Structure of the Carbohydrate Units of IgA, Immunoglobulin

I. COMPOSITION, GLYCOPEPTIDE ISOLATION, AND STRUCTURE OF THE ASPARAGINE-LINKED OLIGOSACCHARIDE UNITS*

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SUMMARY

The carbohydrate composition of an IgA (α1 subtype) myeloma protein has been determined. The carbohydrate, present only on the heavy chain, was found to consist of 3 moles of sialic acid, 9 mole of galactose, 5.4 moles of mannose, 0.8 moles of fucose, 8.8 moles of N-acetylglucosamine, and 5 moles of N-acetylgalactosamine per mole of heavy chain.

Four major glycopeptide-containing fractions were isolated following pronase degradation of the protein. Glycopeptide I consisted of galactose, N-acetylgalactosamine, threonine, serine, and proline in the molar ratio 4:5:4:5:9. Each of the N-acetylgalactosamine residues was found to be involved in an O-glycosidic linkage, demonstrating that there are five O-glycosidically linked oligosaccharide units per heavy chain. The structure of this glycopeptide is presented in the following paper (Baenziger, J., and Kornfeld, S. (1974) J. Biol. Chem. 249, 7270-7281).

The three other glycopeptide fractions contained 1 to 2 residues of sialic acid, 0 to 0.7 residue of fucose, 2 residues of galactose, 3 residues of mannose, 4 to 5 residues of N-acetylglucosamine, and 1 residue of asparagine. Glycopeptide II A was a homogeneous glycopeptide with the following structure:

All immunoglobulins contain carbohydrate in amounts characteristic of their class. While a great deal of information concerning the amino acid sequences of both the heavy and light chains of immunoglobulins has been obtained from myeloma proteins, until recently only limited information has been available regarding the sequence of the sugars in the oligosaccharide units of these proteins. Our laboratory has determined the structures of the oligosaccharide units found on IgG1 (1), IgM (2), and IgE (3, 4) myeloma proteins. In this paper and the following one (5) we present data which establish the structures of the oligosaccharide units found on an IgA myeloma protein of the α1 subtype.

Previous investigators have determined the total carbohydrate composition of the IgA class of immunoglobulins (6) but only one report has appeared dealing with the actual sequence of the oligosaccharide chains and only partial structures could be proposed (7). The IgA1 subclass is of special interest because it contains both N-glycosidically and O-glycosidically linked chains (5, 7). The O-glycosidically linked oligosaccharide units are characteristic of the α1 subtype and are not found in the α2 subtype due to a deletion of the regions in which they occur (8). Recently, Moore and Putnam (9) have succeeded in localizing one of the N-glycosidically linked oligosaccharides to a cysteine-containing peptide.

In this paper we shall deal with the structure of the N-glycosidically linked oligosaccharide units of IgA1 and in the following

"The abbreviations used are: IgG, IgM, IgE, IgA, IgA1, IgA2, immunoglobulin G, etc."
paper with the O-glycosidically linked chains of this immunoglobulin.

**EXPERIMENTAL PROCEDURE**

**Preparation and Isolation of IgA Glycopeptides**—The IgA myeloma protein was isolated from the serum of a patient (Walker) with multiple myeloma by the method of Dawson and Clamp (7). The preparation was judged 95% pure by immunoelectrophoresis. Dr. Howard M. Grey, National Jewish Hospital, Denver, Colorado, kindly classified the protein as being of the α type.

Three preparations of IgA glycopeptides were made by incubating 2 to 9 grams of myeloma protein (approximately 60 mg per ml) with 2% (w/w) pronase in 0.1 M Tris buffer, pH 7.9, containing 2.0 mM CaCl₂. Incubations were carried out at 37°C under a toluene atmosphere. After 48 hours the pH was adjusted to 8 with NaOH, an additional 2% (w/w) pronase was added, and the incubation continued for another 24 hours. The sample volume was reduced to 10 ml and applied to a Sephadex G-25 column (2.0 × 80 cm) and eluted with H₂O. Those fractions containing hexose were pooled, concentrated, and digested again with 1% (w/w) pronase for 24 hours. This material was then applied to a Bio-Gel P-10 column (1.6 × 83 cm) and eluted with 0.1 M pyridine-acetate buffer, pH 6.0. The hexose and sialic acid content of each fraction, determined by the phenol-sulfuric acid (10) and thiobarbituric acid (11) methods, respectively, is shown in Fig. 1A.

The presence of a leading shoulder with a lower sialic acid content than the more retarded portion of the peak indicated the presence of two species of glycopeptide. However, rechromatography of Pool I (Fig. 1A) did not result in complete resolution of the two species (Fig. 1B). The two fractions (either combined or separately) were therefore subjected to ion exchange chromatography on DEAE-cellulose. The result of ion exchange chromatography of the combined Pools I and II from the Bio-Gel P-10 column (Fig. 1A) is shown in Fig. 2. The fractions were pooled as indicated and designated glycopeptides I, IIA, IIB, and IIC. When the two fractions obtained from the Bio-Gel P-10 column were chromatographed separately on DEAE-cellulose, it was found that glycopeptide I was the predominant glycopeptide in the larger molecular weight material (Pool I, Fig. 1A) while glycopeptides IIA, IIB, and IIC were the primary constituents of the lower molecular weight, sialic acid-containing material (Pool II, Fig. 1A).

**Methylation Analysis**—IgA glycopeptides were permethylated by the method of Hakomori (12) as described by Bjorndahl and Lundblad (13). The alditol acetate derivatives of the methylated neutral sugars were separated and identified by gas-liquid chromatography and mass spectrometry as described previously (1).

**Molecular Weight Determinations**—Estimates of the molecular weight of each glycopeptide were made by gel filtration on Sephadex G-50-80 as described by Bhatti and Clamp (15).

**Preparation of Light and Heavy Chains**—The myeloma protein (8 mg) was dialyzed overnight against 0.15 M Tris buffer, pH 8.0, containing 0.15 M NaCl and 2 mM EDTA. Dithiothreitol was then added to 0.01 M and the sample was incubated at 25°C for 2 hours. Iodoacetic acid was then added to 0.02 M and the sample was dialyzed for 12 hours against 250 volumes of 0.02 M KPO₄ buffer and 0.15 M NaCl, pH 7.5, at 4°C. The reduced and alkylated heavy and light chains were separated by gel filtration on a calibrated column of Sephadex G-100 (1.4 × 80 cm) which had been equilibrated with 4.5 M urea-1 M propionic acid. The fractions which contained protein as monitored by A₂₈₀ were pooled and washed free of propionic acid and urea by repeated concentration and addition of 0.1 N acetic acid in a 50 ml Amicon membrane concentrator with a UM-10 filter.

**RESULTS**

**Carbohydrate Composition of IgA, Immunoglobulin and Its Glycopeptides**—Gel filtration of the purified IgA myeloma protein...
FIG. 2. DEAE-cellulose chromatography of the glycopeptide-containing fractions from the Bio-Gel P-10 column. Pools I and II were combined and loaded on a DEAE-cellulose column (1.5 X 20 cm) which was equilibrated with 4 mM phosphate buffer, pH 6.8. After washing the column with 80 ml of starting buffer, elution was carried out with a linear gradient (100 ml each) of 4 to 100 mM phosphate buffer, pH 6.8. Each 6.3-ml fraction was analyzed for hexose (---) and sialic acid (O—O). Fractions were then pooled as noted.

Separation of the heavy and light chains by gel filtration on Sephadex G-200 yielded two peaks corresponding to the monomeric and polymeric forms of this immunoglobulin as judged by molecular weight (7). The polymeric and monomeric forms (Peaks I and II, respectively, in Table I) had virtually identical amino acid (data not shown) and carbohydrate compositions (see Table I). The amount of protein present in these two forms was obtained by summation of the individual amino acids (moles of each amino acid X molecular weight) and indicated that the value obtained by the Lowry colorimetric assay (16) using bovine serum albumin as the standard was approximately a-fold greater than the actual amount present.

The values presented in Table I are therefore given as moles per mole of heavy chain.

The carbohydrate and amino acid compositions of the glycopeptide fractions obtained from the DEAE-cellulose column are given in Table II. The values for the individual residues of the 4 major glycopeptides were determined by averaging the values obtained from three different preparations of these glycopeptides. The small standard deviations are a good indication of the reproducibility and homogeneity of these glycopeptides. The glycopeptide yields obtained in the three preparations are also presented. The relative proportions of I, IIA, IIB, and IIC are quite reproducible. The lower total yield in Preparations I and II is in part a reflection of the fact that in these preparations the material was partially separated on Bio-Gel P-10 (Fig. 1A) as Pools I and II and then fractionated separately on DEAE-cellulose while in Preparation III the pools were combined for the ion exchange chromatography.

* Mannose set equal to 3.00.
* N.D., not determined.
* IIB and IIC emerged as a single peak in Preparation I rather than two peaks as in Preparations II and III.
IIA differs from IIB and IIC in that it contains a single residue of sialic acid and no fucose, while IIB and IIC both have 2 residues of sialic acid and 0.6 to 0.7 residue of fucose. All of the sialic acid present in these glycopeptides was found to be N-acetyleneuraminic acid by gas-liquid chromatography. The other sugars, mannose, galactose, and N-acetylglucosamine are present in very similar amounts in all three glycopeptides, suggesting that if more than 1 species of glycopeptide is present they may be closely related.

Estimation of the molecular weights of the glycopeptides on Sephadex G-50-80 (Fig. 3) gave values of 2350 and 2440 for IIA and IIB, respectively. These values are in good agreement with the calculated value of 2375 for glycopeptide IIA and 2590 for glycopeptide IIB (Table III). The reason for using a value of 5 residues of N-acetylglucosamine for calculating the molecular weight of IIA and 4 residues of N-acetylglucosamine for IIB will be clarified below.

The results of methylation studies of glycopeptides IIA, IIB, and IIC are presented in Table IV. Several additional differences between glycopeptides IIA, IIB, and IIC become apparent. Glycopeptide IIA with only 1 residue of sialic acid displays a complete absence of fucose which correlates with: (a) a much greater proportion of 2-monomethylmannose (0.0 residue) as opposed to 2,4-dimethylmannose, (b) the complete lack of 3-monomethyl-2-N-methyl-N-acetylglucosamine, and (c) the presence of more than a full residue of terminal N-acetylglucosamine. Glycopeptides IIB and IIC with 2 residues of sialic acid each and 0.6 to 0.7 residue of fucose display (as compared to IIA): (a) a greater proportion of 2,4-dimethylmannose as compared to 2-monomethylmannose, (b) 0.3 and 0.8 residue, respectively, of 3-monomethyl-2-N-methyl-N-acetylglucosamine, and (c) much less terminal N-acetylglucosamine.

Table III

| Glycopeptide | Calculated molecular weight | Estimated molecular weight |
|--------------|----------------------------|----------------------------|
| I            | 3280                       |                            |
| IIA          | 2375                      | 2350                       |
| IIB and IIC  | 2590                      | 2440                       |

*Calculated on the basis of a fucose to sialic acid to galactose to mannose to N-acetylglucosamine to asparagine ratio of 0:1:2:3:5:1.

bCalculated on the basis of a fucose to sialic acid to galactose to mannose to N-acetylglucosamine to asparagine ratio of 1:2:2:3:4:1.

Table IV

| Glycopeptide | FucoSe | Galactose | Mannose | 2 N-Methyl- N-acetylglucosamine |
|--------------|--------|-----------|---------|--------------------------------|
|               | 2,3,4,6-| 2,3,4,6-  | 2,3,4,6-|
|               | Trimmethyl | Trimmethyl | Trimmethyl |
| IIA           | 0      | 0.7       | 2.0     | 1.4  | 3.8  | 0     |
| IIB           | 0.6    | 0.1       | 2.0     | 0.6  | 3.2  | 0.3   |
| IIC           | 0.8    | 0.2       | 2.0     | 0.6  | 2.6  | 0.8   |

Proportions were determined by setting 3,4,6-trimethylmannose to 2.0. The values given represent the average of 2 to 3 different methylations.

Fig. 3. Estimation of molecular weights of IgA glycopeptides by exclusion chromatography on Sephadex G-50-80. Samples were applied to a column (1.8 x 85 cm) of Sephadex G-50-80 and eluted with 0.9% NaCl-0.01 M NaHCO3. Fractions of 3.1 ml were collected. The samples contained in addition to the IgA glycopeptides, the following materials: blue dextran to locate the void volume (Vv), [14C]mannose to determine V100, an IgG glycopeptide, and fetuin glycopeptide. The IgG glycopeptide had been labeled by acetylation with [3H]acetic anhydride. The compounds used for calibration were: 1, mannose; 8, lactose; S, IgG glycopeptide (mol wt 1919); and fetuin glycopeptide (mol wt 2898).

Relative proportions of methylated sugars from IgA glycopeptides IIA, IIB, and IIC.

The calculated molecular weights were determined from the compositions of the glycopeptides shown in Table II. The estimated molecular weights were determined by exclusion chromatography as described in Fig. 3.

The calculated molecular weights were determined from the compositions of the glycopeptides shown in Table II.
degree of destruction of N-acetylglucosamine in that only 3.7 residues of N-acetylglucosamine survived periodate oxidation, a value in very good agreement with the 3.8 residues of 3,6-di- 

methyl 2-N methyl N-acetylglucosamine found to be present by methylation (Table IV). According to the methylation results, there should be 1.4 residues of terminal periodate-sensitive N-acetylglucosaminidase in addition to the 3.7 residues of periodate-resistant N-acetylglucosamine. This would add up to a total of 5.1 residues of N-acetylglucosamine in IIA, somewhat more than the 4.4 residues determined by gas-liquid chromatography (Table III). The value of 5 residues obtained by methylation analysis is probably the more accurate since it is in good agreement with the results of the glycosidase treatment of the glycopeptide (see below).

Knowing the species of sugars present and their substitution patterns it remained to establish the sequential relationships and linkages by a combination of enzymatic and periodate degradations which are summarized in Table VI for glycopeptide IIA and in Table VII for IIB and IIC.

Sequential Glycosidase Degradation of IIA—Before considering the data presented in Table VI, several important aspects concerning the structure of glycopeptide IIA can be deduced from the methylation data (Table IV). Since the 2-monomethylmannose is the only sugar residue with multiple substitutions, it must act as the single branch point for glycopeptide IIA. The 3 branches arising from this mannose must have 3 nonreducing termini ending with 1 sialic acid, 1.3 N-acetylglucosamine, and 0.8 galactose residue. Therefore, one could draw the following abbreviated structure:

\[
\text{Gal} \xrightarrow{\beta_1,4} \text{GlcNAc} \xrightarrow{\beta_1,2} \text{Man} \xrightarrow{\alpha} \text{Gal}
\]

where only position 2 of the core mannose is left unsubstituted and no indication is made of the number of residues intervening between the nonreducing triunoi and the core mannose.

Treatment of glycopeptide IIA with α-mannosidase and β-N-acetylglucosaminidase (Table VI, Experiment 1) did not release any mannose or N-acetylglucosamine indicating that either the terminal N-acetylglucosamine residues are not linked β or that the β-N-acetylglucosaminidase cannot release these residues because of steric restrictions. That steric factors are indeed involved is supported by the fact that α-N-acetylglucosaminidase also did not release the terminal N-acetylglucosamine (Table VI, Experiment 1). Neuraminidase (Vibrio cholerae) released 1 residue of sialic acid (Table VI, Experiments 2 and 4) and methylation of the product showed that all of the 2,3,4-trimethylgalactose had been converted to 2,3,4,6-tetramethylgalactose (data not shown). In addition, treatment of the intact and asialo-glycopeptides with β-galactosidase led to the release of 1.1 and 1.8 residues of galactose, respectively (Table VI, Experiments 3 and 4).

Following the removal of the terminal galactose residue, either jack bean or rat epididymal β-N-acetylglucosaminidase was able to release between 0.9 and 1.2 residues of N-acetylglucosamine (Table VI, Experiments 3 and 5). However, the β-mannosidase present in the rat epididymal II enzyme preparation was unable to release any mannose while α-mannosidase was able to release 1.0 residue of mannose (Table VI, Experiment 5). Concomitant with the release of this α-linked mannose was the release of 0.8 residue of N-acetylglucosamine by β-N-acetylglucosaminidase (Table VI, Experiment 5). These experiments indicate that 1 of the 3 branches arising from the core mannose consists of

\[
\text{Gal} \xrightarrow{\beta_1,4} \text{GlcNAc} \xrightarrow{\beta_1,2} \text{Man} \xrightarrow{\alpha} \text{Gal}
\]

and another consists of N-acetylglucosamine linked β to the core mannose. This terminal N-acetylglucosamine can only be released by β-N-acetylglucosaminidase after the α-linked mannose has been removed. The rate of release of the α-linked mannose residue was also slower than that seen for the analogous mannose in the IG E glycopeptide B-1 which does not have a third substituent on its core mannose (4). Thus, it appears that the presence of 3 substituents on a single residue of mannose results in steric problems for several glycosidases, ranging from the complete blocking of β-N-acetylglucosaminidase activity to the partial blocking of α-mannosidase activity.

The glycopeptide which was produced by the enzyme degradations just discussed was reisolated and found to contain 1 residue of sialic acid, 1 residue of galactose, 2 residues of mannose, and 3 residues of N-acetylglucosamine (Table VI, Experiment 5), indicating that the enzymes had indeed removed 1 galactose, 1 mannose, and 2 N-acetylglucosamine residues. Following oxidation of the remaining glycopeptide with sodium metaperiodate, 1 residue of mannose and 3 residues of N-acetylglucosamine remained (Table VI, Experiment 5). This confirmed that 1 of the 2 N-acetylglucosamine residues which had been removed must have been terminal since no periodate-sensitive N-acetylglucosamine remained. This experiment also revealed that complete removal of the 2 branches ending in galactose and N-acetylglucosamine,
respectively, resulted in a mannose substituted at position 3 since it is still periodate resistant. Thus, the other 2 branches which were removed must have occupied positions 4 and 6 of the core mannose.

The sequence of the sugars in the branch which terminates in sialic acid was then determined. Following removal of the sialic acid from IIA, 2 residues of galactose could be released by β-galactosidase (Table VI, Experiments 4 and 6). In addition, 2.0 residues of N-acetylglucosamine could then be released by β-N-acetylglucosaminidase (Table VI, Experiment 6). The mannose and N-acetylglucosamine residues were released by a combination of α-mannosidase, β-N-acetylglucosaminidase, and β-N-acetylglucosamine amidohydrolase. The structure of the core of IIA is therefore:

\[
\text{Man} \xrightarrow{\beta-1,4} \text{GlcNAc} \xrightarrow{\alpha-1,4} \text{GlcNAc} \xrightarrow{\beta} \text{Asn}
\]

and the proposed structure for the complete molecule is given in Fig. 4.

**Sequential Degradation of Glycopeptides IIB and IIC—Glycopeptides IIB and IIC present a more complex situation than glycopeptide IIA in that they appear to be heterogeneous. Neither IIB nor IIC has a complete residue of fucose, terminal N-acetylglucosamine, or 2,4-dimethylmannose. The presence of 0.6 residue of terminal N-acetylglucosamine by methylation and of slightly more than half a residue of 2-monomethylmannose suggests that both IIB and IIC consists of a mixture of two glycopeptides, one of which is identical with IIA except for the presence of a second residue of sialic acid. The other glycopeptide differs from IIA in that it lacks the terminal N-acetylglucosamine which is linked to the core mannose (resulting in a 2,4-dimethylmannose rather than the 2-monomethylmannose seen in glyco-

### Table VI

*Effects of glycosidases and periodate oxidation on glycopeptide IIA*

| Experiments | Sialic acid | Fucose | Galactose | Mannose | N-Acetylglucosamine |
|-------------|-------------|--------|-----------|---------|---------------------|
| Intact glycopeptide IIA. | 1.1 | 0 | 1.8 | 3.0 | 5.0* |
| 1. α-Mannosidase (J. B.)\(^b\). | | | | | |
| Then β-N-acetylglucosaminidase (J. B.). | | | | | |
| Then α-N-acetylglucosaminidase (pig)\(^c\). | | | | | |
| 2. Neuraminidase. | 1.0 | 0 | 0 | 0 | |
| Then α-galactosidase + β-N-acetylglucosaminidase (Ficin). | 1.1 | | | 0.8 | |
| Then α-N-acetylglucosaminidase (J. B.). | | | | | |
| Repeat. | | | | | |
| Then α-mannosidase (J. B.). | | | | 0.1 | |
| 3. β-Galactosidase (J. B.). | 1.8 | 0 | 0.7 | 0.7 | |
| Then β-N-acetylglucosaminidase (J. B.) 2 times. | | | | | |
| 4. Neuraminidase. | 1.0 | | 0.7 | 0.7 | |
| Then α-galactosidase (J. B.). | | | | | |
| Then β-N-acetylglucosaminidase (J. B.) | | | | | |
| 5. β-Galactosidase + β-N-acetylglucosaminidase + β-mannosidase (rat epididymal II)\(^d\). | | | | | |
| Repeat. | 2.0 | | 0 | 0.8 | |
| Then α-mannosidase + β-N-acetylglucosaminidase. | 0.1 | | 0 | 1.0 | |
| Repeat. | | | | 0.2 | |
| Then α-mannosidase + β N acetylglucosaminidase. | 1.7 | | 0.5 | 0.6 | |
| Repeat. | 0.9 | | 0.5 | 0.4 | |
| Reisolate core. | 1.0 | | 0 | 3.0 | |
| (a) Core intact. | 0 | | 0 | 1.2 | |
| (b) Core × NaIO<sub>4</sub>. | 0 | | 1.2 | 3.0 | |
| 6. 0.1 N H<sub>2</sub>SO<sub>4</sub>, 80°, 1 hour. | 1.0 | | 1.0 | 2.2 | |
| Then β-galactosidase + β-N-acetylglucosaminidase + β-mannosidase (rat epididymal II). | | | | | |
| Repeat. | | | | | |
| Repeat. | | | | | |
| Then α-mannosidase + β N acetylglucosamime aminohydrolase (rat epididymal II). | 0.9 | 0.2 | 0.7 | 1.6 | |

* 5.0 is used rather than 4.4 for reasons discussed in the text.
* (J. B.), jack bean (3).
* (pig), pig liver (3).
* See Ref. 3 for details concerning this enzyme preparation made from rat epididymidis.
TABLE VII
Effects of glycosidases and periodate oxidation on glycopeptide IIB

| Experiment | Residues released by glycosidases or surviving periodate oxidation |
|------------|---------------------------------------------------------------|
|            | Sialic acid | Fucose | Galactose | Mannose | N-Acetylglucosamine |
| Intact glycopeptide IIB |             |        |          |         |                     |
| 1. α-Mannosidase |             |        |          |         |                     |
| Then β-N-acetylglucosaminidase |             |        |          |         |                     |
| Then β-galactosidase |             |        |          |         |                     |
| 2. Neuraminidase |             |        |          |         |                     |
| Then β-galactosidase + β-acetylglucosaminidase + α-fucosidase (rat epididymal I) | 2.0 | 0 | 0 | 2.0 | 1.4 |
| Repeat |             |        |          |         |                     |
| 3. 0.1 N H₂SO₄, 80°, 1 hour |             |        |          |         |                     |
| Then β-galactosidase + β-N-acetylglucosaminidase + β-mannosidase (rat epididymal II) | 0.6 | <0.1 | 0.1 | 0.2 | 0.2 |
| Repeat |             |        |          |         |                     |
| Reisolate core |             |        |          |         |                     |
| (a) Core intact |             |        |          |         |                     |
| (b) Core × NaIO₄ |             |        |          |         |                     |
| (c) Core + β-mannosidase + β-N-acetylglucosaminidase + β-N-acetylglucosamine amidohydrolase | 1.8 | 0 | 0 | 1.3 |
| Repeat |             |        |          |         |                     |
| 4. NaIO₄, reduction with NaBH₄, mild acid hydrolysis; separation on G-10 Sephadex (see Fig. 5) |             |        |          |         |                     |
| Fragment |             |        |          |         |                     |
| Core |             |        |          |         |                     |
| (a) Core × NaIO₄ |             |        |          |         |                     |
| (b) Core × α-mannosidase |             |        |          |         |                     |
| (c) Core × β-mannosidase (hen oviduct) |             |        |          |         |                     |
| (d) Core × β-N-acetylglucosaminidase |             |        |          |         |                     |
| (e) Core × β-mannosidase + β-N-acetylglucosaminidase + β-N-acetylglucosamine amidohydrolase |             |        |          |         |                     |

* (J. B.), jack bean.

No sugar was released from the intact glycopeptides by β-galactosidase, α-mannosidase, or β-N-acetylglucosaminidase (Table VII, Experiment 1). Following the removal of 2 residues of sialic acid, 2 residues each of galactose and N-acetylglucosamine could be sequentially removed by β-galactosidase and β-N-acetylglucosaminidase (Table VII, Experiments 2 and 3). At this point β-mannosidase did not release any mannose whereas α-mannosidase released 1.8 residues of mannose concomitant with the release of 0.6 residue of N-acetylglucosamine (Table VII, Experiment 3). The inability of the β-N-acetylglucosaminidase to release the final 0.6 residue of N-acetylglucosamine until after the removal of the α-linked mannose residues is completely analogous to the situation seen with glycopeptide IIA (Table VI, Experiments 5 and 6).

The sequence of the 2 nonreducing termini of IIB and IIC ending in sialic acid must therefore be:

Sialic acid \( \xrightarrow{\text{α2,6}} \) Gal \( \xrightarrow{\text{β1,4}} \) GlcNAc \( \xrightarrow{\text{β1,2}} \) Gal \( \xrightarrow{\text{β1,4}} \) GlcNAc

On the order of 50% of the molecules have an additional third radiations and periodate oxidations does not always rule out all alternative possibilities. The results obtained from sequential enzymatic degradation and periodate oxidation of IIB and IIC were essentially identical except for one difference, which is noted below. For simplicity, only the data obtained with glycopeptide IIB is shown (Table VII).

The sequence of the 2 nonreducing termini of IIB and IIC ending in sialic acid must therefore be:

Sialic acid \( \xrightarrow{\text{α2,6}} \) Gal \( \xrightarrow{\text{β1,4}} \) GlcNAc \( \xrightarrow{\text{β1,2}} \) Man \( \xrightarrow{\text{α1,6}} \) GlcNAc

Since we were unable to separate the 2 species of glycopeptides present in IIB and IIC, the data obtained from enzymatic deg-
nonreducing terminus consisting of $N$-acetylglucosamine linked $\beta$ to the core mannose.

The core produced by these enzymatic degradation steps was reisolated and was found to contain fucose, mannose, and $N$-acetylglucosamine in the ratio of 0.7:1.0:2.0. Periodate oxidation resulted in the destruction of the remaining mannose and fucose but not of the $N$-acetylglucosamine (Table VII, Experiment 3b). Treatment of the core with $\beta$-mannosidase, $\beta$-$N$-acetylglucosaminidase, and $\beta$-$N$-acetylglucosamine amidohydrolase led to the release of 0.6 residue of mannose and 1.5 residues of $N$-acetylglucosamine (Table VII, Experiment 3c). This result could best be explained if the core had the sequence:

$$\text{Man} \overset{\beta l.4}{\longrightarrow} \text{GlcNAc} \overset{\beta l.4}{\longrightarrow} \text{GlcNAc} \overset{\beta}{\longrightarrow} \text{Asn}$$

$$\uparrow \alpha 1.0$$

$$\pm \text{Fuc}$$

The above digest would then result in the production of free mannose, $N$-acetylglucosamine, and asparagine as well as the disaccharide

$$\text{Fuc} \overset{\alpha 1.6}{\longrightarrow} \text{GlcNAc}$$

which is reactive in the Morgan-Elson assay for amino sugar (17). Had the fucose residue been linked to the other $N$-acetylglucosamine residue, no $N$-acetylglucosamine could have been released by the $N$-acetylglucosaminidase and any trisaccharide produced by the amidohydrolase would not have been reactive in the Morgan-Elson assay. IIC did differ from IIB as regards this experiment in that 1.0 residue of mannose and 1.0 residue of $N$-acetylglucosamine were released by the $\beta$-mannosidase and $\beta$-$N$-acetylglucosaminidase, respectively. It may be that IIC has an additional amino acid which would block the $\alpha$-$N$-acetylglucosamine amidohydrolase (18). This possibility is suggested by the fact that IIC has no other obvious characteristic that would be expected to lead to its separation from IIB. Unfortunately other explanations based on selective degradation of 1 or the other form of the core glycopeptides could be raised and some ambiguity must remain regarding the relationship of the fucose to the two $N$-acetylglucosamine residues.

In the case of glycopeptides IIB and IIC it remained to be established what the exact relationship of the 3 mannose residues to each other was since two possibilities existed:

$$\text{Man} \overset{\beta l.4}{\longrightarrow} \pm \text{GlcNAc} \overset{\beta l.4}{\longrightarrow} \pm \text{GlcNAc} \overset{\beta}{\longrightarrow} \text{Asn}$$

$$\uparrow \alpha 1.0$$

$$\pm \text{Fuc}$$

Possibility II was ruled out by the following experiments summarized in Table VII, Experiment 4. Intact glycopeptide IIB was oxidized with sodium metaperiodate. The oxidized glycopeptide was then subjected to reduction with NaBH₄ and the reduced fragments were released by mild acid hydrolysis (0.1 M H₂SO₄, 2 hours, 80°C). The hydrolysate was then passed over a column of Sephadex G-10 and each fraction was assayed for hexose and reducing activity by the phenol-sulfuric acid and Park Johnson methods, respectively (Fig. 6). The retarded material accounted for 1.6 residues of $N$-acetylglucosamine while the material eluting near the void of the column contained 1 residue of mannose and 2.4 residues of $N$-acetylglucosamine (Table VII, Experiment 4). Following periodate oxidation 0.2 residue of mannose and 1.5 residues of $N$-acetylglucosamine remained intact. The mannose in this core molecule was completely resistant to $\alpha$-mannosidase whereas 100% of the mannose was released by $\beta$-mannosidase (hen oviduct). Therefore, it must be the $\beta$-linked mannose which survives periodate oxidation and this mannose residue is linked to the two $N$-acetylglucosamine residues in the core. This finding rules out possibility II since in that structure, the $\beta$-linked mannose would be periodate sensitive. The proposed structures for the 2 glycopeptides found in IIB and IIC are given in Fig. 6.

**DISCUSSION**

IgA is an unusual immunoglobulin in that the $\alpha$, subtype has both $N$-glycosidically and $O$-glycosidically linked oligosaccharide units present on the heavy chain (5, 7). In contrast, the oligosaccharide units of IgG, IgM, and IgE are all of the $N$-glycosidic type (1-4).

The carbohydrate composition of the IgA myeloma protein (Walker) is quite similar to the composition of several other IgA proteins such as IgA (Bra) (7) and IgA (Ha) (19). The total carbohydrate composition of this myeloma protein is most compatible with the presence on each heavy chain of 1 unit of glyco-
peptide I (which contains 5 O-glycosidically linked oligosaccharide chains) and 2 of the N-glycosidically linked units. Assuming this distribution of oligosaccharide chains, it is tempting to speculate that the N-glycosidically linked oligosaccharide units lacking fucose (IIA and part of IIB and IIC) arise from one location on the heavy chain while the oligosaccharide units which contain fucose (present in IIB and IIC) arise from a second location on the heavy chain. The fact that the single sialic acid residue of IIA is not randomly distributed between the nonreducing termini arising from positions 3 and 6 of the core mannose but is found exclusively on the terminus arising from position 3, and the fact that no glycopeptides with a single residue of sialic acid were found which also contained a residue of fucose suggest that glycopeptide IIA is not merely the product of random incompleteness but may indeed represent a glycopeptide originating from a specific location along the heavy chain of IgA.

Some support for this proposal comes from the work of Moore and Putnam (9) who have isolated from an IgAr myeloma protein a tryptic peptide which has an oligosaccharide unit with the composition fucose 0, mannose 3.2, galactose 2.0, N-acetylgalactosamine 4.0, and sialic acid 0. The complete lack of fucose suggests that this glycopeptide may be the equivalent of glycopeptide IIA. The lack of sialic acid could be due to a deficiency of the appropriate sialyl transferase in the plasma cell, or to a loss of sialic acid during the preparation of the peptide which involves several steps utilizing 1 N acetic acid (9). Putnam et al. (20) have also isolated 2 additional tryptic peptides containing asparagine-linked oligosaccharide units. One of these is located in the variable region at the extreme N terminus of the heavy chain and would not be expected to be present in other IgAr myeloma proteins. The other glycopeptide contains an oligosaccharide unit linked to an asparagine which is located 14 residues from the COOH terminus of the α1 heavy chain. Since the complete carbohydrate composition of this glycopeptide has not yet been determined, it cannot be directly compared to the glycopeptides which we have isolated. However, it is interesting to note that the sequence around the asparagine located near the COOH terminus is His-Val-Asn(CHO)-Val Ser-Val Glu, while that around the asparagine in the peptide mentioned previously is Glu-Ala-Asn(CHO)-Leu-Thr-Cys. The fact that glycopeptide IIA contains 0.4 residue of alanine and 0.3 residue of glutamic acid while glycopeptides IIB and IIC contain virtually no alanine and glutamic acid, suggests that glycopeptide IIA does indeed arise from the same location as the fucose-deficient glycopeptide of Moore and Putnam. In addition, the presence of 0.5 residue of valine in glycopeptide IIC indicates that it may have been located on the asparagine found near the COOH terminus of the α1 heavy chain.

During the preparation and isolation of the glycopeptides from this myeloma protein, we were unable to detect any oligosaccharide units similar to the type 3 oligosaccharide (4 mannose and 3 N-acetylgalactosamine residues) described by Dawson and Clamp to be present in an IgA myeloma protein (7). It may be that the type 3 oligosaccharide observed by these authors represents a much less completed form of the type 2 chains (oligosaccharide units such as glycopeptide IIA). It must, however, be pointed out that we also did not observe any of the Type II glycopeptides from IgA (Walker) to have less than 1 residue of sialic acid.

The proposed structures for glycopeptide IIA (Fig. 4) and for the 2 species present in IIB and IIC (Fig 6) differ in several regards from the structure proposed for the type 2 glycopeptide by Dawson and Clamp (7). The data presented to support their tentative structure is consistent with ours; however, the additional information provided by complete glycosidase degradation, methylation, and combination glycosidase-periodate degradation studies have allowed us to unequivocally establish all but a few aspects of the structures.

The fucose-containing glycopeptides of IgA, are identical in structure with the B type glycopeptides of IgE (4) and very similar if not identical with the glycopeptides of this type found in IgG (1) and IgM (2). The glycopeptides of IgA, which do not contain fucose are also very similar to the B type glycopeptides of IgE aside from the absence of fucose and the presence of a third nonreducing terminus in the form of a terminal N-acetylgalactosamine linked to position 4 of the core mannose. Although this may appear to be a minor difference, the effect on the tertiary structure of the glycopeptide could be quite significant. That this in fact may be the case is suggested by the finding that the release of mannose from position 6 of the core mannose in
IIA is much slower than seen with glycopeptide B-1 of IgE (4). In addition, the terminal N-acetylglucosamine can only be released by β-N-acetylglucosaminidase after removal of the α-linked mannose from position 6. It will be of interest to determine whether this alteration will have any effect on the binding of lectins by IIA as compared to glycopeptide B-1 of IgE. The differences between these two glycopeptides should be virtually confined to the effect of the terminal N-acetylglucosamine since in both cases it is the branch arising from position 3 of the core mannose which ends in sialic acid.

In the following paper (5) we discuss the structure of the O-glycosidic units of IgA, and present a discussion of all the oligosaccharide units which have been isolated by our laboratory from IgG (1) IgM (2), and IgE (3, 4).

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