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The Isolation and Structure of the Core Oligosaccharide Sequences of IgM†

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ABSTRACT: Methods are presented for separating the three IgM heavy chain sialoglycopeptides associated with asparagines 170, 332, and 395. The core glycopeptide units containing the disaccharide fucosyl-*N*-acetylglucosamine were obtained through the use of an endo-β-*N*-acetylglucosaminidase from *Diplococcus pneumoniae,* following exoglycosidase treatment of the sialoglycopeptides. In addition to the core glycopeptides, high yields of a tetrasaccharide, $(Man)_3GlcNAc$, were obtained. The fucose in the core disaccharide is glycosidically linked to the 6-O position of the *N*-acetylglucosamine residue in Asn-GlcNAc. This core unit is resistant to glycosyl asparaginase, but becomes susceptible to hydrolysis on removal of the fucosyl residue by a purified hen oviduct α-L-fucosidase. The core sequence of the immunoglobulin M sialoglycopeptides appears to be similar to that of most other asparagine-linked oligosaccharides in consisting of a basic unit composed of β-D-Man(1→4)β-D-GlcNAc(1→4)β-D-GlcNAc(1→4), but with L-fucose linked α-(1→6) to the proximal GlcNAc. The two nonreducing terminal ends of $(Man)_3GlcNAc$ are linked to β-D-Man by α-(1→3) and α-(1→6) bonds, respectively.

Following our demonstration (Tarentino et al., 1970; Sukeno et al., 1971) that the "core" glycopeptide region of ribonuclease B consists of β-D-Man(1→4)β-D-GlcNAc(1→4)β-D-GlcNAc-Asn,[1] studies on glycopeptides derived from several glycoproteins have revealed this sequence to be common to both neutral and acidic oligosaccharides linked to asparagine (Kabasawa and Hirs, 1972; Lee and Socca, 1972; Sugahara et al., 1972; Sukeno et al., 1972; Tarentino et al., 1972, 1973; Baenziger et al., 1974; Baenziger and Kornfeld, 1974; Spik et al., 1975). Pertinent to these findings is the association of one or the other type of oligosaccharide with some glycoproteins (ovalbumin, deoxyribonuclease A, ribonuclease B, and IgG) and of both types with others (thyroglobulin and IgM).

It was shown recently by us (Tarentino et al., 1974) that an endoglycosidase from *Streptomyces griseus* is capable of releasing neutral oligosaccharides from glycoproteins or glycopeptides by hydrolyzing the di-*N*-acetylchitobiosyl residue in the core region. This enzyme, unfortunately, cannot release acidic oligosaccharides from glycoproteins or

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[1] Abbreviations used are: Asn-GlcNAc, 2-acetamido-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; GlcNAc, 2-acetamido-2-deoxy-β-D-glucosamine; GlcN, 2-amino-2-deoxy-β-D-glucosamine; Gal, galactose; Fuc, fucose; Man, mannose; PGA, pyroglutamate; Me_3Si, trimethylsilyl.

glycopeptides. However, another endoglycosidase from *Diplococcus pneumoniae* referred to as endo-*N*-acetylglucosaminidase D (Arakawa and Muramatsu, 1974) will accomplish this feat providing some of the peripheral carbohydrate residues are removed first. Through the use of this enzyme, it will be shown that the core glycopeptides associated with each of the acidic oligosaccharides in the heavy chain of IgM can be isolated and characterized. A preliminary account of these findings was presented recently (Tarentino et al., 1975).

Experimental Section

Analytical Methods. Glycopeptides were hydrolyzed at 110° for 12 hr with 2.0 *N* HCl in evacuated, nitrogen-flushed tubes and glucosamine and amino acids were determined on the amino acid analyzer (Spackman et al., 1958). Reducing sugars were liberated from glycopeptides and oligosaccharides by hydrolysis at 100° for 2.5 hr in 2.0 *N* HCl in evacuated, nitrogen-flushed tubes and quantitated on a Technicon carbohydrate analyzer using a modified Walborg procedure (Walborg et al., 1969). Enzymically released mannose was determined by a spectrophotometric procedure (Tarentino et al., 1970) and *N*-acetylglucosamine by the colorimetric method of Reissig et al. (1955). Column effluents were monitored for carbohydrate by the phenol–H_2SO_4 method (DuBois et al., 1956) and for free amino groups with fluorescamine (Udenfriend et al., 1972).

Chromatographic and Electrophoretic Procedures. Oligosaccharides containing asparagine were subjected to sequential glycosidase digestion and the products were identified chromatographically on the amino acid analyzer (Kimmel and Plummer, 1972), but modified to elute aspartic acid with 0.1 *M* sodium citrate (pH 3.1) containing 0.025 *M* NaCl. Disaccharides were silated with Tri-Sil Z (Pierce Chemical Co.) and subjected to isothermal gas chromatography at 250° on a 6-ft glass-coiled column of diatoport S coated with 3% SE-30 (Supelco Inc.). A Model 5700 Hewlett-Packard gas chromatograph was used with He as the carrier gas.

Alditol acetates were separated isothermally at 180° on a similar column, but with OV-275 on 100/120 Chromosorb W AW. The methylated mannitol acetate standard was kindly provided by Dr. C. E. Ballou.

The methylation of 3 mg of $(Man)_3GlcNAc$ was performed by a micromodification of the procedure of Kuhn and Trischman (1963), followed by acid hydrolysis, borohydride reduction, and acetylation of the isolated alditols with 0.2 ml of pyridine–acetic anhydride (1:1).

Disaccharides containing *N*-acetylglucosamine on the reducing end were reduced with sodium borotritide and subjected to a one-cycle Smith degradation (Garner et al., 1958) to identify the position of attachment of the substituent group. The tritiated acetylated polyol generated by this procedure was identified by descending paper chromatography in 1-butanol–pyridine–H_2O (6:4:3) and by electrophoresis in 1% sodium tetraborate buffer as described previously (Tarentino et al., 1970). The glycosylated asparagine derivatives were electrophoresed for 3 hr at 17.5 V/cm in an electrode buffer containing 70 ml of 98% formic acid and 50 ml of glacial acetic acid/2.5 l. The compounds were detected with ninhydrin.

Combined gas chromatography and mass spectrometry on the Me_3Si derivatives of the indicated disaccharides was performed by Dr. V. Reinhold of the Arthur D. Little Co.

Periodate Oxidation. Core glycopeptides (0.1 μmol) were oxidized at 0° in 200 μl of 0.075 *M* sodium periodate for 48 hr in the dark, and the reactions were terminated with 20 μl of 8 *M* ethylene glycol. Iodate ions were removed from the asparaginyl core glycopeptide, R-3 (Figure 2), by elution of the latter from a small column of Dowex-1 formate with water. The pyroglutamyl core glycopeptide, R-2, was separated from iodate by gel filtration on Sephadex G-10 (0.9 × 230 cm) with 0.1 *M* acetic acid. The aspartic acid and glucosamine content of the iodine-free oxidized glycopeptides was determined on the amino acid analyzer after acid hydrolysis.

Alkaline Borohydride Hydrolysis. The pyroglutamyl core glycopeptide, R-2 (2 μmol), was hydrolyzed in 2 ml of 1 *N* NaOH–1 *N* $NaBH_4$ at 100° for 6 hr in a Teflon-capped vial (Lee and Socca, 1972). The reaction mixture was then immersed in an ice bath, neutralized with glacial acetic acid, and desalted on a column of Sephadex G-10 (0.9 × 230 cm) with 0.1 *M* acetic acid as the eluting agent. Fucosylglucosaminitol was located in the column effluent with fluorescamine.

Enzyme Assays. All exoglycosidases were assayed with the appropriate *p*-nitrophenyl glycosides and the specific activities are reported as micromoles of substrate hydrolyzed per minute per milligram of protein at 37°, unless otherwise indicated.

Exoglycosidases. α-D-Mannosidase and β-D-*N*-acetylhexosaminidase were isolated from jack bean meal, with specific activities of 40 and 90 units/mg, respectively, by a modification of the method of Li and Li (1968). Hen oviduct glycosyl asparaginase (Tarentino and Maley, 1971) and β-D-mannosidase (Tarentino et al., 1970) were purified as described previously to final specific activities of 0.52 and 1.4 units/mg, respectively.

α-L-Fucosidase was isolated from homogenized hen oviducts. The supernatant fraction obtained on centrifugation was subjected to ammonium sulfate fractionation, and the precipitate that formed between 0.35 and 0.70 saturation was dissolved in and dialyzed against 0.01 *M* sodium acetate (pH 5). This fraction was adsorbed to a column of phosphocellulose (3.8 × 30 cm) previously equilibrated in 0.01 *M* sodium acetate (pH 5.0), and eluted with a linear gradient from 0 to 0.5 *M* NaCl (3 l. in each reservoir). Final purification of the enzyme was achieved using an affinity column consisting of ε-aminocaproylfucopyranosylamine covalently linked to Sepharose, similar to that described by Blumberg et al. (1972). α-L-Fucosidase was eluted from the column with 0.1 *M* sodium citrate (pH 6.0) containing 1% α-L-fucose. A 200-fold purified enzyme preparation (specific activity, 3.6) free of contaminating glycosidases was obtained by this procedure.

Endoglycosidase. Diplococcus pneumoniae, type I (strain Q-25), was maintained on blood agar slants at 37°. Endo-*N*-acetylglucosaminidase D production was initiated by inoculating the organism into 2-l. flasks containing 1 l. of medium of Hughes and Jeanloz (1964). After the cells were grown at 37° for 3 days in a New Brunswick rotary shaker, sodium azide was added to each flask to a concentration of 0.02%, and the cells were removed by centrifugation at 6000*g* for 15 min. The enzyme in the supernatant fraction was assayed by the same procedure used for the endoglycosidase from *S. griseus* (Tarentino and Maley, 1974) with [^{14}C]dansyl-Asn-$(GlcNAc)_2(Man)_5$ as the substrate.

The endoglycosidase was precipitated from the clear cultural filtrate with zinc acetate as described previously for the *S. griseus* enzyme (Tarentino and Maley, 1974) and

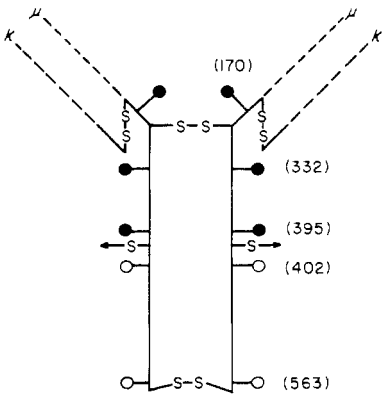

FIGURE 1: Location of the neutral (O) and acidic (●) glycopeptides in IgM (Ga). Adapted from Shimizu et al. (1971) and Putnam et al. (1973). The numbers in parentheses refer to the location of each asparagine from the amino end of the heavy chain.

solubilized with 0.3 *M* sodium citrate (pH 6.0). Ammonium sulfate (0.30–0.70 saturation) was used to precipitate the enzyme which was dissolved in a small volume of 0.01 *M* Tris-HCl (pH 7.5). After exhaustive dialysis against the same buffer, the enzyme was adsorbed to a column of DE-52 (3.8 × 32 cm) previously equilibrated in 0.01 *M* Tris-HCl (pH 7.5) and developed with a linear gradient from 0 to 0.4 *M* NaCl (3 l. in each reservoir). Neuraminidase eluted from the column at about 0.1 *M* NaCl and was purified subsequently by affinity chromatography as described by Cuatrecasas and Illiano (1971). The endoglycosidase eluted at about 0.3 *M* NaCl and was concentrated in an Amicon ultrafilter. Following gel filtration on a column of Sephadex G-200 (3.8 × 140 cm) with 0.01 *M* Tris-HCl (pH 7.5) containing 0.1 *M* NaCl, the endoglycosidase was obtained at a specific activity of 0.21 unit/mg which represents a 30-fold purification above that in the cultural filtrate.

Isolation of IgM. Plasma from a patient with Waldenstrom's macroglobulinemia, designated as Ga (Florent et al., 1974), was generously contributed by Dr. S. Murphy. IgM was isolated by precipitation at low ionic strength followed by solution in 0.15 *M* NaCl. Insoluble material was removed by centrifugation. This process was repeated four times.

Results

Isolation of IgM Glycopeptides. The location of the various neutral and acidic oligosaccharides in IgM was determined by Putnam et al. (1973) and Florent et al. (1974) and is diagramatically presented in Figure 1. To isolate the desired glycopeptides for analysis, the procedure presented in Figure 2 was followed, which employs an initial gel filtration to separate the neutral and acidic glycopeptides (Tarentino et al., 1974). This step is followed by the enzymic removal of peripheral sialic acid, galactose, and *N*-acetylglucosamine residues from the acidic glycopeptides, a procedure which makes the resulting glycopeptides susceptible to hydrolysis by endo-β-*N*-acetylglucosaminidase D. Following hydrolysis by this enzyme, the oligosaccharides and core glycopeptides are separated by ion exchange chromatography. A detailed description of the methodology involved is presented below.

IgM was dissolved in 0.015 *M* $CaCl_2$ to a concentration of 31.4 mg/ml, followed by addition of Pronase to 1% of the weight of IgM. The digestion was conducted at 37°, with the pH maintained at 8.0 with a pH stat. A second addition of Pronase was made at 8 hr and the reaction was terminated after 26 hr by lowering the pH to 4.5. The reaction mixture was lyophilized and the material which was not soluble in distilled water was removed by centrifugation and washed twice with distilled water. The washes were combined with the original supernatant fraction, and the precipitate, containing only 10% of the original phenol-H_2SO_4 positive material, was discarded. The soluble fraction was divided into aliquots equivalent to 2.5 g of the original quantity of IgM, and each aliquot was subjected to gel filtration on a column of Sephadex G-25 (2.54 × 200 cm) equilibrated in distilled water containing 1% butanol. The column was developed at 60 ml/hr and fractions of 10 ml were collected. As anticipated from earlier studies (Tarentino et al., 1974), two carbohydrate-containing peaks were detected by the phenol-H_2SO_4 assay: the first, which eluted at the column void volume, contained sialic acid, as determined by the periodate–resorcinol assay (Jourdian et al., 1971); the second contained the neutral glycopeptide fraction. The peaks were pooled separately and lyophilized.

The sialic acid containing glycopeptides from 15 g of IgM were redissolved in 0.1 *M* Tris-HCl (pH 8.0) containing 0.015 *M* $CaCl_2$ and redigested at 40° with Pronase (1 mg of Pronase/g of IgM, at 0 and 12 hr). After 48 hr the reaction was terminated by lowering the pH to 5.0, and the solution was lyophilized. The glycopeptides were separated on Sephadex G-25 as described above to yield two sialic acid containing peaks (S-1 and S-2) in a ratio of approximately 1:2. Except for a higher amino acid content in S-1, S-1 and S-2 were almost identical. S-2 was lyophilized, dissolved in 0.05 *M* NH_4HCO_3, and chromatographed at a rate of 79 ml/hr on a column of Sephadex G-100 (44–54 μ, 3.7 × 100 cm). The column had been equilibrated previously in 0.05 *M* NH_4HCO_3 containing 1% butanol. Fractions of 10 ml were collected. This procedure separated the carbohydrate-containing fractions from contaminating peptides. The desired fractions were lyophilized and redissolved in a small volume of distilled water.

Preparation of IgM Core Glycopeptides. A mixture of the S-2 sialoglycopeptides from IgM (140 μmol) in a final volume of 12.0 ml was incubated at 37° in 0.1 *M* potassium phosphate (pH 6.5) with the following glycosidases: neuraminidase, 1 unit; *Escherichia coli* β-galactosidase, 900 units; β-*N*-acetylhexosaminidase, 60 units; and *D. pneumoniae* endoglycosidase, 2 units. An additional unit of endoglycosidase was added after 24 hr and the digestion was allowed to continue for another 48 hr. The removal of *N*-acetylglucosamine residues in the peripheral side chains plateaued when about 70% of the total hexosamine had been released. The pH of the reaction mixture was lowered to 2.0 with glacial acetic acid, the solution was divided into two equal parts, and each fraction was chromatographed on a column of Sephadex G-25 (1.9 × 230 cm) equilibrated in 0.1 *M* acetic acid. The column was developed at a rate of 30 ml/hr and fractions of 6 ml were collected. Two carbohydrate-containing and fluorescamine-positive peaks were detected (Figure 3): an unhydrolyzed fraction which eluted in the column void volume (peak 1) and a lower molecular weight component which contained the core glycopeptides (peak 3). Each peak was pooled separately and lyophilized.

Preliminary Separation of IgM Core Glycopeptide Fragments. The core glycopeptides (Figure 3, peak 3) were redissolved in 0.03 *N* HCl and applied to a column of Dowex 50-X2 (H^+, 0.9 × 10 cm). The column was eluted with dis-

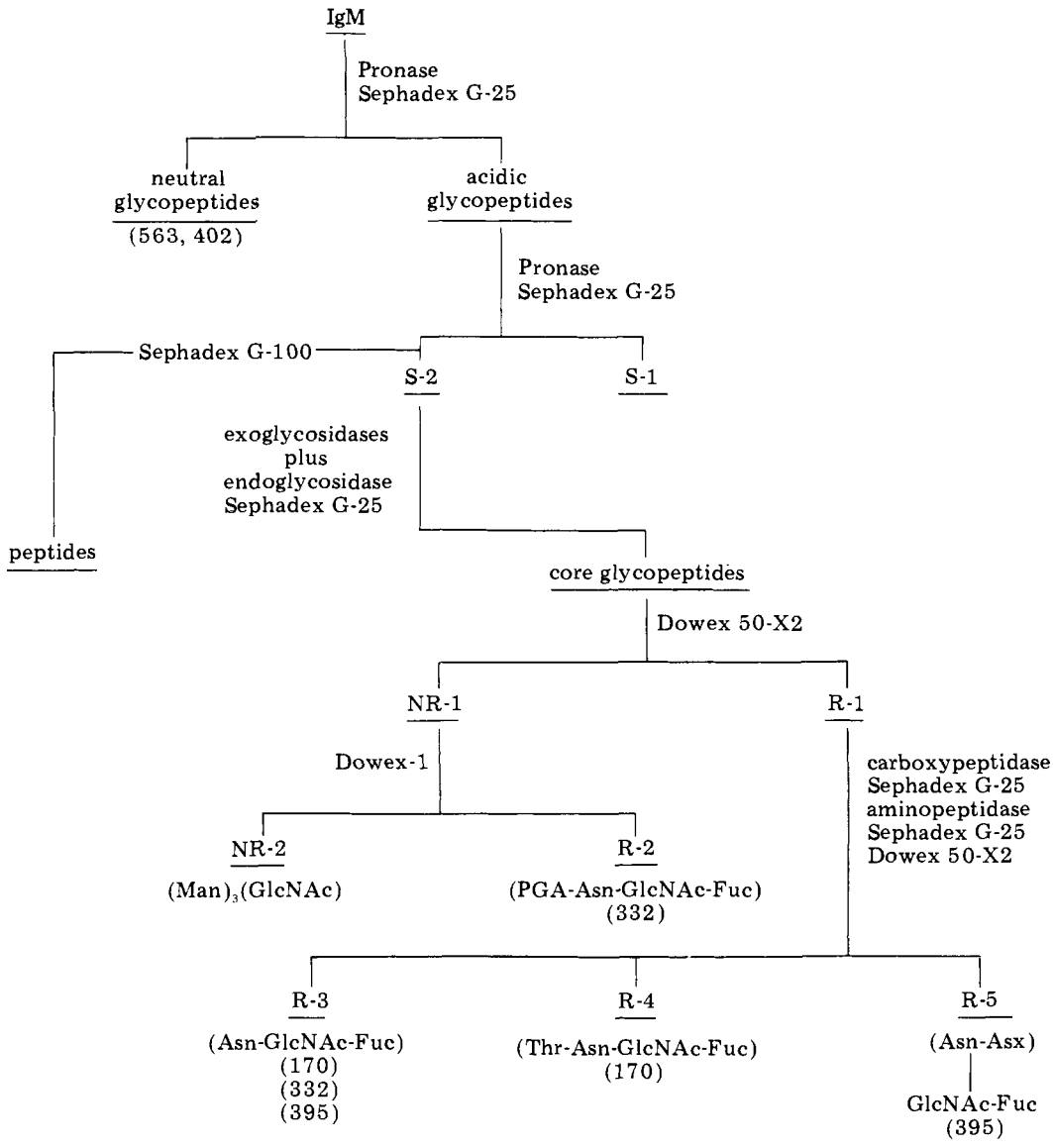

FIGURE 2: Major steps employed in the isolation of IgM glycopeptides. The numbers in parentheses refer to the location of each asparagine from the amino end of the heavy chain.

tilled water at a rate of 12 ml/hr and fractions of 3.0 ml were collected. After 3 hr the eluting solution was changed to 0.5 *M* pyridine. Carbohydrate analyses on suitable aliquots with phenol-H_2SO_4 revealed a fraction (NR-1) that was not retained by the column and one (R-1) that was eluted with 0.5 *M* pyridine. Only the retarded peak was fluorescamine positive. However, amino acid analysis of NR-1 (Table I) indicated the presence of aspartic and glutamic acid at a ratio of unity.

NR-1 was adjusted to pH 9.0 with 0.5 *N* NaOH and applied to a column of Dowex 1-X4 (HCO_3^-, 1.5 × 7.0 cm). The column was washed with distilled water for 3 hr and then with 0.3 *M* NH_4HCO_3 at a rate of 25 ml/hr; 5-ml fractions were collected. Carbohydrate analyses (Table I) revealed a large hexose-positive peak (NR-2) in the column void volume containing 3 mol of mannose and 1 mol of *N*-acetylglucosamine. A fucose-containing fraction (R-2) was retained by the Dowex-1, but was recovered by elution with 0.3 *M* NH_4HCO_3.

On treatment of the tetrasaccharide (NR-2) with α-mannosidase, a dissacharide containing 1 mol each of mannose and *N*-acetylglucosamine was obtained. Evidence supporting the structure as β-D-Man(1→4)-D-GlcNAc was obtained by demonstrating the complete susceptibility of this disaccharide to β-D-mannosidase and also by comparing the retention time of the unknown compound with a series of known disaccharides which were chromatographed on an SE-30 column at 250° (see Experimental Section). That the other two mannosyl residues are linked (1→3) and (1→6) to the β-D-mannose was determined by gas chromatographic analysis of the methylated alditol acetate derivatives formed from the tetrasaccharide (Figure 4). The ratio of peak 1 (2,3,4,6-tetra-*O*-methylmannose) to peak 5 (2,4-di-*O*-methylmannose) was 1.8:1.0 as expected for a compound of the indicated structure. These results essentially confirm those reported by Hickman et al. (1972).

Fraction R-2, with a molar ratio of glutamic-aspartic acid of 1, was not retained by Dowex 50 but was retained by Dowex 1, indicating that pyroglutamic acid was on the amino end of the dipeptide. The carbohydrate associated with R-2 could be released on alkaline borohydride hydrolysis as a disaccharide composed of fucose and glucosaminitol. Following *N*-acetylation and gas chromatographic analysis of the disaccharide on a column of SE-30 at 250°, a

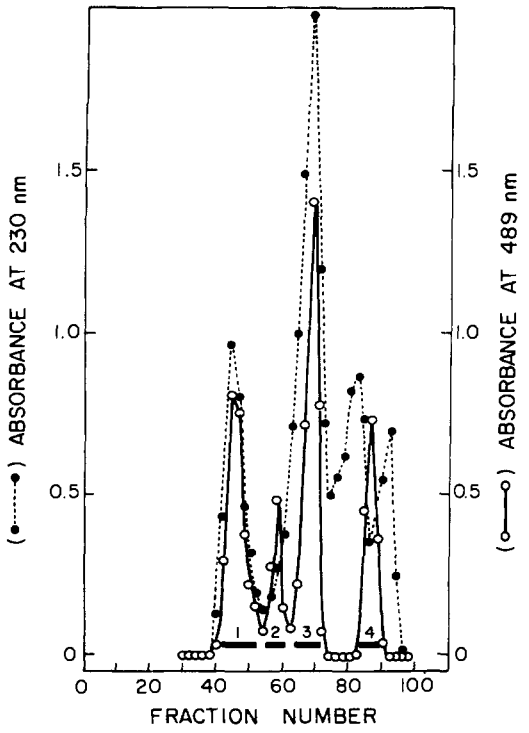

FIGURE 3: Sephadex G-25 chromatography of exo- and endoglycosidase-digested sialoglycopeptide fraction S-2. The 230-nm absorbing regions coincided exactly with the fluorescamine-positive material. The bars signify the fractions that were pooled, with peak 3 containing the core glycopeptides (for details see text and Figure 2).

Table I: Compositional Analyses of IgM Core Glycopeptide Fractions.[a]

	Fraction						
Residue	NR-1	NR-2	R-1	R-2	R-3	R-4	R-5
Asp	1.00		1.87	1.00	1.00	1.00	2.00
Glu	0.82		0.05	0.90			
Thr	0.0		0.57			0.73	
Ser	0.12		0.47				
Ala	0.0		0.09				
Fuc	ND		1.23	0.94	0.75	1.02	0.77
GlcN	3.17	1.00	1.00	0.72	0.87	0.79	0.92
Man	ND	3.15					

[a] The indicated fractions are presented in Figure 2. The molar ratios of the components in each fraction are presented relative to GlcN or Asp. ND, not determined.

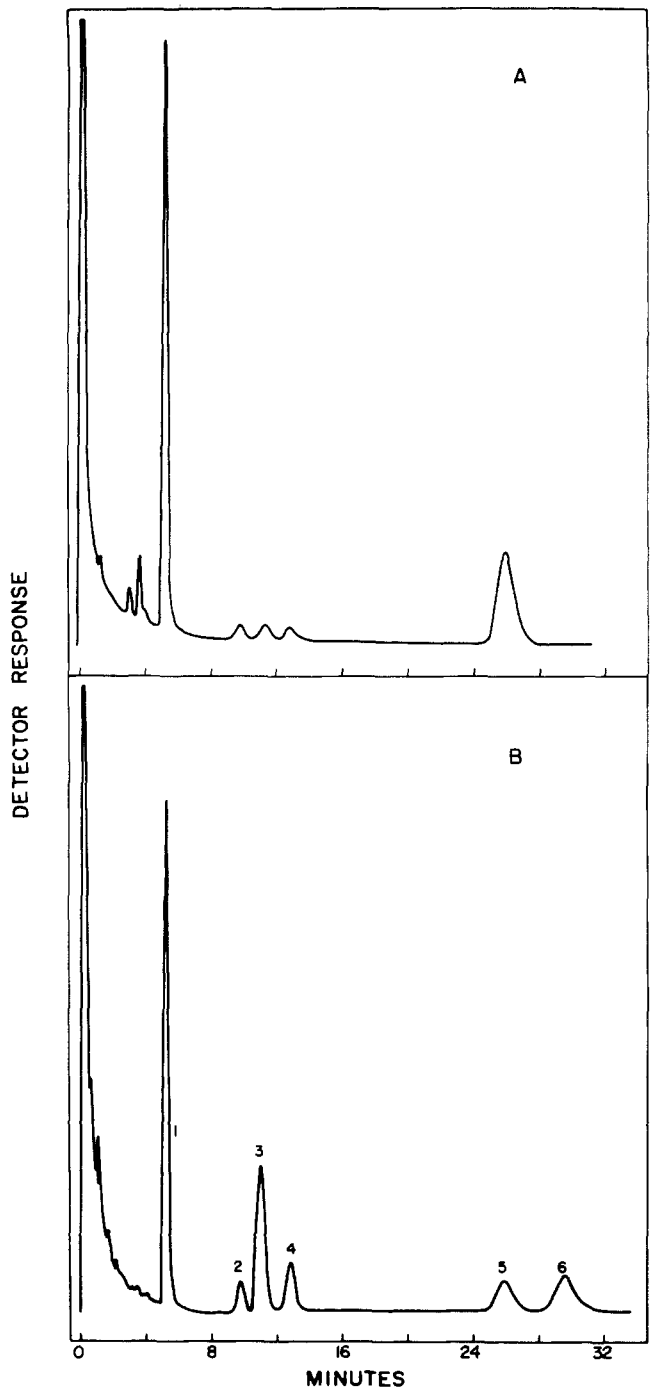

FIGURE 4: Gas chromatography of methylated mannitol acetates obtained from $(Man)_3GlcNAc$ (A). The peaks in (B) represent the following standards: (1) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; (2) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol; (3) 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol; (4) 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol; (5) 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylmannitol; (6) 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol.

single peak was obtained which migrated identically with that of a known sample of α-L-Fuc(1→6)-D-GlcNAc-OH. Susceptibility of the *N*-acetylglucosamine moiety in R-2 to periodate oxidation verified the location of the fucosylglycosidic linkage. Further confirmation for this bond was obtained by subjecting the disaccharide to a Smith degradation. The only labeled product obtained following this treatment was *N*-acetylserinol, which can only be derived from an *N*-acylated hexosaminitol substituted in the 6-O position (Tarentino et al., 1970).

Enzymic Digestion of R-1 Core Glycopeptides. The R-1 glycopeptides (10 μmol/ml) were dissolved in 0.1 *M* potassium phosphate (pH 8.0) containing carboxypeptidase A (7 mg/ml) and the resulting solution, which contained added thymol and toluene, was incubated at 37° for 108 hr. The pH of the digestion mixture was adjusted to 3.0 and frozen overnight. Carboxypeptidase A was removed by centrifugation and the glycopeptides were passed through a column of Sephadex G-25 (1.9 × 230 cm) with 0.1 *N* acetic acid. The glycopeptide peak was detected by monitoring the absorbance of the effluent at 230 nm.

Fractions containing the core glycopeptides were lyophilized, dissolved to a concentration of 10 μmol/ml in 0.16 *M* potassium phosphate (pH 7.1) containing aminopeptidase M (Henley and Co., 1 mg/ml), and incubated at 37° with thymol and toluene. After 48 hr the reaction mixture was

lyophilized, redissolved in 0.1 *N* acetic acid, and centrifuged to clarify the solution. The supernatant fraction containing the core glycopeptides was passed through a column of Sephadex G-25 (1.9 × 230 cm) with 0.1 *N* acetic acid and the major peak detected at 230 nm was lyophilized.

Ion-Exchange Chromatography of Core Glycopeptides. The carboxypeptidase, aminopeptidase-treated R-1 glycopeptides were dissolved in 3.6 ml of 0.075 *M* sodium acetate buffer that had been adjusted to pH 3.0 with 98% formic acid. This solution was applied at a rate of 32 ml/hr to a column of Dowex 50-X2 (30–48 μ, 1.5 × 100 cm) that had been equilibrated in the acetate buffer. Fractions of 7.5 ml were collected. After 11 hr of washing with the acetate buffer, the column was eluted with a convex gradient containing pH 3.0 acetate buffer in the mixing chamber (100 ml) and 0.1 *M* sodium acetate in the reservoir. Column effluents were monitored for glycopeptides with fluorescamine. Three peaks (R-3, fractions 42–50; R-4 and R-5, fractions 92–98) were detected and the desired fractions in each were pooled, lyophilized, and desalted on a column of Sephadex G-25 (1.9 × 230 cm) equilibrated in 0.1 *N* acetic acid. The desalted glycopeptides were concentrated by lyophilization. Their compositions are presented in Table I.

The structure proposed for R-3, Asn-GlcNAc-Fuc, is supported by the migration of this compound on electrophoresis in a formic acid–acetic acid buffer (see Experimental Section) and by chromatography on the amino acid analyzer (Figure 5). The most likely position of this asparagine is 170 in the heavy chain, but some contribution from the glycopeptides associated with Asn-332 and Asn-395 is likely. In contrast to the findings of Baenziger et al. (1974) with an apparently identical compound from IgE, the removal of aspartic acid from R-3 could not be effected by glycosyl asparaginase until fucose was first removed with α-L-fucosidase. This fact is clearly demonstrated by the data in Figure 5 (D and E), and indicates that their preparation of glycosyl asparaginase was contaminated with α-fucosidase. As in the case of R-2, the fucose of R-3 is most probably linked to *N*-acetylglucosamine by a (1→6) bond, as evidenced by the susceptibility of the *N*-acetylglucosamine moiety to periodate oxidation. A similar susceptibility was found in R-4 and R-5.

Final proof that the disaccharide in R-3 is α-L-Fuc(1→6)-D-GlcNAc was obtained by comparing the mass spectrum of the gas chromatographed Me_3Si derivative of Fuc(1→6)GlcNAc-OH isolated from R-3 with chemically synthesized Me_3Si α-L-Fuc(1→6)-D-GlcNAc-OH. As shown in Figure 6, the masses of the ion fragments produced are almost identical and were not compatible with the spectra of other disaccharides such as β-D-Man(1→4)GlcNAc, α-D-Man(1→4)GlcNAc, and α-L-Fuc(1→6)-D-Gal. The peaks at *m/e* 494 and 612 can be assigned to the ion fragments resulting from the cleavage of a 6-O substituted Me_3Si derivative of GlcNAc-OH, which again confirms the proposed linkage point between fucose and *N*-acetylglucosamine.

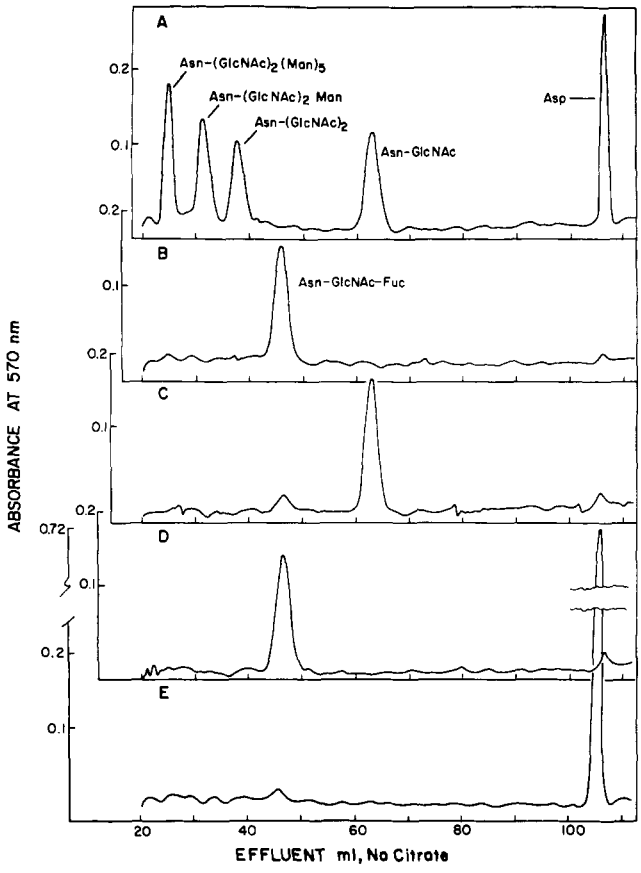

FIGURE 5: Chromatography of R-3 on the amino acid analyzer before and after treatment with glycosyl asparaginase and α-fucosidase. (A) Standard mixture of ovalbumin glycosyl-Asn derivatives; (B) isolated R-3; (C) R-3 treated with α-L-fucosidase (0.36 unit in 0.35 ml of 0.15 *M* sodium citrate (pH 5.4) at 37° for 15 hr); (D) R-3 treated with glycosyl asparaginase (0.12 unit, as in C); (E) R-3 treated with glycosyl asparaginase and α-L-fucosidase (same conditions as in C and D).

Discussion

Unequivocal evidence has been presented in this paper indicating that fucose is glycosidically linked to the 6-O position of the *N*-acetylglucosamines associated with asparagines 170, 332, and 395 of the IgM heavy chain. Earlier studies revealed fucose to be located in the core region of the sialoglycopeptides of IgG (Kornfeld et al., 1971) and IgM (Hickman et al., 1972), but the precise position of this sugar was not defined. More recently it was shown with IgE (Baenziger et al., 1974) and IgA (Baenziger and Kornfeld, 1974) that the fucosyl moiety is most probably attached to the same position as that described in this paper for IgM.

Glycopeptides containing α-L-Fuc(1→6)-D-GlcNAc were shown recently (Tsay and Dawson, 1975) to accumulate in α-fucosidosis an inborn error of glycoprotein metabolism. Related products with structures comparable to the immunoglobulin oligosaccharides, but with a single *N*-acetylglucosamine on the reducing end followed by varying amounts of mannose, have been shown to accumulate in the liver and urine of patients (Nordén et al., 1974) and animals (Phillips et al., 1974) afflicted with α-mannosidosis. Similar oligosaccharides, but with even longer carbohydrate chains, have been shown to be associated with the GM_1 and GM_2 gangliosidoses (Tsay and Dawson, 1975; Wolfe et al., 1974; Ng Ying Kin and Wolfe, 1974), indicating that cleavage was occurring at the core di-*N*-acetylchitobiosyl unit of the parent molecule. The general similarity of these products to those formed by the endo-*N*-acetylhexosaminidases from bacterial sources (Tarentino et al., 1974; Arakawa and Muramatsu, 1974) suggested the existence of a comparable enzyme in animal tissues. Partial purification of such an enzyme from hen oviduct was recently obtained by us (A. L. Tarentino and F. Maley, in preparation), and was also inferred in the studies of Nishigawa et al. (1974) with

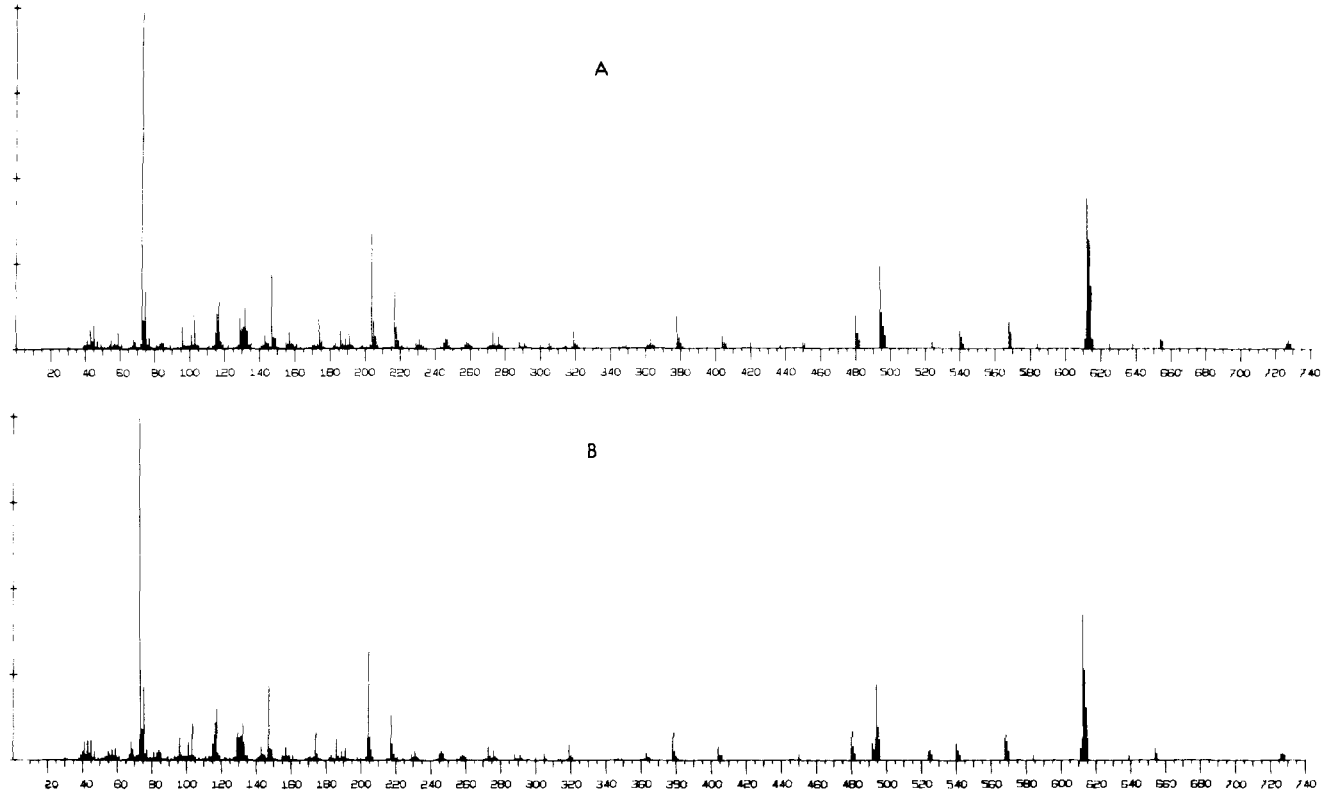

FIGURE 6: Mass spectra of (A) chemically synthesized Me_3Si-α-L-Fuc(1→6)-D-GlcNAc-OH and (B) Me_3Si derivative of reduced disaccharide isolated from fraction R-2 (see Figure 1).

crude extracts from rat and porcine tissues. The existence of this endoglycosidase in animal tissues, coupled with the absence of specific exoglycosidases in the above indicated lysosomal genetic defects, is the most probable explanation for the accumulation of oligosaccharides with *N*-acetylglucosamine on the reducing end.

While fucose appears in most instances to be associated with the core region of complex oligosaccharides of such proteins as the immunoglobulins (Kornfeld et al., 1971; Hickman et al., 1972; Baenziger et al., 1974; Baenziger and Kornfeld, 1974), bovine thyroglobulin (Arima et al., 1972; Toyoshima et al., 1972) and porcine ribonuclease (Kabasawa and Hirs, 1972), it has not been found in the same region of the neutral oligosaccharides also present in these proteins. Since the core regions are identical, a requirement for the addition of fucose to the proximal GlcNAc of the di-*N*-acetylchitobiose units associated with the complex acidic oligosaccharides would appear to be the presence of the peripheral sugars, galactose, *N*-acetylglucosamine, and sialic acid. The presence of these sugars in addition to fucose appears necessary for the secretion of the immunoglobulins from the mouse B lymphocyte (Andersson et al., 1974). Fucosyl transferases (Chester and Watkins, 1969; Grollman et al., 1969) have been described for the biosynthesis of the blood group substances where the fucosyl residues are more peripherally located than in the immunoglobulins, However, the nature of the fucosyl transferases involved in the addition of fucose to the core region of complex oligosaccharides remains to be clarified.

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Mechanism of Action of Adenosylcobalamin: 3-Fluoro-1,2-propanediol as Substrate for Propanediol Dehydrase—Mechanistic Implications†

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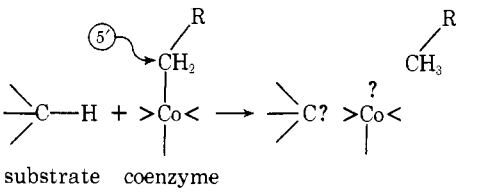
ABSTRACT: 3-Fluoro-1,2-propanediol has been found to be a substrate for propanediol dehydrase and has very similar binding and catalytic constants compared to the natural substrate. The only isolable products of the reaction are acrolein and inorganic fluoride; with 3-fluoro-3,3-dideuterio-1,2-propanediol as substrate, only 3,3-dideuterioacrolein is obtained. These results indicate that the primary product of the reaction is 3-fluoropropionaldehyde which spontaneously loses hydrogen fluoride to yield acrolein. The similar kinetic parameters for the fluorinated as compared to the normal substrate suggest that significant charge does not develop on the fluorinated or, by implication, the natural substrate during any rate-limiting steps of the reaction. These results support a radical, as contrasted to an ionic pathway for reactions involving adenosylcobalamin and diol dehydrase.

Most current mechanisms for rearrangement reactions involving adenosylcobalamin invoke, as one of the first steps, the cleavage of the bond between the cobalt and the 5′-methylene carbon of the deoxyadenosyl residue of the coenzyme with concomitant removal of a hydrogen atom from substrate by the 5′ carbon (which becomes thereby a methyl group).



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