The structures of the predominant high mannose oligosaccharides present in a human IgM myeloma protein (Patient Wa) have been determined. The IgM glycopeptides, produced by pronase digestion, were fractionated on DEAE-cellulose and CM-cellulose which, respectively, to which high mannose oligosaccharides are attached in IgM (Patient Ou) (Putnam, F. W., Florent, G., Paul, C., Shinoda, T., and Shimizu, A. (1973) Science 182, 287-290). The high mannose glycopeptides in IgM (Wa) exhibit heterogeneity in the oligosaccharide portion. Structural analysis of the major oligosaccharides indicates that the simplest structure is:

\[
\begin{align*}
\text{Man}_{4} & \rightarrow 6\text{Man}_{4} \rightarrow 6\text{Man}_{4} \rightarrow 4\text{GlcNAc} \beta_{1} \rightarrow 4\text{GlcNAc} \\
\uparrow \text{Al}3 & \uparrow \text{Al}3 \\
\text{Man} & \text{Man} \\
\text{Asn} & 
\end{align*}
\]

The larger oligosaccharides present have additional mannose residues linked 1 → 2 to terminal mannose residues in the above structure. Glycopeptide I contains primarily \text{Man}_, and \text{Man}_, species, while glycopeptide II contains \text{Man}_, and \text{Man}_, species. The two \text{Man}_, oligosaccharides have different branching patterns.

Human IgM immunoglobulins contain both complex and high mannose-type oligosaccharides linked to asparagine residues on the heavy chain of the protein. Amino acid sequence and carbohydrate analysis performed on the human IgM (Ou) by Putnam and co-workers, has shown that this myeloma protein contains two high mannose oligosaccharides, one attached to Asn 402, and the other to Asn 563 (1, 2).

Structural studies carried out by Hickman et al. (3) on a high mannose glycopeptide derived from the Asn 563 region of a different human IgM myeloma protein indicated that the oligosaccharide moiety was heterogeneous in respect to the number of mannose residues per chain. The methods used in that study provided partial characterization of the oligosaccharide structure of the glycopeptide, but the mixture of high mannose chains was not separated. Subsequently the enzyme endo-\(\beta\)-N-acetylgalactosaminidase was isolated from several sources and shown to have the very useful property of cleaving the \(N,N\)_-diacetylmuramoditol linkage in the core of most high mannose glycopeptides, thereby releasing the oligosaccharide chains and leaving the linkage \(N\)-acetylgalactosamine residue attached to asparagine in the peptide (4).

Thus, it is now possible to release such high mannose oligosaccharide chains, to separate them according to size, and, by employing a variety of techniques including acetylation, methylation and periodate oxidation, to determine their complete structure. Using this approach, we have now determined the structures of the high mannose oligosaccharides that occur on two glycopeptides isolated from the human IgM myeloma protein (Wa) and shown that both glycopeptides display heterogeneity in their oligosaccharide moieties.

**EXPERIMENTAL PROCEDURES**

Isolation of IgM Glycopeptides—Serum was obtained from a patient (Wa) with Waldenstrom's macroglobulinemia. Purification of IgM from this serum was accomplished as described by Hickman et al. (3). The purified protein was shown to be greater than 99% pure by zone electrophoresis and immunoelectrophoresis. One gram of purified IgM was subjected to pronase digestion with 1% (w/v) pronase in 0.1 M Tris buffer, pH 7.9, at 60°C for 25 h, with a further addition of pronase at 12 h. At the end of the incubation time, the sample was boiled, the precipitate was spun out, and the supernatant was applied to a Sephadex G-25 column (3.5 x 80 cm). The column was eluted with water, and the hexose-containing fractions, which eluted in the void volume, were pooled. This material was retreated with pronase and was fractionated again on the G-25 column. The hexose-containing material from the second G-25 column was pooled and subjected to ion exchange chromatography on a column of DEAE-cellulose (Whatman DE52) as shown in Fig. 1A. Fraction I from the DE52 column contained both high mannose and complex glycopeptides and was further fractionated on a column of CM-cellulose (Whatman CM52) to separate a high mannose type glycopeptide, I, from a complex glycopeptide, A, as shown in Fig. 1B.

Enzymes—\(\alpha\)-Mannosidase and \(\beta\)-N-acetylglucosaminidase were purified from jack bean meal by the method of Li and Li (5). \(\beta\)-Mannosidase was purified from hen ovomucoid by the method of Sulkove et al. (6). Clostridial endo-\(\beta\)-N-acetylglucosaminidase \(C_{1}\) was a kind gift from Dr. Y. T. Li (Tulane University), and was also prepared in this laboratory by the method of Ito et al. (7). Both preparations were found to be free of \(\alpha\)-mannosidase and \(\beta\)-N-acetylglucosaminidase. Pronase, grade D, was purchased from Calbiochem.

**Analytical Methods**—Analysis of the monosaccharides present in the glycopeptides was performed by gas-liquid chromatography of their trimethylsilyl derivatives, as described previously (8). Mannose liberated by exoglycosidase digestion of the glycopeptides as well as sialic acid, total hexose, and \(N\)-acetylglucosamine were measured as described previously (9). Radiochromatogram scanning was performed with a TMC Vanguard Instrument Corp. dual channel autoscanner, model 880-D. Radioactivity was determined with a Beckman liquid scintillation spectrometer on samples in 1 ml of water and 3 ml of 3a70 scintillation mixture (Research Products, International). Amino acid analysis was performed as previously described (9).
Paper Chromatography—Descending paper chromatography was performed on Whatman No. 1MM using the following solvent systems: Solvent I, ethyl acetate/pyridine/acetic acid/water (5:5:1.3); and Solvent II, n-butyl alcohol/pyridine/water (4:3:4).

Paper Electrophoresis—Paper electrophoresis was performed using the following systems: A, 0.06 M borate buffer, pH 9.5, at 40 V/cm for 24 h with Whatman No. 1MM paper, which was used to separate mannitol from glucosaminol after methanalysis of reduced samples in 1.5 M methanolic HCl for 4 h at 37°C (10); B, 0.1 M molydate buffer, pH 5.5, at 20 V/cm for 1 h (with Whatman No. 3MM paper) to be used to separate Man1 → 2 mannitol from Man1 → 3 mannitol (11).

Exoglycosidase Digestion—All exoglycosidase digestions were performed at 37°C as follows: a-Mannosidase digestions were performed with 20 μl of enzyme (50 units/ml) in 0.05 M sodium acetate buffer, pH 4.6, for 6 h; β-mannosidase digestion was performed with 50 μl of enzyme (1.6 units/ml) in 0.05 M citrate phosphate buffer, pH 5.0, for 48 h; and β-N-acetylglucosaminidase digestions were performed with 50 μl of enzyme (2.4 units/ml) in 0.05 M sodium citrate buffer, pH 4.6, for 48 h. A unit of activity is the amount of enzyme which cleaves 1.0 μmol of the appropriate p-nitrophenyl glycoside in 1 min.

Endoglycosidase Digestion—One micromole of glycopeptide was subjected to digestion with 80 μl of endo-β-N-acetylglucosaminidase C1 (60 millunits/ml in 400 μl of 0.05 M citrate/phosphate buffer, pH 7.0, at 37°C) for 48 h. Over 90% of the oligosaccharide portion of glycopeptides I and II were cleaved under these conditions.

Reduction of Oligosaccharides—The pH of the reaction mixture was adjusted to 10.0 with 0.2 N NaOH. For endoglycosidase-digested samples, one-fourth of the sample was transferred to a separate tube and reduced with 30 μl of NaBH4 (210 mCi/ml) in dimethylformamide (8 mCi/ml). To the remaining sample, 100 μl of NaBH4 (50 mg/ml in 0.05 N NaOH) was added. The mixtures were kept at 30°C for 5 h, then 25% or 75% of glacial acetic acid were added to destroy excess borohydride. The samples were evaporated with 1% acetic acid at 60°C on a rotary evaporator (Buchler Instruments, New York) in order to remove methylborates. The samples were then combined, and the reduced oligosaccharides were separated from buffer salts and digested peptide material on a column of Amberlite CG-50 (60 milliunits/ml) in 400 μl of 0.05 M citrate/phosphate buffer, pH 7.0, at 37°C for 48 h.

Acetylation—Fifty nanomoles of oligosaccharide was subjected to acetylation as described by Ta et al. (12), except that the incubation period was shortened to 12 h at 35°C and, at the end of the incubation period, the sample was extracted directly into 0.35 mM sodium bicarbonate.

Periodate Oxidation—Periodate oxidation of the glycopeptides was performed as described by Baenziger et al. (8).

Smith Periodate Degradation—Samples were oxidized as described by Spiro (13). Hydrolysis of the samples was carried out in 0.1 M H2SO4 at 80°C for 80 min. Hydrolysates were desalted over columns (0.5 × 1.5 cm) of Dowex AG-3 X4 (100 to 200 mesh, acetic form).

Methylation Analysis—The oligosaccharides and glycopeptides were methylated by the method of Hakomori (14). The permethylated products were subjected to acetylation, reduction, and acetylation as described by Stettler et al. (15). The alditol acetates of the partially methylated sugars were separated and analyzed on a Finnigan gas chromatograph-mass spectrometer (model 3200 or 3300). Separation was achieved on columns of either 1% OV-17 or 1% OV-1 on 80/100 mesh Q (Applied Science). ECNSS-M was operated at 170°C for 20 min and then programmed at 4°C/min to 240°C to separate 2,3,4-, and 2,3,4,6-trimethylmannose. OV-17 runs were programmed at 4°C/min from 140 to 240°C. Mass spectra were recorded with a separator temperature of 240°C, ionization potential of 70 eV, ionization current of 60 μA, and ion source temperature of 270°C. Injection temperature was 260°C.

Identification of sugar derivatives was achieved by comparison of retention times and mass spectra with those known for standard compounds (16-18). Molar ratios of sugar derivatives from methylation of intact glycopeptides, released oligosaccharides, and periodate fragments were determined by measuring the area under the peaks of the F & M and F & Q detector scan obtained by gas chromatography using an F & M 402 gas chromatograph with either a 1% OV-17 column or 3% ECNSS-M column at 140 + 3°C/min (use of the ECNSS-M column with this program provided separation of the 2,4-dimethylmannose peak from a non-sugar contaminant which led to anomalous ratios in the OV-17 runs).

When reduced oligosaccharides were methylated (Tables IV and V), the two derivatives of 4-monosubstituted N-acetylgalactosaminol were observed. They were 1,3,5,6-tetra-O-methyl-N-methyl-N-acetylglucosaminol, which is the expected derivative, and 1,3,5,6-tetra-O-methyl-N-diacetylgalactosaminol, which elutes later on gas-liquid chromatography and has a different mass spectrum than the N-methyl-N-acetyl derivative. Finne and Ruvala (19) have shown that permethylated 2-acetamido-2-deoxyhexosyl ethers, in contrast to permethylated 2-acetamido-2-deoxyhexosylamino derivatives, undergo some N-demethylation during the acetylation and acid hydrolysis steps of the isolation process. Used in this study. During the subsequent acetylation step, to prepare derivatives for gas-liquid chromatography, the N-demethylated product is converted to the N-acetylatedamino derivative.

Materials—NaBH4 was purchased from New England Nuclear. Other chemicals were reagent grade and were purchased from commercial sources. Mannose-[1H]glucosaminol standards were purified from Chinese hamster ovary cell glycopeptides by E. Li, who determined their composition and structure.

RESULTS

Fractionation of the pronase-digested IgM glycopeptides on DEAE- and CM-cellulose columns resulted in six major glycopeptide fractions (Fig. 1). Fractions A, B, C, and D consist of complex type glycopeptides containing mannose, N-acetylglucosamine, galactose, fucose, and (except for Fraction A) sialic acid. The structural analysis of these glycopeptides will be the subject of a future report.

Fractions I and II are high mannose-type glycopeptides, as shown by their monosaccharide and amino acid compositions (Table I). In order to determine the sequence of sugars and the linkages involved in these two glycopeptides, they were subjected to methylation analysis, exoglycosidase digestion, and periodate oxidation. The methylation results shown in Table II indicate that both glycopeptides contain 3 terminal mannose residues, and that none of the N-acetylgalactosamine is terminal. Both glycopeptides contain fractional residues of mannose substituted at position 2, indicating heterogeneity of the oligosaccharides. Based on the total number of mannose residues and the pattern of methylated mannose derivatives, 2 disubstituted mannose residues (branch points) are expected for each glycopeptide. The value of 2.3 residues of 2,4-dimethylmannose in glycopeptide I is most likely due to a non-sugar contaminant that co-chromatographs with 2,4-dimethylmannose. To confirm this explanation, the glycopeptides were subjected to periodate oxidation, which destroys sugars containing vicinal hydroxyls. The disubstituted mannoses and the 2 N-acetylgalactosamine residues should be the only sugars resistant to oxidation. Results in Table III show that in each case 2 residues of mannose and both residues of N-acetylgalactosamine survived periodate oxidation.

Exoglycosidase Digestion of Intact Glycopeptides—α-Mannosidase digestion was performed at 37°C on each glycopeptide in each glycopeptide (Table II). The remaining mannosidase was partially removed with β-mannosidase treatment of the α-mannosidase-digested core with both β-mannosidase and β-N-acetylglucosaminidase, but not with β-N-acetylglucosaminidase alone, resulted in release of N-acetylgalactosamine and mannose. These results, in conjunction with the methylation results, indicate that the structure of the product of α-mannosidase digestion in each glycopeptide is:

\[ \text{Man}(1 \rightarrow 4 \text{GlcNAc}2 \rightarrow 4 \text{GlcNAc} \rightarrow \text{Asn} \]

1. The Man-[3H]GlitolNAc standard was derived from a glycopeptide with the structure shown in the discussion as the common oligosaccharide structure (20). The structures of the Man, Man, and Man2 standards have also been determined by methylation analysis, acetylation, and periodate oxidation and will be published (E. Li, and S. Kornfeld (1979) J. Biol. Chem., in press).
Major High Mannose Oligosaccharides of IgM

Fig. 1. Top, DEAE-cellulose chromatography of hexose-containing fractions from the Sephadex G-25 column. The DEAE-cellulose (Whatman DE52) column (2 X 15 cm) was equilibrated in 0.0075 M Tris, pH 7. Four-milliliter fractions were collected. After washing the column with 200 ml of starting buffer, elution was carried out with a linear gradient (200 ml each) of 0.2 M NaCl in 0.0075 M Tris. Each 4-ml fraction was assayed for hexose (O--O) and sialic acid (0--0). Fractions were then pooled as noted. Bottom, CM-cellulose chromatography of DEAE-fraction IDE. The CM-cellulose column (0.9 x 14 cm) was equilibrated in 0.005 M sodium acetate buffer, pH 5.5. Elution was carried out with 200 ml of starting buffer. Every other tube was assayed for hexose, and fractions were then pooled as indicated.

### TABLE I

Carbohydrate and amino acid compositions of high mannose glycopeptides I and II

| Residues | I            | II           |
|----------|--------------|--------------|
|          | mol/mol glycopeptide |
| Man      | 5.8 ± 0.3    | 6.7 ± 0.2    |
| GlcNAc   | 2.0          | 2.0          |
| Amino acid analysis |
| GlcNAc   | 1.9          | 2.0          |
| Asx      | 1.0          | 1.0          |
| Ser      | 0.5          | 1.0          |
| Thr      | 0.60 ± 0.15  | 0.60 ± 0.15  |
| Glx      | 0.30 ± 0.05  | 0.30 ± 0.05  |
| Ala      | 0.80 ± 0.20  | 0.80 ± 0.20  |
| Val      | 0            | 1.13         |
| Gly      | 0.65 ± 0.23  | 0.65 ± 0.23  |
| Pro      | 1.0          | 0            |
| His      | 0.8          | 0            |

"In the carbohydrate analysis, GlcNAc was set to 2.0, and in the amino acid analysis Asx was set to 1.0.

### TABLE II

Methylation of glycopeptides I and II

| Glycopeptide | Glycopeptide methylmannosyl | Glycopeptide methylmannosyl | Glycopeptide methylmannosyl | Glycopeptide methylmannosyl |
|--------------|----------------------------|----------------------------|----------------------------|----------------------------|
|              | 2,3,4,6-Tetramethylmannose  | 3,4,6-Tri-methylmannose    | 2,4-Dimethylmannose        | 3,6-Dimethylmannosyl(GM1)  |
| I            | 2.0                        | 0.8                        | 2.5                        | 2.0                        |
| II           | 2.0                        | 2.4                        | 2.0                        | 2.0                        |

"The proportions were determined by setting 3,6-dimethyl-N-acetylglucosamine = 2.0 residues.

Separation of Oligosaccharides Released from Glycopeptides I and II—Results obtained by methylation of the intact glycopeptides suggested that heterogeneity existed in the oligosaccharide portion of the glycopeptides. Therefore, glycopeptides I and II were digested with endo-β-N-acetylglucosaminidase C10, which cleaves the di-N-acetylchitobiose core in most high mannose glycopeptides. The released oligosaccharides were reduced with NaBH₄, and fractionated on a Bio-Gel P-4 column. As shown in fig. 2, both glycopeptide I and glycopeptide II gave rise to two radioactive oligosaccharide fractions. Each of the four oligosaccharide fractions was then subjected to descending paper chromatography in Solvent I for 4½ days to further resolve the component oligosaccharide chains. In Fig. 3, the radioactivity profiles of the chromatograms reveal that the major oligosaccharide components originating from glycopeptide II co-migrated with Man₅GlcitolNAc (IIA), and Man₅GlcitolNAc (IIB). The oligosaccharide chains in Fraction IB separated into two components, which co-migrated with Man₅GlcitolNAc (IB-2) and Man₅GlcitolNAc (IB-1). Fraction IA was resolved into four components which co-migrated with oligosaccharides of varying size from Man₅ to Man₁₅GlcitolNAc.

### TABLE III

Effects of glycosidases and periodate oxidation on glycopeptides I and II

| Residues released or destroyed | Residues remaining* |
|-------------------------------|---------------------|
| Mannose | GlcNAc | Mannose | GlcNAc |
| Intact glycopeptide I | Periodate oxidation | 3.8 | 0 | 5.8 ± 0.3 | 2.0 |
| α-Mannosidase | Then β-mannosidase | 0.5 | 0.43 | 0.43 | 0.41 |
| Or then β-mannosidase + β-N-acetylglucosaminidase | | | | |
| Intact glycopeptide II | Periodate oxidation | 4.7 | 0 | 6.7 ± 0.2 | 2.0 |
| α-Mannosidase | Then β-mannosidase | 0.5 | 0.41 | 0.41 | 0.40 |
| Or then β-mannosidase + β-N-acetylglucosaminidase | | | | |
| Or then β-N-acetylglucosaminidase | | | | |

* Determined by isolation of product and sugar analysis by gas-liquid chromatography of the trimethylsilyl derivatives.

Fig. 2. Elution profile of oligosaccharides on Bio-Gel P-4 (200 to 400 mesh). Glycopeptides I and II were treated with endo-β-N-acetylglucosaminidase C10 to release the oligosaccharides which were reduced with NaBH₄, and applied to a column (1.5 x 90 cm) of Bio-Gel P-4 equilibrated with 0.1 M NH₄HCO₃. One-milliliter fractions were collected. An aliquot of every other fraction was assayed for radioactivity, and fractions were pooled as indicated.
low complete elucidation of the oligosaccharide structures. Fig. 4 shows a schematic diagram for the structural studies performed using oligosaccharide IIA as an example.

Exoglycosidase Digestion of Released Oligosaccharides—In order to confirm that each of the released oligosaccharides had the expected composition \((\alpha\text{-Man})_7\beta\text{-Man}\text{GlcitolNAc}\), samples were digested first with \(\alpha\)-mannosidase and then with \(\beta\)-mannosidase. The products of glycosidase digestion were chromatographed in Solvent II for 19 h with appropriate standards. Results for all the oligosaccharides were identical to those shown in Fig. 5 for oligosaccharide IIA. One treatment with \(\alpha\)-mannosidase converted the oligosaccharides to \(\text{Man}_3\beta\rightarrow\text{Man}_4\text{GlcitolNAc}\) (Fig. 5A). One subsequent treatment with \(\beta\)-mannosidase released about 50% of the \(\beta\)-linked mannose, giving rise to a radioactive product that co-migrated with GlcitolNAc (Fig. 5B). When the residual disaccharide was pooled as shown and retreated with \(\beta\)-mannosidase, one-half of it was cleaved and migrated with GlcitolNAc on rechromatography.

Smith Degradation—In order to determine the linkage of the outer branch point mannose to the inner one (Fig. 4), oligosaccharides were subjected to Smith degradation. The product isolated after one round of treatment in every case was a component that migrated as a trisaccharide in Solvent II (Fig. 5C). Methylation of the isolated product, in each case, gave rise to 2,3,4,6-tetramethylmannose and 2,3,4-trimethylmannose in a 1:1 ratio, indicating a 1→6 linkage between the 2 branch point mannose residues (see Table V for the methylation results).

Further Structural Studies of Oligosaccharide IIA (\(\text{Man}_3\text{GlcitolNAc}\))—Methylation of IIA reveals the presence of 3 terminal nonreducing mannose residues, two 3,6-disubstituted mannose residues, and 3 mannose residues substituted only on position 2 in addition to N-acetylglucosaminitol substituted on position 4 (Table IV). Acetolysis which cleaves 1→6 linkages preferentially (12) was performed on the oligosaccharide (Fig. 4). Fig. 6 (Panel A) shows the paper chro-

![Fig. 3. Paper chromatography of Bio-Gel P-4 fractions IIA, IIB, IA, and IB. Each fraction was subjected to paper chromatography in Solvent I for 4½ days. A small aliquot was spotted as an indicator strip and after chromatography the paper was cut into 1-cm segments which were counted for radioactivity. The remainder of each sample was streaked on the paper which was scanned for radioactivity, and the areas corresponding to the bracketed peaks indicated in the figure were eluted. Standards M₅₀ are Mannoses.][1]

![Fig. 4. Scheme for characterization of oligosaccharide IIA. In the original structure (left): M, mannose residues resistant to Smith periodate degradation and * sugar has been reduced with NaBH₄. Right: X, xylosaminitol, which is the Smith degradation product of N-acetylglucosaminitol.][2]

![Fig. 5. Exoglycosidase digestion and Smith degradation of oligosaccharide IIA. A, \(\alpha\)-mannosidase alone; B, \(\beta\)-mannosidase digestion of the product of the \(\alpha\)-mannosidase digestion; C, fragment resulting from Smith degradation of oligosaccharide IIA. The samples were chromatographed for 19 h in Solvent II with the following standards: 1, oligosaccharide IIA; 2, \(\text{Man}_1\rightarrow\text{Man}_3\rightarrow4[^3\text{H}]\text{GlcitolNAc}\); 3, \(\text{Man}_3\beta\rightarrow4[^3\text{H}]\text{GlcitolNAc}\); 4, \([^3\text{C}]\text{N-acetylglucosaminitol}\).][3]
matographic separation of the unreduced acetolysis fragments which are labeled only in N-acetylglucosaminitol. The major labeled species migrated as a pentasaccharide and a minor species migrated as a tetrasaccharide. Methylation (Table V) of the pentasaccharide indicates that it has the structure Man \( \rightarrow 2 \) Man \( \rightarrow 2 \) Man \( \rightarrow 3 \) Man \( \rightarrow 4 \) GlcitolNAc. The tetrasaccharide has the structure Man \( \rightarrow 2 \) Man \( \rightarrow 3 \) Man \( \rightarrow 4 \) GlcitolNAc and probably results from overdegradation of the pentasaccharide. Reduction of the acetolysis products with NaB\( _3 \)H\(_4\) and fractionation in Solvent II (Fig. 6B) resulted in the appearance of a large disaccharide peak and a smaller mannitol peak. When the disaccharide peak was subjected to paper electrophoresis in molybdate buffer, which separates Man \( \rightarrow 3 \) mannitol from Man \( \rightarrow 2 \) mannitol, two peaks were observed as shown in Fig. 7. Methylation (Table V) of the material in these two peaks confirmed that disaccharide 1 was Man \( \rightarrow 3 \) mannitol and disaccharide 2 was Man \( \rightarrow 2 \) mannitol as expected. Spectra for the two different methylated mannitol species are shown in Fig. 8, with their fragmentation patterns. Both of these methylated mannitolos have the same retention time relative to tetramethylmannose (Trel = 0.45) on 1% OV-17 (160°C isothermal). The monosaccharide peak from the reduced acetolysis mixture (Fig. 6B) is mannitol which is the expected by-product from overdegradation of the pentasaccharide fragment. The structure proposed for oligosaccharide IIA on the basis of these results is shown in Fig. 9.

Further Structural Studies on Oligosaccharide IIB

Methylation of this oligosaccharide indicates that it contains 3 terminal nonreducing mannose residues, two 3,6-disubstituted mannose residues, and 1 mannose residue substituted on position 2 in addition to N-acetylglucosaminitol substituted on position 4. Acetolysis produced a

### Table IV

| Methylated sugar | IB-1 | IB-2 | IIA | IIB |
|------------------|------|------|-----|-----|
| Mannose          |      |      |     |     |
| 2,3,4,6-Tetramethyl | 2.9 | 2.9  | 2.9 | 2.9 |
| 3,4,6-Triethyl   | 1.0  | 0    | 3.0 | 1.0 |
| 2,4-Dimethyl     | 2.0  | 2.0  | 2.0 | 2.0 |
| N-Acetylglucosaminitol |      |      |     |     |
| 1,3,5,6-Tetra-O-Me-N-Me | +  | +    | +  | +  |
| 1,3,5,6-Tetra-O-Me-N-AC | +  | +    | +  | +  |

Proportions were determined by setting 2,4-dimethylmannose = 2.0 residues.

a. +, sugar is present but not quantitated.

### Table V

| Fragments | 2,3,4,6-TetraMe | 3,4,6-TriMe | 2,4,6-TriMe | 2,3,4-TriMe |
|-----------|----------------|-------------|-------------|-------------|
| IIA | 1.0 | 1.7 | 1.1 | 1.1 |
| Disaccharide 1 | + | + | + | + |
| Disaccharide 2 | + | + | + | + |
| Smith periodate fragment | 1.0 | 1.0 | 1.0 | 1.0 |
| IIB | 1.0 | 0.9 | 0.9 | 1.1 |
| Disaccharide | + | + | + | + |
| Smith periodate fragment | 1.0 | 1.0 | 1.0 | 1.0 |
| IB-1 | 1.0 | 1.0 | 1.0 | 1.0 |
| Disaccharide | + | + | + | + |
| Smith periodate fragment | 1.0 | 1.0 | 1.0 | 1.0 |
| IB-2 | 1.0 | 1.0 | 1.0 | 1.0 |
| Disaccharide | + | + | + | + |

a. Peak area of the methylated sugar species which is set to 1.0.

b. +, sugar species is present.
tetrasaccharide labeled in N-acetylglucosaminitol (Fig. 6C) as well as a disaccharide and monosaccharide which, after NaB₃H₄ reduction, could be detected as a disaccharide alditol and mannitol (Fig. 6D). Methylation of the tetrasaccharide (Table V) showed that it has the structure Man₁ → 2Man₁ → 3Man₁ → 4GlcitolNAc. Methylation of the disaccharide showed that it was Man₁ → 3 mannitol. The structure of oligosaccharide IIB is shown in Fig. 9.

Further Structural Studies of IB-1 (Man₃GlcitolNAc)

Methylation analysis (Table IV) of this oligosaccharide indicates that it has 3 terminal nonreducing mannose residues, two 3,6-disubstituted mannose residues, and 1 mannose residue substituted on position 2 in addition to N-acetylglucosaminitol substituted on position 4. Acetolysis was performed and Fig. 6 (Panel E) shows that the GlcitolNAc-labeled fragment migrated as a trisaccharide. The product of α-mannosidase treatment of this trisaccharide co-migrated with Manβ₁ → 4 GlcitolNAc. Data from periodate oxidation and methylation of intact IB-1 showed that the linkages in the inner structure must be:

\[
\text{Man₁ → 6Man₁ → 6Man₁ → 4GlcitolNAc}
\]

and since 1 → 6 bonds are broken in acetolysis, the GlcitolNAc containing trisaccharide from the acetolysis procedure must be Man₁ → 3Man₁ → 4GlcitolNAc. Paper chromatography of the acetolysis mixture after reduction with NaB₃H₄ produced the pattern seen in Fig. 6 (Panel F). The trisaccharide peak is now larger, and there is also a disaccharide peak and mannitol. Treatment of the trisaccharide material with α-mannosidase gave rise to radioactive mannitol and Man₁ → 4GlcitolNAc, indicating that it was a mixture of two trisaccharides, one containing mannitol and the other GlcitolNAc. Since the two trisaccharides could not be separated adequately by chromatography in Solvent II or by borate electrophoresis, the mixed trisaccharide material was methylated directly. Methylation gave rise to tetramethylmannose, 3,4,6-trimethylmannose, 2,4,6-trimethylmannose, the two methylated derivatives of 4-monosubstituted GlcitolNAc, and 1,2,4,5,6-pentamethylmannitol. These data indicate that the trisaccharides have the following structures: Man₁ → 3Man₁ → 4GlcitolNAc and Man₁ → 2Man₁ → 3Man₁. When the reduced disaccharide fragment was subjected to α-mannosidase treatment, all of the radioactivity had the mobility of mannitol. When the disaccharide was subjected to molybdate electrophoresis, two peaks were observed, which co-migrated with Man₁ → 2Man and Man₁ → 3Man (varying amounts of these two components were observed in three different acetolysis experiments.) These disaccharides are presumed to arise from overdegradation of the Man₁ → 2Man₁ →

![Fig. 7. Molybdate electrophoresis of IIA acetolysis disaccharide fragment. The reduced disaccharides resulting from acetolysis of IIA (see Fig. 6, Panel B) were eluted and subjected to electrophoresis in 0.1 M sodium molybdate buffer, pH 5.5, at 20 V/cm for 1% hours on Whatman No. 3MM paper. The paper was then scanned for radioactivity. Peaks 1 and 2 were eluted, desalted, and methylated.](image1)

![Fig. 8. Mass spectra and fragmentation schemes of (A) 1,2,4,5,6-pentamethylmannitol acetate and (B) 1,3,4,5,6-pentamethylmannitol acetate. Both samples had a retention time relative to 2,3,4,6-tetramethylmannitol acetate of 0.45 on 1% OV-17 at 160°C.](image2)

![Fig. 9. Structures present in glycopeptides I and II from IgM (Wa).](image3)
3-Mannitol trisaccharide at positions 1 → 2 or 1 → 3. The large free mannose peak suggests that overdegradation is occurring. Still, the presence of a small amount of a Man₆ species which has a branching pattern containing the two disaccharides cannot be excluded. The amount of mannotol label in the tri- and disaccharide peaks is roughly equal. If there were no overdegradation in acetylation, this would mean that two-thirds of the Man₆ fraction contained an oligosaccharide with trisaccharide reducing end. Reduction of the size of the trisaccharide peak and increase in the disaccharide peak sizes due to overdegradation in acetylation would suggest that the trisaccharide structure accounts for more than two-thirds of the total, perhaps all. The proposed structure of this predominant oligosaccharide is shown in Fig. 9.

Further Structural Studies of IB-2 (Man₃GlcitolNAc)

Methylation analysis of oligosaccharide IB-2 (Table IV) shows that it has 3 terminal mannose residues and two 3,6-di-substituted mannose residues, in addition to N-acetylgalactosaminid substituted on position 4. Acetylation of the oligosaccharide shows that the glucosaminitol labeled fragment (Fig. 6G) moves as a trisaccharide. After reduction of the acetylation products, paper chromatography (Fig. 6H) shows two additional peaks, a disaccharide, and mannitol. Methylation of the trisaccharide from acetylation (Table V) shows that it has the structure Man₁ → 3Man₁ → 4GlcitolNAc. Methylation of the disaccharide shows that it has the structure Man₁ → 3mannitol. The only possible structure for oligosaccharide IB-2 is shown in Fig. 9.

Discussion

The amino acids present in glycopeptide I (Asn, Pro, Ala, Thr, and His) and glycopeptide II (Asn, Ser, and Val), correspond to those found by Putnam et al. (1) in the sequences around Asn 402 and Asn 563 to which high mannose oligosaccharides are attached in the human IgM (11). The amino acid sequence of the Fe region of five pathological human IgM chains have now been compared and shown to be the same and to be substituted with oligosaccharide on the same asparagine residues (21). Therefore, we conclude that the oligosaccharides of glycopeptide I are attached to Asn 402 and the oligosaccharides of glycopeptide II are attached to Asn 563 in the μ chain of IgM (Wa). Studies on the carbohydrate of two of these IgM molecules by the same authors (21) and on a third pathological IgM by Jonneau and Bourillon (22) show a high mannosetype glycopeptide appears at Asn 563 in these proteins as well as in those studied by Shimizu and co-workers earlier (2). The major oligosaccharides of glycopeptide I are (Man₆GlcNAc)₂ (IB-1) and (Man₆GlcNAc)₂ (IB-2), and the major oligosaccharides of glycopeptide II are (Man₆GlcNAc)₂ (IIA), and (Man₆GlcNAc)₂ (IIB). The structures of these four oligosaccharides are similar or identical to asparagine-linked high mannose oligosaccharides found in several other glycoproteins including ovalbumin, α-amylase, Chinese hamster ovary cells, calf thyroglobulin, and yeast mannan (4, 20, 23–25). All of these oligosaccharides have a common structure of:

Man₁ → 6Man₁ → 6Man₁ → 4GlcNAc₁ → 4GlcNAc₁ → 4GlcNAc₁

a     b     c

with additional mannose residues linked α,2 at positions a, b, or c. High mannose oligosaccharide structures have been studied in detail in two other human myeloma immunoglobulins, with results suggesting that the oligosaccharides in these cases are somewhat different. The structure for the IgE high mannose oligosaccharide chain:

\[
\text{Man}₁ → 6\text{Man}₁ → 4\text{GlcNAc}₁ → 3\text{Man}₁ → 4\text{GlcNAc}₁ → \text{Asn}
\]

lacks the N,N-diacylchitobiose core present in the other high mannose oligosaccharides (8). It also contains an α-linked GlcNAc residue and displays no microheterogeneity. In an earlier study of another human IgM myeloma protein, Hickman et al. (3) partially characterized a high mannose glycopeptide with the structure:

\[
(\text{Man})₃(GlcNAc)₂\text{Asn}
\]

This oligosaccharide displays heterogeneity in the number of mannose residues but differs from the structures found in IgM (Wa) in having an additional mannose attached to N-acetylglucosamine, probably in β linkage.

Although all four of the IgM (Wa) high mannose chains contain the same structure for the 5 innermost mannose residues, oligosaccharide IB-1 has its sixth mannose attached to position b of the common structure, whereas IIB has its sixth mannose attached to position c. Clearly, the biosynthesis of these oligosaccharides proceeds with specificity and yet heterogeneity is observed in the size of the chains attached to a particular asparagine residue. Since myeloma proteins such as IgM (Wa) are products of a single clone of cells, this heterogeneity cannot be attributed to diversity in the cells of origin. Since plasma contains α-mannosidase activity, this heterogeneity could possibly arise from degradation of the oligosaccharides as the IgM circulates. However the amount of α-mannosidase activity at the physiologic pH of 7.4 is very low (26). The other explanation for the heterogeneity is that it arises during the biosynthesis of the molecules. We now know that the biosynthesis of high mannose oligosaccharides occurs by transfer of a large lipid-linked high mannose oligosaccharide to asparagine followed by “processing” of the sugar chain by removal of mannose residues (27–29). An interruption of this sequence of events could result in a spectrum of oligosaccharide chain lengths. In fact glycopeptide I contained, in addition to the two major high mannose oligosaccharide chains described here, small amounts of four other oligosaccharides whose structures have been analyzed. These results and their possible relevance to oligosaccharide processing are reported in the following papers.

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*J. Biol. Chem.* 1979, 254:816-823.

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