeptide involves positions 3 and 6 of mannose, with slightly more 1→2-linked mannose in GP-563. Neither terminal nor branched GlcNAc was observed.

Isolation of IgM Mannose-Rich Oligosaccharides. After digestion of GP-402 and GP-563 with endo-β-*N*-acetylglucosaminidase, the reaction mixtures were desalted on Dowex AG-1 ($CH_3CO_2^-$) and Dowex AG-50 (H^+) columns, and the products were chromatographed on Bio-Gel P-4. The patterns (Figure 7) show that both glycopeptides were heterogeneous and that the oligosaccharides from GP-563 were, on the average, larger. Tubes were pooled as shown in Figure 7 and rechromatographed to give four fractions (I, II, III, and IV) from each glycopeptide. The elution positions of III and IV coincided with reference compounds **22** ($Man_6GlcNAc$) and **21** ($Man_5GlcNAc$), respectively.

Methylation Analysis of IgM Mannose-Rich Oligosaccharides. Several of the IgM oligosaccharides were methylated (Table V). The data are normalized to two branched mannoses to reflect the conclusion, based on the composition (Table III) and gel filtration, that these compounds are in the hexa- to decasaccharide size range. Each oligosaccharide contains two 3,6-di-O-substituted and three terminal mannoses, and the progressively larger fragments include additional →2Man1→ units. Insufficient GP-563-IV was obtained for study, and the heterogeneity observed in the H-1 spectra of GP-402-I and II (see below) precluded their analysis.

^1H NMR Spectra of IgM Mannose-Rich Oligosaccharides. The IgM oligosaccharide NMR spectra revealed in each a

Table V: Sugar Linkages in IgM (Ca) Mannose-Rich Oligosaccharides Determined by Methylation Analysis

oligosaccharide	molar ratios of linkage types[a]			
	Man1→	→2Man1→	→6 →3Man1→	→4GlcNAc1→
GP-402-III	3.02 (3)[b]	1.14 (1)	2.00	+[c]
GP-402-IV	3.22 (3)	0.32 (0)	2.00	+[c]
GP-563-I	2.58 (3)	3.36 (3)	2.00	+[c]
GP-563-II	3.34 (3)	1.82 (2)	2.00	+[c]
GP-563-III	3.16 (3)	1.22 (1)	2.00	+[c]

[a] Ratios are normalized to two 3,6-di-O-substituted mannose residues. [b] The values in parentheses are the nearest integral. [c] This sugar was present but not quantitated.

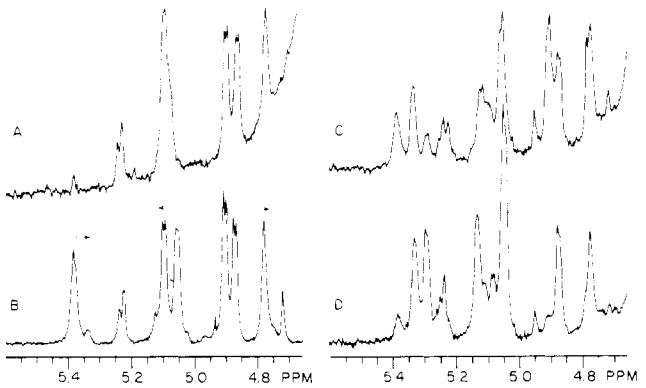

FIGURE 8: Anomeric proton NMR spectra of the IgM core oligosaccharides derived from glycopeptide GP-402. The samples were (A) GP-402-IV, (B) GP-402-III, (C) GP-402-II, and (D) GP-402-I. The arrows in (B) indicate the magnitude and direction of change found for three signals upon comparison with the spectrum (Figure 4A) for **22**. For clarity, spinning sidebands were removed from (A). All spectra were acquired at a probe temperature of 40.0 °C.

single reducing end GlcNAc (δ_α 5.23) and one β-linked mannose (δ 4.77). Because the parent glycopeptides were susceptible to endo-β-*N*-acetylglucosaminidase, which acts only on substrates containing the sequence βMan1→4βGlcNAc1→4GlcNAc (Kobata, 1979), these features were expected. With the exception of fraction IV from each glycopeptide, however, the pairs of spectra differed for each oligosaccharide size class. Furthermore, the assortment of H-1 resonances for GP-402-I and GP-402-II appeared particularly complex, having nonintegral peak intensities that indicated heterogeneity. The spectra for the core fragments derived from GP-402 are shown in Figure 8.

The spectrum of GP-402-IV (Figure 8A) was virtually identical with that for the ovalbumin hexasaccharide (compound **21**, Table II). This corroborates the methylation data which showed that GP-402-IV contained five mannoses, of which two were 3,6-di-O-substituted and three were terminal, and we conclude that the IgM fragment has the same structure as **21**. An isomer of **21** in which the β-mannosyl residue is not 3-O-substituted would agree with the methylation data, but the β-mannosyl H-1 signal of such a compound should be at a slightly higher field since 3-O-substitution of this sugar has a deshielding effect of ~0.02 ppm (compare the β-mannosyl H-1 of compound **16** with compounds **17** and **20** in Table II). Acetolysis of ^3H-reduced GP-402-IV (see below) is consistent with 3,6-substitution of the β-linked mannose.

Oligosaccharide GP-563-IV gave an H-1 spectrum similar to GP-402-IV (not shown), but the lack of material (<60 μg) prevented acquisition of a spectrum of sufficient quality to rule out contamination by other isomers. The main components of each class IV IgM oligosaccharide, however, appear to be

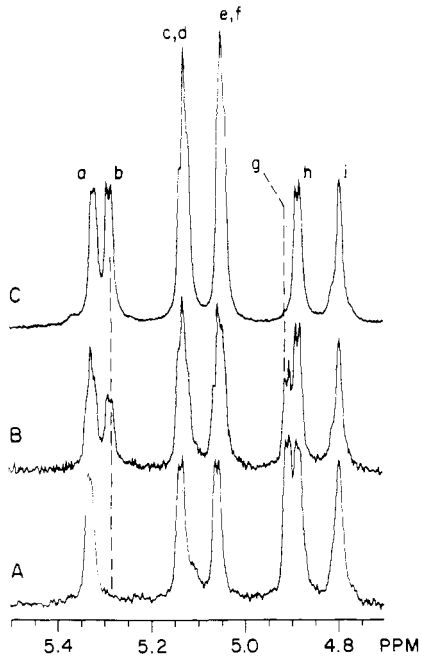

FIGURE 9: Anomeric proton NMR spectra of reduced IgM core oligosaccharides derived from glycopeptide GP-563. Peaks are identified by the small letters, and their chemical shifts and relative areas are listed in Table VI. Assignments are indicated in Figure 10. The spectrum in (A) is for rGP-563-III, (B) is for rGP-563-II, and (C) is for rGP-563-I. The samples were maintained at 55.0 °C.

Table VI: Anomeric Proton Chemical Shifts for Reduced GP-563 Oligosaccharides[a]

peak[b]	δ rGP-563-III	δ rGP-563-II	δ rGP-563-I
a	5.328 (0.96)[c]	5.322 (0.87)	5.316 (1.06)
b		5.282 (0.61)	5.282 (1.07)
c,d	5.132 (1.15)	5.125 (1.39)	5.122 (2.08)
e,f	5.055 (1.05)	5.049 (1.40)	5.042 (2.12)
g	4.905 (1.02)	4.904 (0.79)	
h	4.885 (1.01)	4.881 (0.80)	4.880 (1.06)
i	4.796 (1.00)	4.792 (1.00)	4.791 (1.00)

[a] Chemical shifts were determined with a precision of ±0.002 ppm. [b] Peaks correspond to those in Figure 9. [c] Integrations are indicated in the parentheses and are normalized to the highest frequency anomeric proton (peak i) for each spectrum. This signal corresponds to the single β-mannosyl residue in each oligosaccharide.

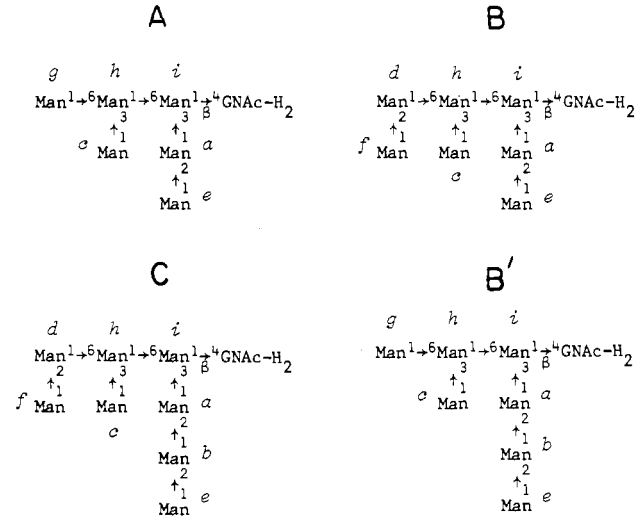

FIGURE 10: Postulated structures for the reduced GP-563 core oligosaccharides. (A) is rGP-563-III, (B) and (B′) are the two components in rGP-563-II, and (C) is rGP-563-I. The small letters correlate individual sugar residues with the anomeric proton resonances in the spectra shown in Figure 9. GlcNAc-H_2 is *N*-acetylglucosaminitol and, unless indicated otherwise, linkages are α.

identical and have the structure of compound **21**.

The H-1 signals for GP-402-III closely resemble those from ovalbumin oligosaccharide **22**, although three of the resonances show small reproducible differences in chemical shifts (Figure 8B). The IgM fragment has the composition $Man_6GlcNAc$, from integration of the mannose H-1 peaks, which agrees with the methylation analysis (Table V). The results also indicate that, although GP-402-III and **22** have the same linkages, they differ in sequence.

To simplify the H-1 spectra, several of the IgM oligosaccharides were reduced with borohydride, thus eliminating the H-1 signals for the reducing-end GlcNAc. The similarly treated reference heptasaccharide **22** is shown in Figure 4B. As anticipated, the GlcNAc H-1 signals disappear, but some of the mannose protons are also affected and not all of the shift changes have the same magnitude or direction. It is unlikely that this is an artifact caused by residual borate in the sample after reduction. First, borate complexation induces downfield shifts of H-1 signals (Gorin et al., 1968), and this effect is much less pronounced than the accompanying line broadening (see Figure 3). Peak f in Figure 4B shows a slight upfield shift, and none of the resonances is very broadened in comparison with the reducing oligosaccharide. Second, the peaks that do shift do not always correspond with the sugars that can bind borate ion. Thus, peaks a and g, assigned to mannoses expected to bind borate, are unchanged, whereas peaks b and c are shifted although they are from residues that lack vicinal hydroxyls. Finally, analogous results were obtained with reduced IgM core oligosaccharides, and we would not expect the same levels of borate to contaminate these different preparations.

^{1}H NMR Spectra of the Reduced Oligosaccharides. The three major oligosaccharide fractions from glycopeptide GP-563 were reduced, and the H-1 portions of their spectra are shown in Figure 9; Table VI lists the chemical shifts and integrations. By normalization of peak areas to the single β-mannosyl H-1 resonance at δ 4.79, chain length and homogeneity were estimated. The integral ratios suggest that rGP-563-III and rGP-563-I are homogeneous and have the compositions $Man_6GlcNAc\text{-}H_2$ and $Man_8GlcNAc\text{-}H_2$, respectively. On the other hand, the nonintegral ratios of rGP-563-II indicate that it must be a mixture. Because GP-563-II was eluted from a Bio-Gel P-4 column between a heptasaccharide and a nonasaccharide (Figure 7A), it was likely to be an octasaccharide of the form $Man_7GlcNAc$. The purified material was eluted as a single, symmetrical peak upon rechromatography, and its size homogeneity was confirmed by the NMR integrations. The sum of all α-mannosyl H-1 peaks relative to the β-mannosyl signal is 5.86, in close agreement with the predicted value of 6.00. We conclude that rGP-563-II is a mixture of isomers with the formula $Man_7GlcNAc\text{-}H_2$ and that it is composed of the two compounds that could serve as intermediates for the biosynthetic interconversion of GP-563-I and -III.

Both as the reducing compounds and after reduction, oligosaccharides GP-563-III and **22** gave similar NMR spectra. The absence of the shoulder, designated x in the ovalbumin fragment spectrum (Figure 4A), from the IgM oligosaccharide spectrum is the only significant difference. Therefore, GP-563-III probably is identical with **22**, and this conclusion is supported by the methylation results in Table V. Figure 10A

correlates the sugars in the reduced oligosaccharide with the H-1 NMR signals. From these data alone, it is unclear whether the terminal mannose unit labeled e in Figure 10A is 2-O-linked to residue c or residue a. Acetolysis studies described at the end of Results prove, however, that the structure given here is correct.

Four differences are apparent between the H-1 spectra of rGP-563-I (Figure 9C) and rGP-563-III (Figure 9A). The larger oligosaccharide (Figure 9C) has lost resonance g, and three new signals are observed at positions b, c,d, and e,f, a net gain of two anomeric protons on going from oligosaccharide III to I. This is consistent with the addition of two →2Man1→ units which are shown by methylation analysis to be present in GP-563-I.

Assigning the H-1 resonances for rGP-563-I was simplified by the constraint of having to fit these additional sugars to rGP-563-III. Inspection of the reference oligosaccharides in Table I indicates that substitution at the 2-hydroxyl of an α-mannosyl residue causes a large (0.10–0.25 ppm) downfield shift of the H-1 resonance. Thus, on comparison of the reduced oligosaccharides I and III (Figure 10), two new resonances should be apparent in the low-field portion of the H-1 spectrum of I. Peak b in Figure 9C is a candidate for one of these. Furthermore, the fact that the chemical shift for peak b (δ 5.282) is identical with that found for the internal sugar of α1→2-mannotriose (compound **6**, Table I) suggests that peak b arises from a similarly linked mannose; such a unit could be formed by 2-O-substitution of the terminal α1→2-linked mannose in rGP-563-III (residue e in Figure 10A). The second additional 2-O-substituted mannose in GP-563-I must come from what had been the terminal α1→6-linked sugar (residue g, Figure 10A) in the smaller oligosaccharide, because the corresponding resonance g in Figure 9A has disappeared from Figure 9C. Its new position would be similar to that found for the branched mannose in tetrasaccharide **15** in Table I (δ 5.107), and resonance c,d (δ 5.122) is nearest to this. Peaks c,d and e,f each integrate as two protons, however, and half of the latter peak could conceivably correspond to a 2-O-substituted mannose. It is more likely that peak e,f represents the two terminal α1→2-mannosyl H-1's, because its position (δ 5.042) is closer to that of H-1 for the nonreducing residues of compounds such as **2**, **6**, and **10**. We have therefore assigned the H-1 resonances of rGP-563-I to the structure in Figure 10C.

The H-1 spectrum of rGP-563-II (Figure 9B) appears to be a composite of the resonances found in the spectra of the smaller and larger rGP-563 oligosaccharides (parts A and C of Figure 9). Because of its homogeneous size, this sample must be a mixture of isomers of $Man_7GlcNAc$, and the methylation results (Table V) indicate that these are related to GP-563-I by the loss of a single →2Man1→ unit. Postulated structures for these isomers are given in Figure 10B,B′. If rGP-563-II were exclusively the compound in Figure 10B, only peaks a, c,d, e,f, h, and i in the ratio 1:2:2:1:1 would be expected, whereas the compound in Figure 10B′ would give a, b, c,d, e,f, g, h, and i all of equal intensity. By integrating the resonances in the spectrum in Figure 9B, the fraction of each isomer in rGP-563-II can be determined. Because peak i, assigned to the β-mannosyl H-1, arises from a single residue in both isomers, the ratio b/i or g/i gives the fraction of one component (Figure 10B′) in the mixture, and either c,d/i − 1 or e,f/i − 1 gives the fraction of the other (Figure 10B).[3]

[3] The value g/i was not used because overlap between peaks g and h made their integrations unreliable.

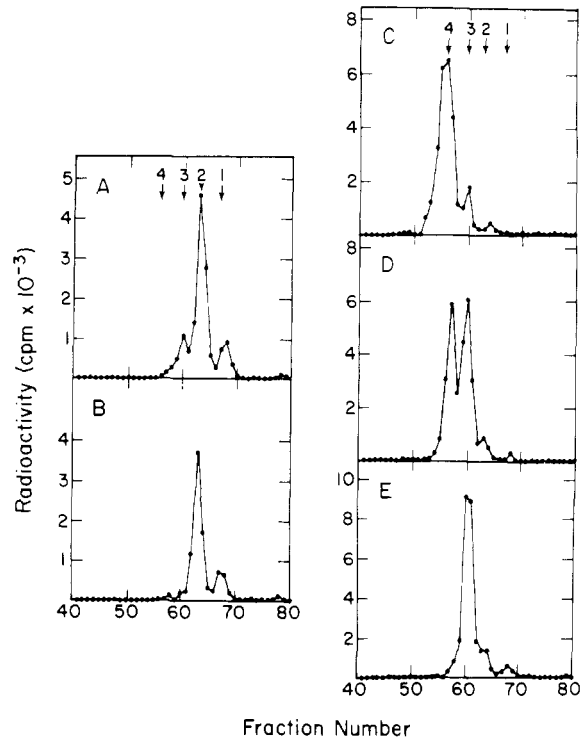

FIGURE 11: Gel filtration chromatography of partial acetolysis products from ^{3}H-reduced IgM mannose-rich oligosaccharides. Approximately 5×10^4 cpm of each oligosaccharide was acetolyzed for 10 h at 40 °C, and portions of the deacetylated products were applied to a Bio-Gel P-2 (−400 mesh) column (0.4 × 100 cm) and eluted with 0.01 M acetic acid. Fractions of 2 drops were collected and counted. The patterns are for (A) [^{3}H]rGP-402-III, (B) [^{3}H]rGP-402-IV, (C) [^{3}H]rGP-563-I, (D) [^{3}H]rGP-563-II, and (E) [^{3}H]rGP-563-III. The arrows indicate the elution positions of standards which were (1) reduced ManGlcNAc, (2) reduced $Man_2GlcNAc$, (3) reduced $Man_3GlcNAc$, and (4) reduced $Man_4GlcNAc$.

In this fashion, we calculate that structures B and B′ of Figure 10 comprise 40 and 60%, respectively, of the oligosaccharides in rGP-563-II.

Acetolysis of ^{3}H-Reduced IgM Mannose-Rich Oligosaccharides. Oligosaccharide fractions I, II, and III from GP-563 and III and IV from GP-402 were reduced with sodium borotritide, the products were acetolyzed, and the fragments thus obtained were separated by gel filtration (Figure 11). The major radioactive products from [^{3}H]-rGP-563-I and [^{3}H]rGP-563-III had the elution position of $Man_4GlcNAc$-H_2 and $Man_3GlcNAc$-H_2, respectively, whereas acetolysis of [^{3}H]rGP-563-II gave a mixture of both. These results are consistent with the structures in Figure 10, although the ratio of acetolysis fragments from [^{3}H]rGP-563-II ($Man_4GlcNAc$-H_2/$Man_3GlcNAc$-H_2 is ~1.0) does not agree exactly with the value of 1.5 predicted from the NMR data. Because the acetolysis reaction does not have absolute specificity for 1→6 linkages (Rosenfeld & Ballou, 1974), we consider the H-1 peak integrations to be more reliable.

The major radioactive product from the acetolysis of ^{3}H-reduced GP-402-III and GP-402-IV was $Man_2GlcNAc$-H_2 (parts A and B of Figure 11). The data support the conclusion that GP-402-IV has the structure of oligosaccharide **21**, whereas GP-402-III cannot have the same structure as GP-563-III, even though the two latter compounds have the same size and linkage composition. This agrees with the observation that the NMR spectra for GP-402-III and **22** differ, and a comparison in Figure 12 of spectra for the reduced class III oligosaccharides from each IgM (Ca) glycopeptide substantiates this. Unlike its GP-563 counterpart, GP-402-III cannot

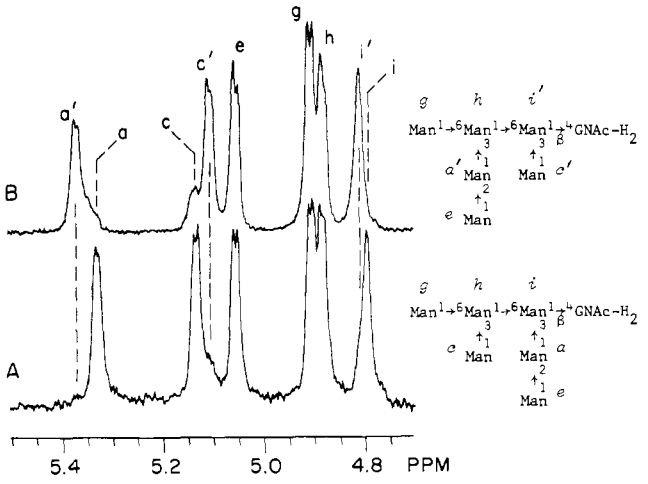

FIGURE 12: Anomeric proton NMR spectra for two isomeric IgM mannose-rich oligosaccharides. Spectrum A, rGP-563-III, is reproduced from Figure 9A, and spectrum B is for rGP-402-III. The small letters correlate the resonances with sugar residues for the structures shown. Chemical shifts (δ) for the major resonances in (B) are (a') 5.367, (c') 5.104, (e) 5.049, (g) 4.902, (h) 4.880, and (i') 4.805. Both spectra were acquired at a probe temperature of 55.0 °C. A minor component of GP-402-III (~15%) is the isomer shown for (A). This is suggested from peaks a and c in spectrum B and acetolysis experiments and is discussed in the text. Unless indicated otherwise, linkages are α; GNAc-H_2 is *N*-acetylglucosaminitol.

have a terminal α1→2-linked mannose attached to the residue identified as peak a in the figure; instead, the acetolysis and methylation results mandate 2-O-substitution of residue c or g. The effect of 2-O-substitution of residue g may be estimated by considering the analogous compound, rGP-563-I (Figure 10C). Here, substitution of the terminal α1→6-linked mannose resulted in a downfield shift of its H-1 resonance from δ 4.905 to 5.122 (see Figure 9). No such effect is seen in the spectrum for rGP-402-III (Figure 12B), so the oligosaccharide must have the structure and assignments shown. Note that, in the spectrum for rGP-402-III (Figure 12B), two shoulders correspond to resonances a and c of rGP-563-III (Figure 12A). This suggests the presence of some material identical with the GP-563 isomer and may explain why the acetolysate of ^{3}H-reduced GP-402-III contains some $Man_3GlcNAc$-H_2.

Discussion

Because mannose conforms to the general rule that equatorial hydrogens resonate at lower frequencies than their axial counterparts (Lemieux et al., 1958; Lemieux & Stevens, 1965), α and β anomers of either D-mannopyranose or D-mannopyranosides can be easily distinguished. For a given anomer, the H-1 resonance is also sensitive to the nature of the aglycon and ring substituents. These effects were first noted in glucose oligosaccharides (Van der Veen, 1963) and dextrans (Pasika & Cragg, 1963). 2-O-Substitution of β-glucosyl (Dedonder & Hassid, 1964) and α-mannosyl (Lee & Ballou, 1965a) residues deshields H-1, and differences attributed to reducing end substituents allowed Lee & Ballou (1965b) to distinguish α1→6 from other α linkages in myoinositol mannosides.

Comparison of the results in Table I illustrates the effects of ring substitution and glycosidic linkage. Relative to the shift for H-1 of α-D-mannopyranose (δ 5.165), the α-anomeric proton is deshielded by 0.20 ppm upon 2-O-substitution (**2**, residue A), whereas it is shielded by 0.02 ppm upon 3-O-substitution (**3**, residue A); neither 4- nor 6-O-substitution (**4** and **5**, residues A) has a significant effect, probably because these positions are too distant from the anomeric center. The axial 2-hydroxyl of mannose shields the equatorial H-1 (Descotes et al., 1970), and it is reasonable that 2-O-substitution should decrease the electron density about O–2, thereby deshielding H-1. The mechanism by which 3-O-substitution shields H-1 is not apparent.

Except for attachment to the 4-hydroxyl, which results in a 0.07-ppm downfield shift, formation of an α-glycosidic bond to position 2, 3, or 6 of a mannose shields H-1. These effects may reflect the relative electronegativity of reducing end substituents (Glass, 1965). The primary 6-hydroxyl is more electropositive than the ring hydroxyls, and therefore H-1 of an α1→6-mannosyl residue is most shielded. Chemical reactivities of pyranose secondary hydroxyls indicate that the 2-hydroxyl is the most acidic (Sugihara, 1953), a fact contrary to the greater shielding afforded by linkage to position 2 than to position 3 or 4. Moreover, the shielding of H-1 that results from glycosylation to neighboring sugars does not parallel the methoxyl proton chemical shifts in permethylated α-D-mannopyranoside (Haverkamp et al., 1975). It is unlikely, therefore, that the glycosidic proton chemical shift can be predicted from hydroxyl electronegativity alone; rather, the long-range influences of neighboring substituents, their configurations, and the linkage conformation[4] all need to be considered. A study of glucooligosaccharides led Usui et al. (1974) also to conclude that steric factors are an important determinant of H-1 chemical shifts.

The application of ^{1}H NMR to the study of oligosaccharide structure is empirical and is limited in accuracy by the variety of reference compounds available. We emphasize that the effects of substituents upon mannose H-1 resonances are not necessarily applicable to other sugars and should be extended to mannose in heteropolymers with caution. The fact that subtle sequence changes can produce effects upon distant mannosyl residues is a mixed blessing. One can distinguish between very similar oligosaccharide isomers such as the IgM core fragments GP-402-III and GP-563-III, and under favorable circumstances, even mixtures of isomers can be evaluated and quantitated, as was done with GP-563-II. Yet, because the H-1 resonance is so sensitive to overall structure, even a set of mannotriose standards encompassing all nearest-neighbor sequences would not suffice to establish empirical rules with which to predict the spectrum for every mannooligosaccharide.

Evidence that structural alterations can affect chemical shifts for anomeric protons of sugars three or more residues away includes (a) the 0.01-ppm deshielding experienced by both α1→3-mannosyl H-1 signals upon conversion of glycopeptide **23** to **21**, (b) the 0.02-ppm shift difference for H-1 of the terminal α1→3-linked mannose in **22** compared to the homologous residue in **21**, (c) the effect of reduction upon H-1 of distal sugars (Figure 4), (d) the different H-1 shifts of the isomeric IgM oligosaccharides in Figure 12, and (e) the 0.04-ppm variation of the GlcNAc α-anomeric proton shifts for the oligosaccharides in Table II. The results suggest that the H-1 resonance is sensitive to conformational differences between these compounds.

Three mechanisms by which H-1 can reflect oligosaccharide conformation are apparent. Though two residues may be widely separated along the primary sequence, the tertiary structure of an oligosaccharide may dictate that they be close together, thereby promoting through-space interactions that affect the H-1 resonance frequency. An alteration of tertiary structure could also influence the conformation of the sugar

[4] *Linkage conformation* is defined as the set of torsion angles that describes the orientation of two sugars connected by a glycosidic linkage (Rees & Scott, 1971).

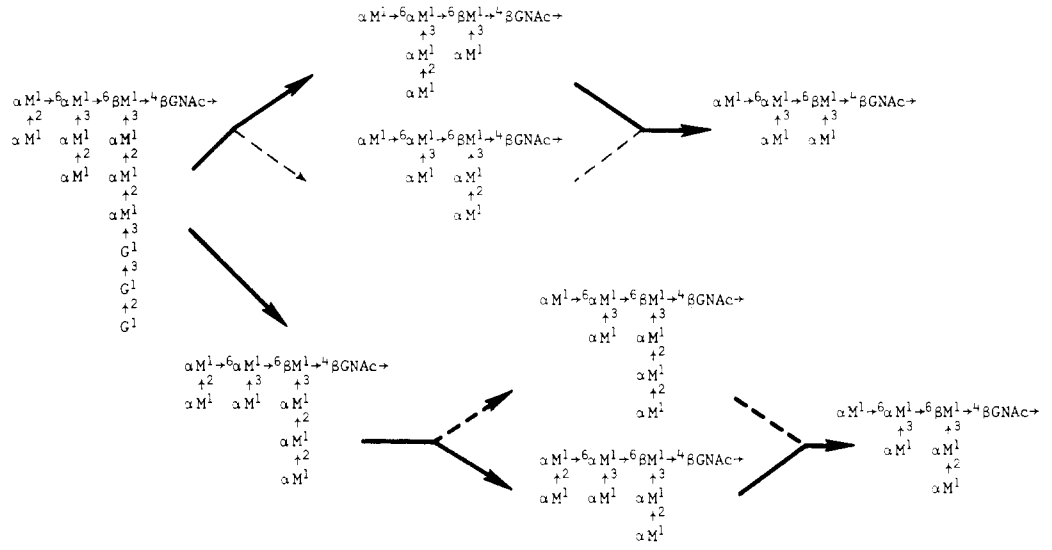

FIGURE 13: Hypothetical scheme depicting the final stages of mannose-rich oligosaccharide processing on human IgM. The glucose-containing precursor oligosaccharide was characterized by Li et al. (1978) and is from Chinese hamster ovary cells infected with vesicular stomatitis virus. A similar fragment is apparently transferred to the IgM protein, and processing of the carbohydrate on Asn-402 and Asn-563 proceeds along different paths. The upper route is for the Asn-402 oligosaccharide and the lower one for Asn-563. For IgM (Ca), each path contains a step that can give rise to two isomers; with Asn-402, one of these is minor, as signified by the lighter dashed arrows. IgM (Wa) may lack the alternative intermediates (Chapman & Kornfeld, 1979b) and also the secondary pathway (dashed arrows). M, mannose; G, glucose; GNAc, *N*-acetylglucosamine.

ring and ring substituents, either of which can affect the environment of H-1. Because we observed no increase in H-1/H-2 coupling constants, however, a gross change in ring conformation from $^4C_1$ to $^1C_4$ does not occur. Finally, the H-1 chemical shift is known to be a function of linkage conformation, as has been demonstrated with linear and cyclic α1→4-glucans (Casu et al., 1966). Constraining these oligosaccharides from acyclic to progressively tighter cyclic forms affects rotation about the glucosidic linkages, which changes the dihedral angle between H-1 and the O′-4–C′-4 bond of the linkage. This increases the diamagnetic shielding of H-1, moving its NMR resonance upfield. A similar phenomenon has been reported for 3-*O*-methylmannose oligosaccharides for which a ligand-promoted conformational change, interpreted as helix formation, is accompanied by an upfield shift of H-1 resonances (Yabusaki et al., 1979). In both examples the shift changes range from 0.20 to 0.34 ppm, which suggests that the relatively modest changes cited in the present study could easily arise from such effects.

Endo-β-*N*-acetylglucosaminidases are used widely for the analysis of glycoprotein carbohydrate structure (Kobata, 1979), and we have focused on fragments of the type produced by these enzymes. βMan1→4GlcNAc, a unit common to these oligosaccharides, can be identified by its ^{1}H NMR resonances. H-1 signals for the GlcNAc residue can be recognized by their reduced heights due to the a mixture of anomers and to the broadening effect of spin coupling. As the oligosaccharide is lengthened, the GlcNAc α-anomeric proton is deshielded from δ 5.20 to 5.23 (compare compounds **16**–**22**, Table II), a shift attributable to conformational differences. The β-mannosyl H-1 signal is identified by its upfield position. 3-O-Substitution of this mannose deshields its H-1 by 0.01–0.02 ppm, whereas 6-O-substitution has no effect. 2-O-Substitution, in analogy with its effect on the β anomers of reducing residues (compare **1** with **2**, **6**, and **9**, Table I), is expected to deshield ~0.02 ppm, but no fragment containing Man1→2βMan1→4GlcNAc has been analyzed. The β-mannosyl H-1 peak is a convenient reference for integration of a core oligosaccharide spectrum, particularly after reduction of the compound to remove interference by the small H-1 signal of the GlcNAc β anomer.

The major H-1 signals for the ovalbumin glycopeptides (**23** and **24**) and oligosaccharides (**21** and **22**) are consistent with the reported structures (Tai et al., 1975; Conchie & Strachan, 1978). A minor peak in the spectrum of **24** (not shown) and **22** (Figure 4A, peak x), however, could not be assigned. This resonance has been detected by other workers[2] and probably is not an artifact of our preparation. Because **22** is homogeneous in size, we suspect that it and the parent glycopeptide (**24**) are mixtures of isomers. Another possibility, that the minor resonance arises from a second stable conformer, has precedence from studies of permethylated glycolipids (Falk et al., 1979a,b). Such cannot apply here because peak x was absent from the spectrum of GP-563-III, which has the same structure as **22**. An ovalbumin glycopeptide corresponding to **24** has been fractionated into two components by concanavalin A–agarose affinity chromatography (F. Wold, personal communication). One of the components constitutes ~15% of the mixture and may be responsible for resonance x.

The purification of mannose-rich oligosaccharides from IgM (Ca) was aided by a fortuitous separation of the glycopeptides by gel filtration. This was due to the acidic character of Bio-Gel P-6, such that when eluted with water the gel tends to exclude negatively charged and retain positively charged molecules. Consequently, the *N*-acetylneuraminic acid containing glycopeptides were eluted first, followed by the mannose-rich species. Resolution of the latter into two fractions was a consequence of their differing amino acid compositions (Table III); the two His residues of the components in peak d caused it to elute behind peak c. Amino acid sequencing established that peaks c and d contained the oligosaccharides originating from Asn-563 and Asn-402, respectively, of the IgM μ chain.

Endo-β-*N*-acetylglucosaminidase digestion and gel filtration of glycopeptides GP-402 and GP-563 showed that each was heterogeneous in size, which agrees with observations of Chapman & Kornfeld (1979a,b) for another human IgM. Although our structural studies are in general agreement, Chapman & Kornfeld (1979b) found a single isomer of $Man_7GlcNAc$ among the Asn-563 core oligosaccharides whereas we find two in a 60:40 ratio. Their structure is

identical with that in Figure 10B. Similarly, the $Man_6GlcNAc$ fragments from Asn-402 of both proteins have the structure in Figure 12B, but we also detected a minor component (~ 15%) identical with GP-563-III (Figure 12A). These discrepancies could reflect a difference between the IgM proteins, or some isomers from IgM (Wa) may have escaped detection. The latter is possible because, of the techniques employed by Chapman and Kornfeld, only acetolysis had the ability to distinguish the isomers. This procedure is not absolutely selective for 1→6 linkages (Rosenfeld & Ballou, 1974), which makes it difficult to differentiate fragments generated by 1→6 bond breakage from other degradation products. Under such circumstances, ^{1}H NMR is clearly a useful adjunct for sequencing.

The discovery of these additional IgM core fragments necessitates modification of the scheme presented by Chapman & Kornfeld (1979b) for oligosaccharide processing. Work from several laboratories (Robbins et al., 1977; Tabas et al., 1978; Hunt et al., 1978; Staneloni & Leloir, 1978; Chen & Lennarz, 1978; Kornfeld et al., 1978) indicates that the mannose-rich oligosaccharides are formed by glycosidase digestion of a common precursor, $Glc_3Man_9GlcNAc_2$, after it is transferred en bloc from dolichol pyrophosphate to protein. Chapman & Kornfeld (1979b) proposed that because each set of Asn-linked mannose-rich oligosaccharides in IgM (Wa) appeared unique, processing of the carbohydrate at each site may also follow a unique pathway. Our results generally corroborate this and support the idea that the mode of processing may be influenced by the protein structure at the glycosylation site. However, the isomers of $Man_7GlcNAc$ for Asn-563 and $Man_6GlcNAc$ for Asn-402 indicate that these pathways may proceed through alternative intermediates, as shown in Figure 13. The possibility was considered by Chapman & Kornfeld (1979a) that, rather than representing biosynthetic intermediates, the assortment of IgM mannose-rich oligosaccharides may result from degradation of the molecules after secretion into the serum. The close similarity of our structural results with theirs makes this explanation of the oligosaccharide heterogeneity less likely.

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